Investigation of Methemoglobin Contribution in Host Pathology and Drug Toxicity During Malaria

A thesis submitted in partial fulfilment of the requirement for the degree of

Doctor of Philosophy

By

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Dedicated to my Parents, Siblings and my beloved Wife

who have stood beside me, with patience and faith in all my journeys and ventures towards success…

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Statement

I hereby declare that the matter embodied in this thesis entitled **"Investigation of Methemoglobin Contribution in Host Pathology and Drug Toxicity During Malaria"** is a cumulative account of the results of investigations carried out in the Department of Biosciences & Bioengineering, Indian Institute of Technology, Guwahati, India, under the supervision of **Dr. Vishal Trivedi.**

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work of other investigators are referred.

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Certificate

It is certified that the work described in this thesis entitled, **"Investigation of Methemoglobin Contribution in Host Pathology and Drug Toxicity During Malaria",** by **Mr. S.N. Balaji** (Roll no: 126106029), submitted to Indian Institute of Technology, Guwahati, India, for the award of the degree of Doctor of Philosophy, is an authentic record of results obtained from the research work carried out under my supervision at the Department of Biosciences & Bioengineering, Indian Institute of Technology, Guwahati, Assam, India. This work has not been submitted elsewhere for a degree.

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Dr. Vishal Trivedi
(Supervisor)

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S. N. Balaji

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ABBREVIATIONS

5-HPQ	5-hydroxyprimaquine
ACT	Artemisinin Combination Therapy
ARM	Age Related Markers
CAT	Catalase
CDNB	1-chloro-2,4-dinitrobenzene
CLT	Clotrimazole
CNBr	Cyanogen bromide
CPrq	Carboxy Primaquine
CQ	Chloroquine
CR1	Complement Receptor 1
CSA	Chondroitin Sulphate A
DCFH_DA	2',7'-Dichlorofluorescin Diacetate
DDT	Dichlorodiphenyltrichloroethane
DNPH	Dinitrophenylhydrazine
DTNB	5,5'-Dithio-bis-2-nitrobenzoic acid
EDTA	Ethylene diacetate tetra acetic acid
FAP	Focal Adhesion Protein
G6PD	Glucose-6-phosphate dehydrogenase
GPI	Glycosyl Phosphatidyl Inositol
GSH	Glutathione
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
Hb	Hemoglobin
HCG	Human Chorionic Gonodotropin
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte Growth Factor
ICAM-1	Intercellular Adhesion Molecule-1
ICP	Intracranial Pressure
IL	Interleukins
INF-γ	Interferon-γ
IRBC	Infected Red Blood Cells
KCN	Potassium cyanide
LDL	Low Density Lipoproteins
MAQ	6-methoxy-8-hydroxyaminoquinoline
MetHb	Methemoglobin
MMP-9	Matrix Metalloproteinase-9
NAC	N-Acetyl cysteine
NBT	Nitroblue Tetrazolium
NF-κB	Nuclear Factor of kappa light chain enhancer of activated B cell
NMDA receptor	N-methyl D-asparate receptor
NO	Nitric oxide
PBN	N-tert-butyl-α-phenylnitrone
PfEMP-1	*Plasmodium falciparum* Erythrocyte Membrane Protein-1
PQ	Primaquine
PS	Phosphatidylserine
Q	Quinine
RBC	Red Blood Cells
RMS	Red Blood Cell Derived Microparticles
ROS	Reactive Oxygen Species
RPMI-1640 Medium	Roswell Park Memorial Institute 1640 Medium
SOD	Superoxide dismutase
TCA	Trichloro Acetic Acid
TEMPO	(2,2,6,6-tetramethylpiperidine-1-yl)oxy
TLR	Toll Like Receptor
TNF-α	Tumour Necrosis Factor-α

Materials Used in the Current Study

Methemoglobin, N-acetylcysteine, Mannitol, 2,7, Dichlorofluroscein Diacetate, Glutaraldehyde [EM grade] 25%, Clotrimazole, Thiobarbituric acid, 1,1',3,3' tetraethoxy propane, Guanidine hydrochloride, 5,5'dithiobis[2-nitrobenzoic acid], Annexin-V-FITC apoptosis detection kit, Primaquine bisphosphate, 2,2',6,6'-tetramethylpiperidin-1-yl-oxyl, α-phenyl N-tertiary-butyl nitrone, Sodium nitrite, Glutathione reduced, Hemin, 5,5-dimethyl-2-(8-octanoic acid)-1-pyrroline N-oxide were procured from Sigma.

Hydrogen peroxide, Hydrochloric acid 35 %, Acetone, Ethanol, Magnesium oxide (MgO), Vitamin C, Dinitrophenyl hydrazine, Ethylacetate, DMSO, Thiourea were procured from Merck.

Tetrazolium nitro blue (TNB), 3,4-dinitro phenylhydrazene (DNPH), Ethylene diamine tetra acetic acid (EDTA), Sodium chloride, Dihydogen potassium phosphate, Dipotassium hydrogen phosphate, Disodium hydrogen phosphate, Methionine, Riboflavin, 1-chloro 2,4 dinitro benzene (CDNB), Ethylene diamine tetra acetic acid disodium salt, Ammonium sulfate, Potassium chloride, Potassium iodide, Potassium dihydrogen orthophosphate, Sodium phosphate dibasic anhydrous, Dimethyl sulphoxide, Guaiacol, L-methionine, N-acetyl L-methionine was procured from Sisco research laboratory (SRL), India.

Anti-Hb antibodies and an isotype control antibody were purchased from santa-cruz biotechnology, inc.

Other chemicals where used in the current studies were analytical grade.

Chapter 1. Malaria from Historic Period to Current: In View of Pathophysiology

1.1 Introduction

Malaria infects over 20 million and kills at least 0.4 million peoples every year. The sub-tropical zones especially Africa, Asia and South America are the most affected regions. Malaria influences the economy of the affected regions and causes reduction of 1-2 % economic productivity. Malaria is caused by *Plasmodium* genus and it has five different species such as *P. falciparum, P. malaria , P. ovale, P. vivax* and *P. knowlesi* to develop disease in humans (Mueller et al., 2007). Among them, *P. falciparum* is the most pathogenic species to account for 60 % death. Though various malaria eradication programs have reduced malaria infection and mortality, increasing drug resistance among the different malarial species is an alarming situation. Multiple organ defects observed during malaria and the cyto-toxic effect of anti-malarial drugs are the other challenges (Sarkar et al., 2010). It drives the researchers to develop safe and non-toxic anti-malarial drugs, additional adjuvant therapies and also to reduce vector burden in the environment to control the spreading of disease. In the current chapter, we are discussing different aspects related to the malaria such as historical discoveries, epidemiology, control strategies, clinical manifestation of malaria. In addition we have discussed in detail the patho-physiology in host during malaria especially the contribution of different factors (host or parasite derived) and their molecular mechanism. Lastly, we have outlined the objectives and significance of the research work presented in the current thesis.

1.2 Historical account of malaria

Malaria infection was observed in Egyptian mummies dating from 3200 and 1304 BC (Nerlich et al., 2008). Indian ancient scripts of the Vedic period (1500 to 800 BC) called malaria the “king of diseases.” In 270 BC, ‘Nei Chin’ the Chinese medical catalogue linked quartan and tertian fevers with splenomegaly which blamed malaria's headaches, fever, and intense chillness (Bruce-Chwatt et al., 1988). The Greek poet Homer, Aristophanes (445-385 BC), Aristotle (384-322 BC), Plato (428-347 BC), and Sophocles (496-406 BC) were described malaria symptoms in their writings. Hippocrates (450-370 BC) was noticed connection between appearances of Sirius the Dog Star with malarial fever (Nerlich et al., 2008; Sherman, 1998). Besides these popular daily life observations, several scientific discoveries are made in different time periods to understand diverse aspects of the disease which are outlined in Table 1.1. In 1880, Charles Louis Alponse Laveran reported first time existence of malaria parasite from patient. In 1891, Dimitri

Leonidovitch Romanowsky accidentally discovered methylene blue – eosin as a suitable stain to identify the parasites in blood smears. In 1897, Sir Ronald Ross reported that the mosquitoes are the vector for avian malaria. Life cycle of human malaria parasite (*P. falciparum, P. vivax*, and *P. malariae*) was reported by Giovanni Battista Grassi and his colleagues. In 1937, Sydney James and Parr Tate revealed that there is pre-erythrocytic multiplication stage after sporozoite infections in chickens. In 1947, Henry Shortt and Cyril Garnham reported pre-erythrocytic liver stage of Malaria parasites, exo-erythrocytic forms of *P. vivax.* In 1948, first rodent malaria parasite *P. berghei* was identified by Ignace Vincke and Marcel Lips. In 1976, William Trager and J.B. Jensen successfully performed in-vitro continuous parasite culture, which accelerated molecular level studies in the malaria field. Since then, molecular, cellular and immunological aspects of malaria, discoveries of important anti-malarial drugs and vaccines are done and they are outlined in Table 1.1.

1.3 Life cycle of malaria parasite

The malaria parasite shows a complex life cycle consisting of an invertebrate vector (mosquito) and a vertebrate host (human). Parasite develops sexual stages in mosquitoes and it multiplies asexually in humans. Parasitic asexual stage further divided into hepatic (pre-erythrocytic) and blood (erythrocytic) stages (Figure 1.1).

1.3.1 Asexual stages of parasites: In humans, female *Anophelus* mosquito injects malarial sporozoites during their blood meal (Figure 1.1). After insertion, sporozoite enters the blood stream from the site of bite and thus they can reach to infect the hepatocytes (Amino et al., 2006). With the infection of hepatocytes, parasites don't express the clinical symptoms but divides to produce thousands of merozoites (cryptozoites). Simultaneously, small number of merozoites develops into hypnozoites and later they cause the malaria relapse in the host (Prudencio et al., 2006; Soulard et al., 2015). Sporozoite to merozoite conversion takes 7-24 days and it depends on the *Plasmodium* species and host immunity. After the release of merozoites from the hepatocytes, each merozoite infects a new red blood cell and it start to develop and multiply within the red blood cell through different stages known as early trophozoites (rings), late trophozoites and schizonts. Shortly after infection parasites form a parasitoporous vacuole in the RBC cytoplasm. Within the parasitoporous vacuole, parasite resides, grows and divides mitotically without cytokinesis until schizont formation. During schizont stage cytokinesis take place which results into new merozoites. Mature schizonts may contain 16-24 merozoites which egress from erythrocytes after lysis of the cells to begin new erythrocytic cycle. Erythrocytic schizogony of malaria parasites is ~48 hr generally but the life cycle of *P. malariae* and *P. knowleski* are 72 hr and 24 hr respectively.

1.3.2 Sexual stages of parasites: Few erythrocytic parasites emerge as gametocytes to start the sexual life cycle in mosquitoes (Figure 1.1)**.** Mosquitoes receive the gametocytes during its blood

meal from the infected human host. After reaching the stomach male and female gametocytes fuse together and form motile ookinetes. Ookinetes escape from the mosquito gut to peripheral side of the gut wall and turn into oocyst. From oocyst numerous sporozoites release and perpetuates

Table 1.1: Historical proceedings of malaria related major scientific findings.

S.No.	Year	Scientists	Discovery/Invention	Reference
1	1820	Pierre Pelletier & Joseph Caventou	Quinine	(Rosenthal, 2001)
2	1880	Charles Louis Alponse Laveran	Malaria parasites.	(Nye, 2002)
3	1891	DL Romanowsky	Methylene blue – eosin stain.	(van't Veer and Haferlach, 2014)
4	1897	Sir Ronald Ross	Mosquitoes role in avian malaria transmission.	(Ross, 1897)
5	1898–1900	Giovanni Battista Grassi	Life cycle of *P. falciparum*, *Pm* and *Pv* Malaria transmission from mosquito to human.	(Cox, 2002)
6	1922	Henry Shortt & Cyril Garnham	Pre-erythrocytic and exo-erythrocytic forms of malaria parasites.	(Cox, 2002)
7	1934	Hans Handersag et al.	Chloroquine synthesis.	(Thomé et al., 2013)
8	1939	Paul Hermann Muller	Insecticidal action of dichlorodiphenyltrichloroethane.	(Gunasekaran, Sahu et al. 2005)
9	1940	Robert Elderfield	Primaquine synthesis.	(Edgcomb, Arnold et al. 1950)
10	1942	Paul F Russell & B. N. Mohan	First malaria vaccine test attempt in Fowls	(Russell and Mohan, 1942)
11	1947	Ignace Vincke & Marcel Lips	*P. berghei*, first rodent malaria parasite, identification.	(Cox 2010)
12	1956	Fulton & Flewett	First electron microscopy studies of *Plasmodium* (*P.berghei* and *P.knowleski*)	(Fulton and Flewett, 1956)
13	1966	William Trager et al.,	Demonstration of *P. falciparum* fine structure.	(Trager et al., 1966)
14	1971	Tu Youyou	Isolation of artemisinin from *Artemisia annua* leaves.	(Cui and Su 2009)
15	1976	William Trager & J.B. Jensen	In vitro continuous parasite culture technique.	(Trager and Jensen 1976)
16	2002	Malcolm J Gardner et al	Completion *P. falciparum* genome sequencing	(Gardner, Hall et al. 2002)
17	2002	E. Lasonder et al.	First *P. falciparum* proteomic analysis.	(Lasonder et al., 2002)
18	2014	J.L. Ramirez et al	*Chromobacterium Csp_P* - reducing the life span of mosquitoes.	(Ramirez et al., 2014)
19	2015	SmithKline Beecham Biologicals	Development of malaria vaccine RTS.S/AS01- Approval of RTS.S/AS01 by WHO.	(RTS, 2015)

towards the salivary gland. During next blood meal sporozoites infect the healthy human (Angrisano et al., 2012; Sherman, 1979; White et al., 2014).

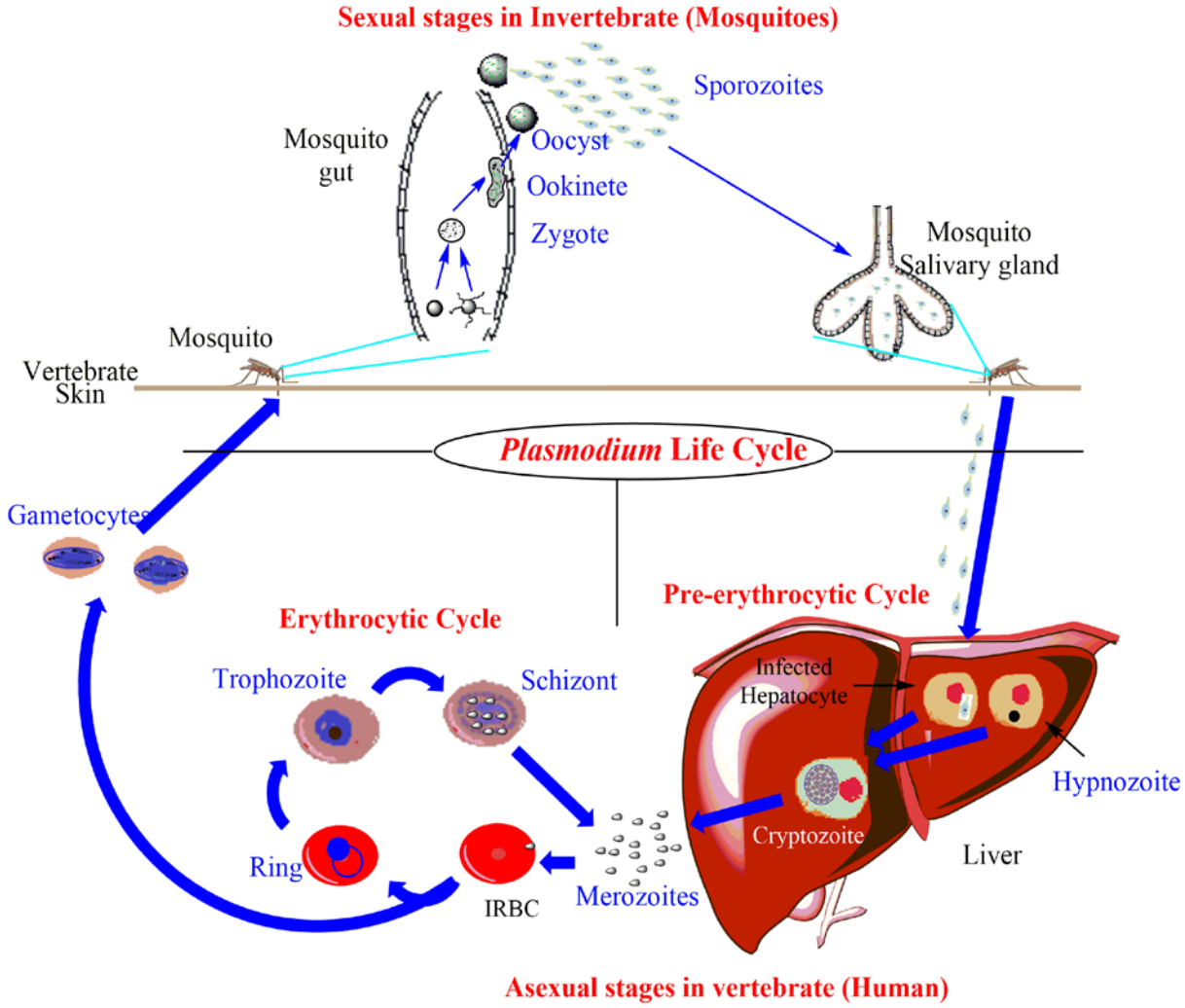


Figure 1.1: Life cycle of malaria parasite in vertebrate and invertebrate host. The sequence malaria parasite life cycle in invertebrate host (mosquito) and vertebrate host (human).

1.4 Clinical Symptoms of malaria

Clinical symptoms of malaria appear during erythrocytic stage of the Malaria parasites. Malarial fever elicits headaches, muscle aches, dry cough, nausea, vomiting, tiredness, etc., along with typical paroxysmal fever (Figure 1.2). Paraoxysomal feature of malaria develop during the merozoites egress from the RBC along with the release of toxic by-products which initiates initial chillness and followed by sudden rise the body temperature (fever) unusually in the malarial patient. In advanced condition, malaria also develops various unusual clinical features which include severe anaemia, lactic acidosis, hypoxia, splenomegaly, liver diseases, kidney diseases, visual defects, cerebral malaria with neuronal damages (Fairhurst and Wellems, 2010; Zaki and Shanbag, 2011). Untreated malaria leads to the coma stage prior to the death.

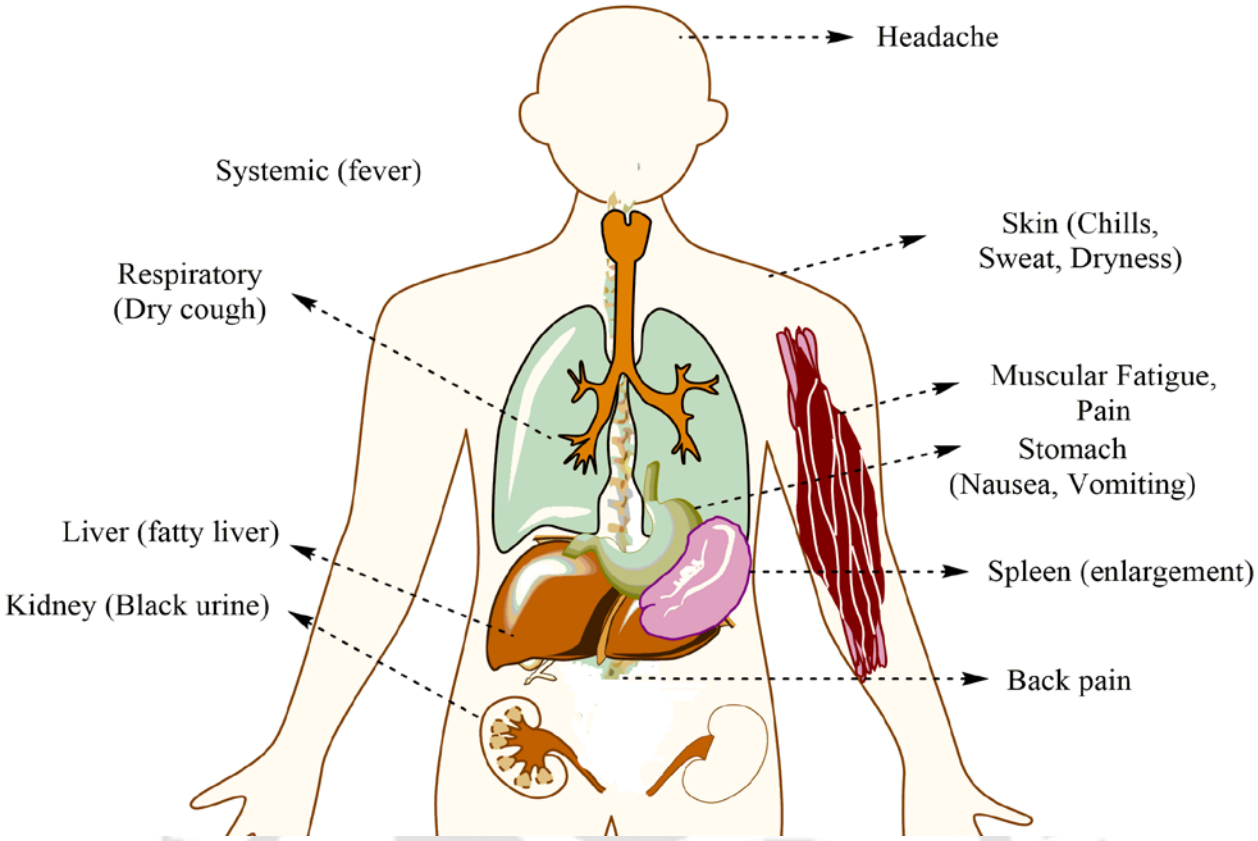


Figure1.2: Common symptoms during the malaria. Clinical symptoms arise during malaria and different organs involvement in host.

1.5 Global malaria risk and distribution

Currently malaria transmission is prevalent in five WHO regions. They are sub-Saharan Africa, tropical region of the South America, Indian sub-continent, South-east Asia and Indo-Pacific islands. Sub-Saharan Africa region is the major malaria infection region and ~70 % malarial deaths would be recorded in 2015. India and South East Asia are followed the sub-Saharan Africa in malaria incidences. Various government and non-government agencies are working together to control the malaria from many decades. Due to the continuous effort malaria transmission and mortality together were reduced significantly. The assessment of malaria control program efforts by WHO which displayed in Figure 1.3. Except the South American country Venezuela, in most of the regions malaria incidence is decreasing. Malaria risk is emerging around the world in the form of resistant development and climate change. Climate change can re-establish the malaria in Mediterranean and Middle East Asia during winter by raising the temperature. Temperature elevations also favour to spread the malaria in the higher altitudes like Nairobi. Drug resistance development against the anti-malarial may lead to the increase in mortality rate.

1.6 Malaria eradication programs and achievements

Till 19th century, there was no recommended drug for malaria and disease remains as fatal

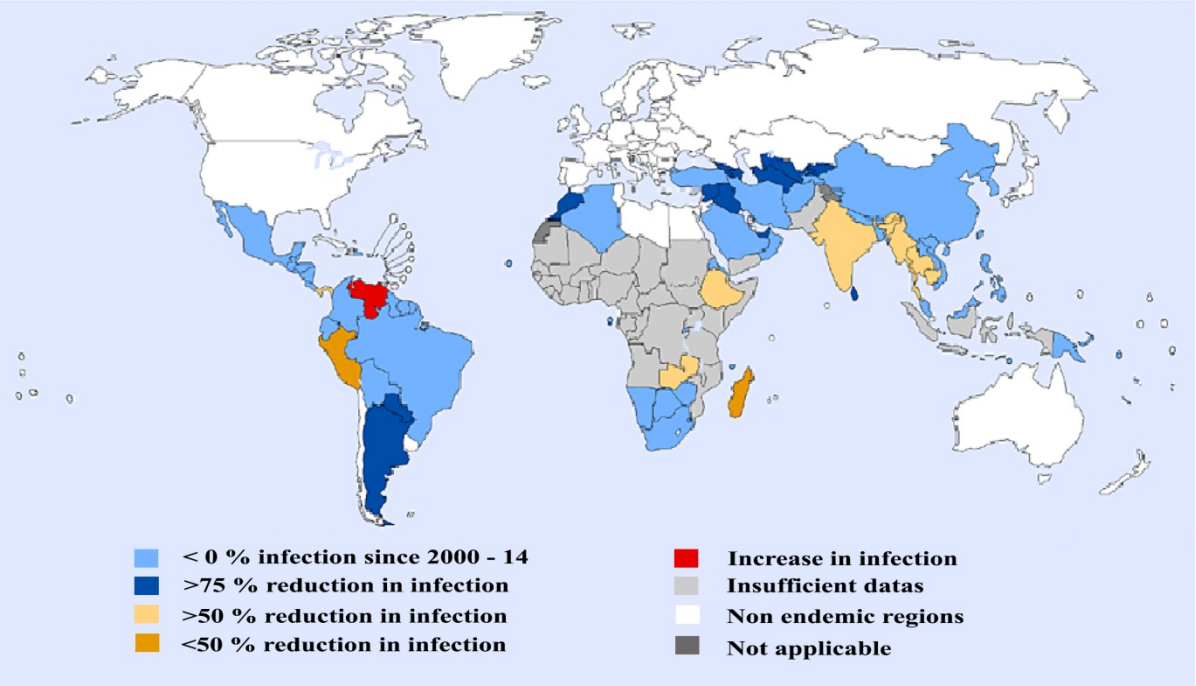


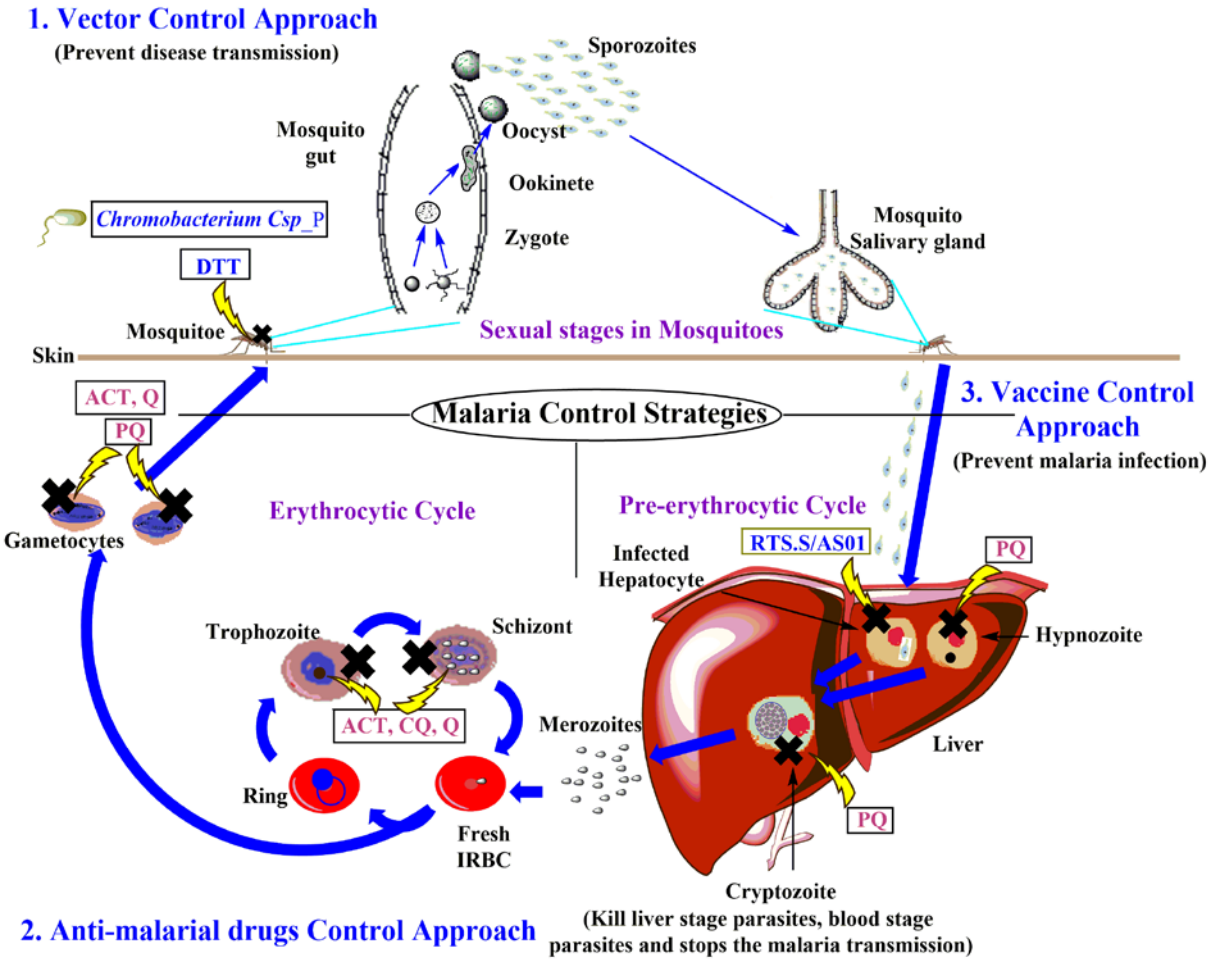
Figure 1.3: Account of Malaria episodes in the world between 2000 -2015. (WHO, 2016)

infectious disease. In order to control the malaria incidences, several approaches are proposed and practiced in society. These approaches and their outcome are discussed below:

1.6.1 Vector control approaches: Since role of mosquito was established by Ronald Ross in malaria transmission, mosquitoes were targeted to reduce the malarial infections. Environment management, such as draining the stagnant water around the residential area, results in reduction of mosquito and malaria infection incidences. Introduction of DDT (dichlorodiphenyltrichloroethane) like pesticides were helped lot all around the world. But due to the environmental hazard issues, DDT was not allowed in developed countries after 1970s. To avoid pesticide pollution, biological methods like mosquito larval destruction by using either larvivorus fishes or infecting mosquitoes with environmental friendly bacteria like *Bacillus thuringiensis israelensis* (Dambach et al., 2014), and *Chromobacterium Csp_P* (Ramirez et al., 2009; Ramirez et al., 2014). These methods were helped to reduce the mosquito population. Recently, sterile insect technique was developed to control the mosquito population (Lees et al., 2015). Vector control approach to prevent the malaria is depicted in Figure 1.4**.**

1.6.2 Anti-malarial drugs in malaria control and their mechanism of action: Anti-malarials are the agents which kills the causative agents *Plasmodium sp.* to stop spreading of disease in the host. They are either natural or synthetic compounds. Chemical structures of few selected anti-malarial agents are given in Figure 1.5 and their mechanism of action is elaborated in Table 1.2. The role of anti-malarial in malaria control is shown in the Figure 1.4. The different anti-malarials and their mode of action are discussed briefly:

Quinine: In the year 1500, Jesuit priests learned from the people of Peru that the powder from the Cinchona tree bark relieved shivering with cold. It was also proposed that it may also offer relief from the chills of malaria. Its activity against the parasite, later shown to be due to bioactive quinine present in Cinchona bark. In 1820, Quinine was successfully separated by Pierre Pelletier and Joseph Caventou from Cinchona bark and was used to treat intermittent fever (Rosenthal, 2001). The white crystalline aryl alkaloid quinine is used as an important anti- malarial drug during Second World War. Currently, it is used for uncomplicated malaria treatment. The recommended dosage is 10 mg/kg of body weight thrice per day for seven days. Quinine essentially target late rings, trophozoites and schizonts stages of parasite. In combination with other drugs, such as clindamycin, tetracyclin, quinine is effectively targeting and killing the different stages of parasites. Oral quinine is indicated in conjunction with another blood stage schizonticides such as doxycycline, clindamycin or sulfadoxine/pyrimethamine for the treatment of uncomplicated chloroquine-resistant



ACT - Artemisinin Combination Therapy; **CQ** - Chloroquine; **PQ** - Primaquine; **Q** – Quinine; **DTT** – Dichlorodiphenyltrichloroethane; **RTS.S/AS01** – Vaccine.

Figure 1.4: Different Strategies to control Malaria transmission. Malaria control is achieved by different methods. They are (1) Vector control approach (to kill the invertebrate vector by insecticides (DTT) and bio-control method (*Chromobacterium CSp_P*); (2) Anti-malarial drugs (to kill the parasites in human host) and (3) Vaccine control approach (to generate antibodies against sporozoites to prevent the liver infection).

malaria caused by *P. falciparum* and *P. vivax* (WHO, 2016). The precise molecular mechanism of quinine action is not conclusive but several hypothesis are proposed for its anti-malarial action. During erythrocytic development, malaria parasite endocytose the RBC cytoplasm into parasitoporous vacuole. Endocytozed RBC cytoplasm reach to the food vacuole and there, they undergo proteolytic degradation by cysteine and aspartic proteases. RBC cytoplasmic digestion results into amino acids and toxic heme. Amino acid is utilized for parasitic protein synthesis but heme start to accumulate within the food vacuole (Deans et al., 1983). Heme is a known toxic compound to generate free radicals and thus to avoid the oxidative stress, parasites converts the heme into less toxic hemozoin (Combrinck et al., 2013). Quinine essentially thought to block the heme to hemozoin mineralization process and lead the accumulation of free heme within the digestive vacuole which may cause the parasite death (Panisko and Keystone, 1990). The basic nature of quinine leads them to accumulate within the parasitic acidic vacuole may lead to alteration of intravascular pH. The alteration in pH may disrupt electrolyte balance in the parasitoporous vacuole and arrest the parasite growth (Panisko and Keystone, 1990).

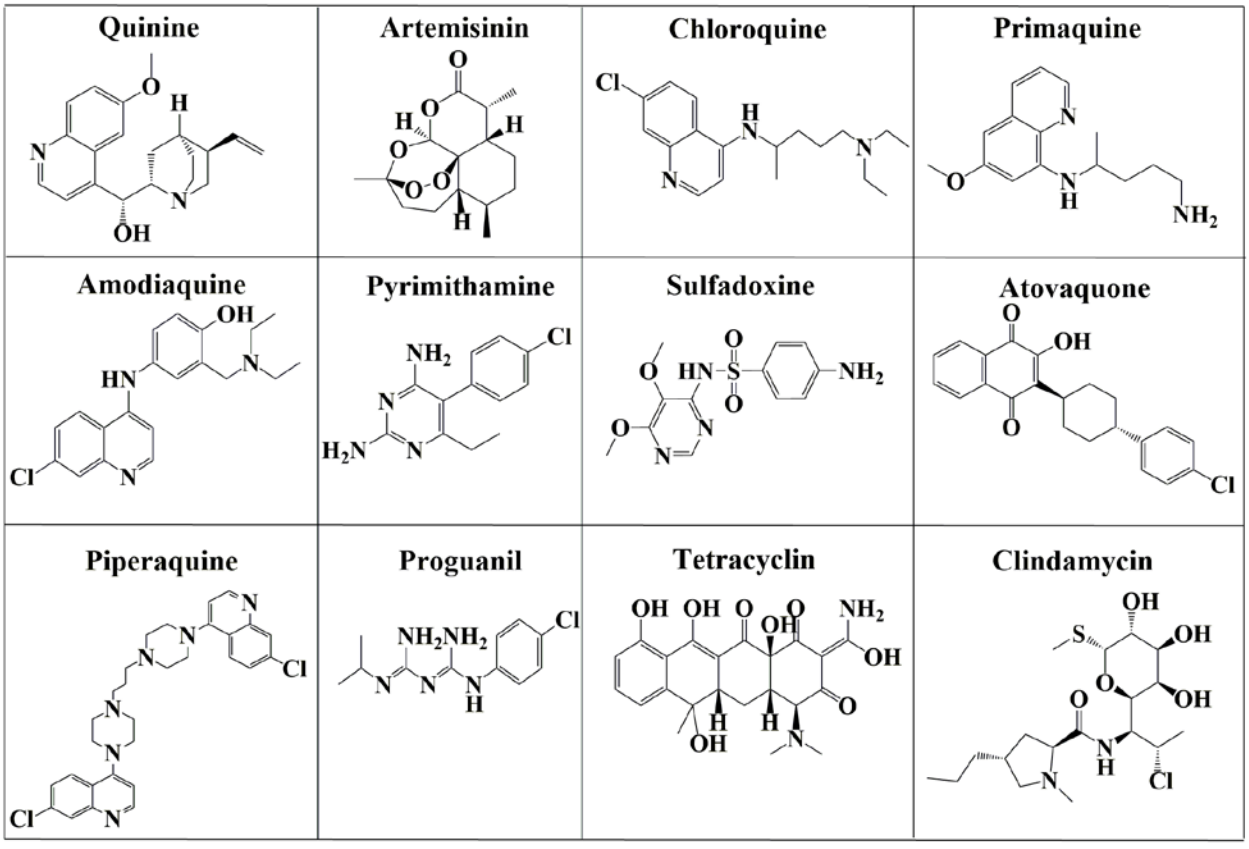


Figure 1.5: Chemical structure of few selected anti-malarial agents.

But this hypothesis is not validated thoroughly and has several unclear observations. In contrast to Chloroquine, quinine binds weakly with free heme and it doesn't correlate with the anti-parasitic activity of the molecule. Instead, quinine may be interacting with the recycling process of digestive vesicle phospholipids and reduce the hemozoin formation process (CD Fitch, 2003). Quinine

inhibits TNF-α level in plasma during malaria and avoids the severe malaria development (Maruyama et al., 1994).

Chloroquine: The increasing demand of quinine led to synthesis of chloroquine, an artificial synthetic derivative. It is a better anti-malarial agent than the quinine. Chloroquine was discovered by Hans Handersag and his group in the year 1934 (Cook and Zumla, 2009). It was widely used during Second World War as replacement of quinine. It is the first choice of anti-malarial due to low cost and less toxicity than quinine. The recommended dosage is 10 mg base/kg of body weight/day for first 2 days and 5 mg base/kg of body weight/day for next 7 days. Chloroquine is used to kill erythrocytic stages of parasites and to some extend it is used for the prophylaxis as well. Chloroquine mechanism of action is not completely understood but several hypotheses were put forward as discussed below:

1. Chloroquine accumulates in food vacuole?: Chloroquine accumulation in parasitic food vacuole may be associate with its anti-malarial activity (Yayon et al., 1984).
2. Chloroquine alters pH of the food vacuole?: Chloroquine is a weak base. Thus, the accumulation of chloroquine raise the PH in food vacuole and disturb food vacuole function (Yayon et al., 1985).
3. Chloroquine interacts with heme?: Chloroquine binds with heme and delay heme dimerization process. It lead to the heme accumulation within the food vacuole and kill the parasites (Fitch, 2004).
4. Chloroquine disturbs Hb digestion?: Unless quinine, chloroquine also leads to the accumulation of within the food vacuole. It suggests that chloroquine may interfere the digestion process by either inhibiting or reduce the synthesis of digesting cysteine/aspartate proteases (Slater, 1993; Surolia and Padmanaban, 1991).
5. Hypothesis of polyamine metabolism inhibition by chloroquine: In vitro studies of chloroquine mediated parasitic ornithine decarboxylase inhibition is the other mechanism proposed for its anti-malarial activity (Konigk et al., 1981).
6. Hypothesis of chloroquine mediated interference in gene expression by binding with DNA: In vitro studies of DNA binding with chloroquine suggested that it may lead to the poor transcription and replication in the parasites and ultimately lead to parasite growth arrest (Parker and Irvin, 1952). However, this hypothesis was rejected when specific binding nature of chloroquine with guanine was demonstrated whereas parasitic genome contains almost 75 % of A:T base pairs (Allison et al., 1965).

In recent time, the chloroquine resistance malaria parasite was observed all around the world and forced to develop alternate malaria therapeutic agents.

Artemisinin: Artemisinin is the most effective anti-malarial drug at present to combat malaria. It is a natural anti-malarial present in the leaves of herbal plant *Artemisia annua*. In 1971, anti-malarial activity of artemisinin was shown by Youyou Tu (Tu, 2011) and received the Nobel prize in the year 2015. The sesquiterpene lactone structure of artemisinin contains unusual peroxide bridge. This peroxide bridge is also known as 1,2,4-trioxane ring and it may the important reaction centre for the artemisinin bioactivity. Artemisinin derivatives such as artesunate, arteether and artemether are better soluble compounds than the artemisinin. The recommended dosage of artesunate is 2.4 mg/kg intravenously for every 12 hr interval for 4 days. Mechanism of artemisinin action is still unclear but free radical generation by artemisinin may be the responsible for the anti-malarial activity. Interaction of free iron or heme with 1,2,4-trioxane ring of the artemisinin lead to the generation free radicals which may demise the parasite (Cui and Su, 2009; Meshnick et al., 1991; O'Neill et al., 2010). Artemisinin is also modulating pro-inflammatory cytokines expression and helps to suppress the neuro-degeneration during severe malaria (Miranda et al., 2013). Other hypothesis such as protein synthesis inhibition, mitochondrial damage are not explained well (O'Neill et al., 2010). Currently artemisinin is used in different combination to give better results and to avoid the development of drug resistance in malaria parasite. The different combinations such as artesunate with amodoquine, artesunate with Sulfadoxine-Pyrimethamine (Spottiswoode et al.). Clinical trial of dihydroartemisinin-piperaquine combination in cerebral malaria patients indicate that the therapy is giving better outcomes (Group et al., 2016).

Primaquine: In 1940, PQ was synthesized by Robert Elderfield to target relapses in *P. vivax* and *P. ovale* (Edgcomb et al., 1950). Relapse of malaria in the recovering patient from disease is common with *P. vivax* and *P. ovale* infections. It can be avoid by an anti-malarial PQ which specifically kills the parasite liver stage hypnozoites by generating free radicals through an unknown mechanism (Legorreta-Herrera et al., 2010). The recommended dosage of PQ is 30 mg/day for 14 days or 21 days. PQ administration is limited due to the severe hemolytic effects in glucose-6-phosphate dehydrogenase deficient patients (Lopez-Antunano, 1999). Gametocidal activity of the PQ is useful to prevent the transmission of malaria (Frischer et al., 1991; Lopez-Antunano, 1999).

Other optional anti-malarial agents: Other antimalarial agents like amodiaquine, mefloquine, halofantrine, lumefantrine, piperaquine, pyronaridine, pyrimethamine, sulfadoxine, atovaquone, clindamycin, tetracycline, etc are used mostly in combination with established anti-malarials due to their less efficacy and high toxicity. Antimalarials mode of actions of selected drugs was briefly given in Table 1.2.

1.6.3 Adjuvant Therapy: Adjuvant therapy is an approach to enhance the efficiency of the primary drugs or to reduce the toxic effects of the primary drugs to provide faster recovery in

patients. Anti-malarials primarily target the parasites and effectively eliminate it from the host but they cause severe complications in off target site due to drug associated toxicity. Different adjuvant therapies are directed towards specific complications in the target organ of host. There are no

Table 1.2: Anti-malarial agents and their mode of action.

S.No.	Compounds	Mechanism	Mode of Action	Reference
1	Quinine, Quinoline, Quinindine, Cinchonine, Cinchonidine.	Interfere with the heme detoxification process, inside the digestive vacuole of the parasite	Parasiticidal, Schizonticidal, Gametocidal	(Panisko and Keystone, 1990)
2	Chloroquine, Amodiaquine.	Similar to quinine	Parasiticidal, Schizonticidal	(Thomé et al., 2013)
3	Pyrimethamine, proguanil, sulpha-diazine.	Inhibits parasitic dihydroopteratesynthetase & dihydrofolatereductase	Parasiticidal, Schizonticidal	(Legorreta-Herrera et al., 2010)
4	Tetracycline, clindamycin.	May be inhibits protein biosynthesis as like as in prokaryotes	Parasiticidal	(van Dyke et al., 1970)
5	Quinacrine,	Inhibit DNA synthesis	Parasiticidal	(van Dyke et al., 1970)
6	Atovaquone	Inhibits malarial electron transport system and dihydroorotate DHase	Parasiticidal	(Siregar et al., 2015)
7	Artemisnin, artesunate, arteether and artemether	Unknown. (hypothetical actions: i) generating oxidative stress, ii) blocking Hemoglobin degradation and heme detoxification	Parasiticidal, Schizonticidal	(Cui and Su, 2009)
8	Primaquine	Kills hypnozoites	Relapse, Tissue schizontizidal	(Legorreta-Herrera et al., 2010)

proper guidelines for malarial adjuvant therapy and till date different agents are under evaluation for the therapy (John et al., 2010; Nuchsongsin et al., 2007b). Various agents such as carticosteriods, mannitol, glycerol, sorbitol, N-acetyl cysteine (NAC), pentoxyfyline, dexamethasone, dichloroacetate, anti-TNF-α monoclonal antibody, ascorbate, tocopherol and ulinastatin and curcumin are used randomly as different malaria adjuvant therapy in different clinical trials (Mimche et al., 2011; Mohanty et al., 2006a). Among them curcumin, NAC, ascorbate and tocopherol showed better activity in preventing vascular complication but they didn't provide any success to reduce malaria associated mortality.

Corticosteroids: Cerebral malaria is the most severe complication during *P. falciparum* infection. Cerebral malaria develops clinical signs such as intracranial pressure (ICP), moderate cerebral oedema, oxidative stress, apoptosis, etc. The mechanism of corticosteroids action in ICP is

unknown. To reduce the ICP, catecholamines are the choice but it also lead to the gastrointestinal bleeding and seizures without having impact on mortality. In addition, clinical trials performed with corticosteriods studies were not effective against cytotoxic oedema and the beneficial effects is difficult to conclude due to small number of trial group (Prasad and Garner, 2000).

Mannitol: Mannitol, an osmotic agent, can't cross the blood brain barrier and metabolize ~ 20 %. It readily diffuses in kidneys and draws the water to increase the urinary output. This mechanism may also applicable to reduce the intracranial pressure, intraocular pressure. Study by Newton and his group with mannitol, as adjuvant therapy within 23 Kenyan children infected with malaria, indicates decrement of ICP. But this small number of population, design of experimental analysis and data were not sufficient to stand for his conclusion (Newton et al., 1997).

Desferrioxamine: Iron chelator desferrioxamine shown the faster parasite clearance, but failed to prove the faster recovery from the deep coma (Smith and Meremikwu, 2003).

Pentoxyfyline: Different studies with pentoxyfyline showed a mixed effect on TNF-α level, i.e., either reduction or no change, in severe malaria patients (Looareesuwan et al., 1998).

TNF-α antibody: Fab fraction of anti-TNF-α is one of the promising agent in reducing TNF-α level in the malaria patient and promoting the faster cure with the artesunate administration (Looareesuwan et al., 1999).

Thiazolidindione: Phagocytosis enhancing agents such as thiazolidindione enhance the CD36 mediated phagocytosis in macrophages and parasite induced TNF α secretion (Asada et al., 2004).

Vitamin E & Ascorbate: Anti-apoptotic factors such as ascorbate, tocopherol may reduce the endothelium phagocytosis and reduce endothelium damage. Along with the protease deactivator ulinastatin, they may useful to protect the kidney damages during malaria (Hemmer et al., 2005).

Dichloroacetate: The common malarial manifestation lactic acidosis can be manage by dichloroacetate which may avoid or reduce the severity of the various malarial complication (Pasvol, 2005).

Curcumin: Curcumin is a natural and a potent bioactive molecule exhibiting wide range of bioactivity. Several studies on curcumin effect on mice experimental cerebral malaria was resulted delay of death, suppressed parasitemia and slightly improved survival by 29% (Aditya et al., 2012; Akhtar et al., 2012; Martinelli et al., 2008; Nayak et al., 2010; Reddy et al., 2005).

N-Acetyl Cysteine (NAC) in adjuvant therapy: Antioxidant NAC is used as an adjuvant during severe malaria to reduce oxidative stress related complications. NAC are thought to reverse the RBC rigidity to avoid RBC clearance and reduction of oxidative stress mediated RBC lysis (Mohanty et al., 2006b). NAC administration was found to reduce the TNF-α level during severe malaria (Watt et al., 2002a). In another clinical trial, intravenous NAC administration in patients (n=15) showed faster lactate clearance than placebo control (Watt et al., 2002b). The underline

mechanism of NAC mediated lactate clearance is not well understood. The oral NAC administration is not effective as compared to intravenous administration. In another clinical trial, malaria patients (n=108) were divided into two groups; First group consists of 56 malaria patients (severe *falciparum* malaria) receiving NAC as adjunct therapy and second group of 52 patients were on placebo. They compared the malaria mediated mortality, lactate clearance time, coma recovery time, urine oxidative marker F2-isoprostane, and parasite clearance time. They have observed no difference in mortality, lactate clearance, and coma recovery time. In NAC treated patients, parasite clearance time was extended from 30 to 36 hrs. Urinary oxidative marker F2-isoprostane derivatives was observer higher in NAC administered patient. (Charunwatthana et al., 2009). Neutral effect of NAC therapy during severe malaria is unclear and need more investigations.

1.6.4 Malaria vaccines: Development of vaccine is the simple way to develop immunity against malaria infection, but even after decades of research, malaria vaccine development is not been feasible. The traditional approach to develop malaria vaccine focused on specific single stage of parasite life cycle i.e., vaccine targets either pre-erythrocytic or the asexual erythrocytic stages. Pre-erythrocytic vaccine strategies aim to generate an antibody response to neutralize sporozoites and prevent them from invading the hepatocyte. Also, to elicit a cell mediated immune response to target the parasites multiplication inside liver (Hill, 2006; Schofield et al., 1987). Erythrocytic stage vaccine strategies aim to elicit antibodies that will inactivate merozoites to block invasion into fresh RBCs. It targets malarial antigens expressed on the RBC membrane through antibody dependent cellular cytotoxicity to potentiate the T-cell response against the infected RBCs (Ahmed Ismail et al., 2014; Reed et al., 2006). By decreasing the exponential multiplication of merozoites, this type of vaccine would mostly serve as a disease reduction vaccine in endemic countries (Hill, 2006). As for vaccines that target the sexual stage of the parasite, they do not aim to prevent illness or infection in the vaccinated individual but to prevent or decrease transmission of the parasite to new hosts (Kristoff, 2007). The initial trials of malaria vaccine had given promising results, but subsequent trials did not meet the predicted expectations (Valero et al., 1996). In Table 1.3, different malaria vaccine candidates are given with the target stage of the parasites and their advantages. RTS.S/AS01 is a first malaria vaccine received positive opinions worldwide. RTS is a genetically engineered peptide which made up of repeating unit of P. falciparum circumsporozoite protein central region with T-cell epitope and hepatitis B surface antigen (HBsAg). RTS is co-expressed along with free HBsAg in yeasts. Spontaneous binding of RTS with HBsAg results RTS,S vaccine. It effectively prevents the infection by sporozoites to liver cells. Phase III Clinical trial of RTS.S/AS01 vaccine showed 26 % and 39 % less infections in children after third and fourth dosage respectively. Further studies are going on to test the extend of efficacy provided by

the RTS.S/AS01 vaccine. As like other vaccines, after every administration of RTS.S vaccine cause the fever as the inflammatory responses. Clinical trials have not shown any other adverse effects in children (RTS, 2015).

Table 1.3: Different vaccines designed to provide protection against malaria

Target stage	Antigens	Salient features	Current status	Reference
Pre-ery thro cytic	Irradiated sporozoites Circumsporozoite protein or peptides liver stage antigens-1, RTS.S/AS01	Stage and species specific; antibody blocks infection of liver. RTS.S evokes humoral & cellular immunity.	RTS.S/AS01-Completion of phase 3clinical trial. LSA-1:Under clinical trials phase 1	(Reed et al., 2006; RTS, 2015)
Meroz oites & erythro cytic	Erythrocyte Binding Antigen, Merozoite Surface Antigen1 & 2, Ring Infected Erythrocyte Antigen, Serine Repeat Antigen, Rhoptry Associated Protein, Histidine Rich Protein, Apical Membrane Antigen1	Stage and species specific; prevents the erythrocytic infection; lead to the destruction of infected RBC	Short term protection. Under development for increasing efficiency.	(Ahmed Ismail et al., 2014; Reed et al., 2006)
Gamet ocytes	*P. falciparum* s25, 48/54k, *P. falciparum* 230.	Prevents the infection of mosquitoes thus blocks transmission cycle; antibody to this antigen prevents maturation of gametocytes, zygotes	Failed	(Kristoff, 2007)
All stages	*P. falciparum* s66 (combination of pre-erythrocytic and erythrocytic stage of *P. falciparum*	Blocks all the stages of parasit development in human host	Failed	(Graves and Gelband, 2006)

1.7 Malaria induced pathological complications in host and its mechanism.

P. falciparum infection causes the severe burden to the host. Malaria parasite cleverly hides within the host cells and multiply safely. Parasites expose for a short span to the host immune system at the time of invasion to new RBC. Such brief exposure is relatively not adequate to generate effective immune response to eliminate the parasite from the host. Mean while, parasitic metabolic wastes damage the host cellular and organelle systems such as reticulo-endothelial systems, placenta, visual defects, etc. These effects are schematically depicted in Figure 1.6 and discussed in this section.

1.7.1 Severe anemia: Anemia is a clinical condition in which Hemoglobin (Hb) level falls below 10g/dl. Severe anemia is very common during malaria often the level decrease less than 6.0 g/dl (Zaki and Shanbag, 2011). Anemia develops in malaria patient by following three mechanisms.

They are (a) intravascular hemolysis, (b) increased clearance of RBC and IRBC, (c) decrease in RBC production through dysregulated erythropoiesis (Figure 1.7). Altogether these factors cause the severe anemia during malaria.

(a) Intravascular hemolysis: Intravascular hemolysis refers the rupture of RBCs within the blood

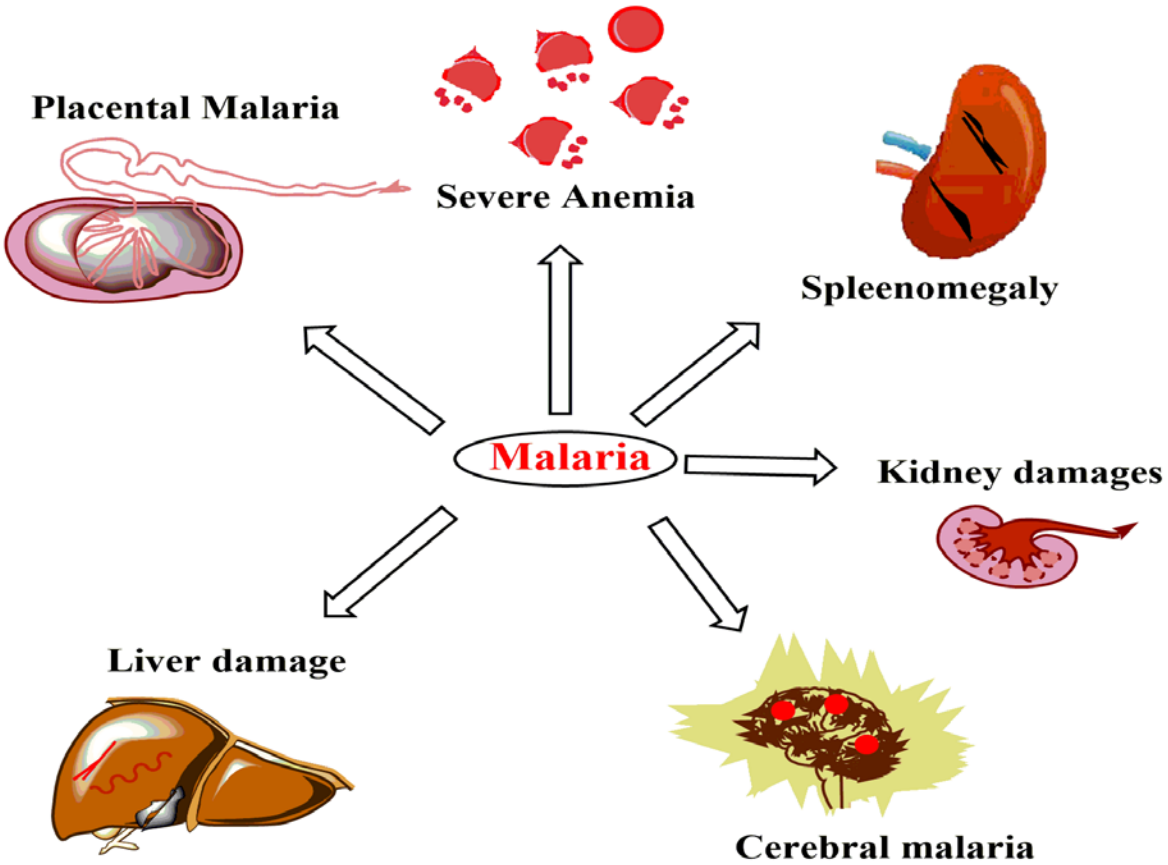


Figure 1.6: Different complications observed in host during malaria. The major organs often undergo for partial or complete dysfunction during malaria to cause the death in host.

vessels. Contribution of intravascular hemolysis in anemia development in malaria patient is depicted in the Figure 1.7. During malaria, parasites lyse the IRBC to release merozoite to complete their life cycle by infecting fresh RBCs. This cyclic process leads to the reduction RBCs in the blood and develop anemia like condition. IRBC lysis also release Hb derived products such as hemin, methemoglobin (MetHb), degraded Hb peptides and malarial pigment hemozoin (Nuchsongsin et al., 2007a). Metabolites released from IRBC are capable to generate the free radicals and cause the oxidative damages to the blood cells. They also induce the NO synthesis by endothelium to contribute into additional oxidative stress. TNF-α also involve in this process and increase the NO level in blood. Exposure of free radicals leads to the RBC death by oxidizing the RBC membrane lipids, proteins and inducing phosphatidylserine (PS) expression on RBC outer membrane (Rifkind et al., 2014). Non-oxidative stress mediated RBC lysis during malaria is not pursued extensively. Complement mediated hemolysis was reported based on in-vitro experiments

but it lack in-vivo support to draw fruitful conclusions (Ansar et al., 2006; Biryukov and Stoute, 2014).

(b) RBCs & IRBCs clearance: Outline of RBC & IRBC clearance pathways are showed in Figure 1.7. During malaria, increased RBC clearance rate was observed. It may be due to the oxidative stress mediated RBC structural and functional changes, reduced deformability, PS externalization. RBCs life span is ~120 days in healthy humans. Old RBCs express different age related markers (ARM) which can be used by macrophages to identify and phagocytose them in spleen, liver and bone marrow (Bratosin et al., 1998; de Back et al., 2014b). Binding products on RBC surfaces also enhances the macrophagic clearance (de Back et al., 2014b). In IRBC, Malaria parasites expressed markers (like RSP-2, PS) on IRBC membrane which lead to the macrophagic clearances (de Back et al., 2014b). Altogether, they cause the abnormal sequestration of RBCs in spleen and this condition lead to anemia with reduction of RBC count in blood (Awah et al., 2009).

(c) Dysregulated erythropoiesis: Outline of erythropoietic dysregulation is schematically illustrated in Figure 1.7. Bone marrow is the prime site to produce and mature erythropoietic cells. Reticulocytes in bone marrow further mature into RBC in blood vessels. Spleen and liver also involve in erythropoietic process as secondary site. Any interference in erythropoietic process causes reduced number of RBC in blood to develop anemia. During malaria, reduced erythropoiesis was observed in correlation with increased level of hemozoin, TNF-α, NO, NO mediated apoptosis in $CD34^+$ cells and reduced level of erythropoietin (Awandare et al., 2011; Thawani et al., 2013).

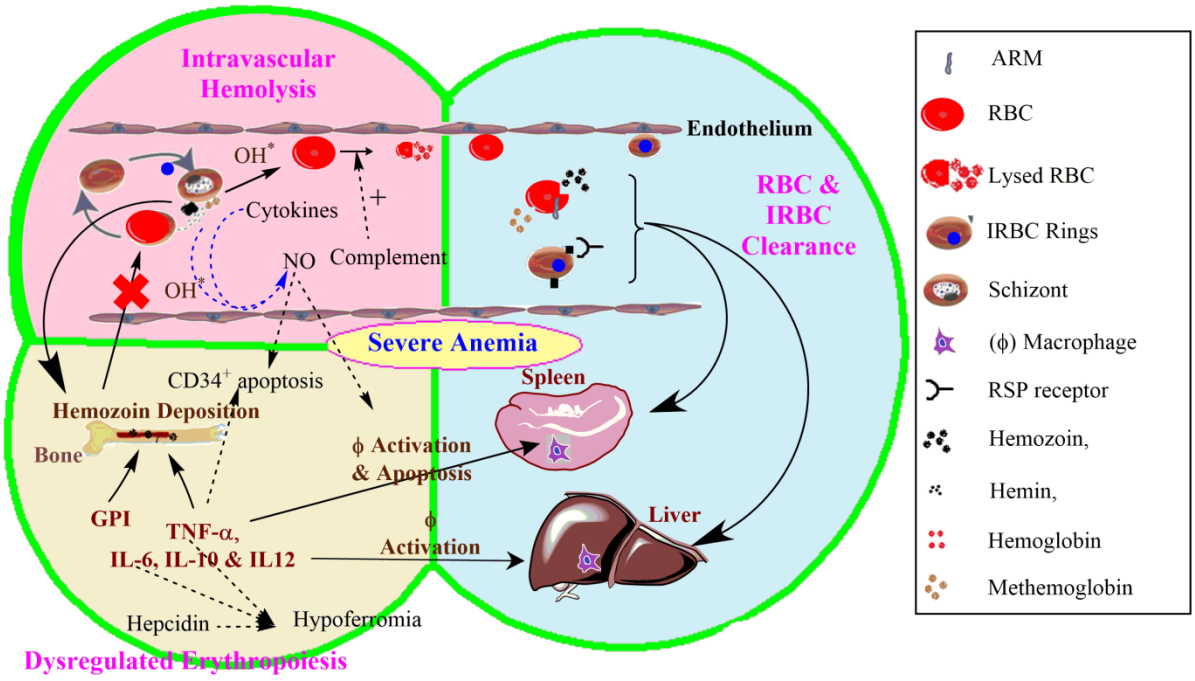


Figure 1.7: Pathophysiology of severe anemia. During malaria severe anemia develops by enhancing the intravascular hemolysis, RBC and IRBC clearance. Suppression of erythropoiesis also causes severe reduction of RBCs in blood. For details see description.

Inflammation develops during different diseases such as tuberculosis, meningitis, rheumatoid arthritis and malaria (Nweneka et al., 2010; Spottiswoode et al., 2015). Abnormal levels of cytokine cause the hepcidin release in liver. Hepcidin is a hormone which regulates iron metabolism through its absorption and storage. Hyperhepsidinemia causes the reduction of intestinal iron absorption. On the other hand, hepcidin promotes the iron release from macrophages and lead accumulation of iron in liver. This process known as iron delocalization and it causes hypoferromia to indirectly disturb the erythropoiesis in bone marrow (Nweneka et al., 2010; Spottiswoode et al., 2015). Reduction in level of cytokine RANTES by hemin also impairs the erythropoiesis during malaria (Perkins et al., 2011; Roumenina et al., 2016).

1.7.2 Cerebral malaria: Cerebral malaria is defined as severe neurological complication. Coma is the hallmark symptom during cerebral malaria along with brain swelling, intracranial hypertension, retinal changes, brainstem signs, bleeding disorders and multi-organ failure (Newton et al., 2000). The incidences of cerebral malaria in children increases mortality rate by 10-20% even after receiving the malarial therapy. In adults, death can be avoided with the intravenous artesunate injection. The patients recovered from cerebral malaria were suffering throughout their life-span with different neurological complications such as blindness, ataxia, central hypotonia, cognition, motor function, behavioural changes and epilepsy. Mechanism of cerebral malaria and associated complications are poorly understood. Postmortem study of the cerebral malaria patients indicates parasite sequestration in the brain vessels, endothelial injury, blood brain barrier dysfunction and intracranial hypertension. The possible pathology of cerebral malaria is schematically accounted in Figure 1.8. IRBC sequestration was thought to have a great role in the development of cerebral malaria. Initially, PfEMP-1 ligand expressed on infected RBCs (IRBC) interacts with ICAM-1 present on endothelial cells to adhere firmly. Adhesion of IRBC to endothelial cell promotes further adhesion of IRBC to endothelium lining and agglutination of blood cells in the sequestered area leads to the blockage of the capillary. Blockage of the capillary develops the hypoxia and hypoglycaemic condition in the local area which leads to the brain cells damage. Lactic acidosis condition adds up cell damages further. Products released from IRBC causes inflammation and oxidative damages of the endothelium. Hemozoin or IRBC phagocytised macrophages secrete high level of matrix metalloprotease-9 (MMP-9) which can damage the basal lamina around the blood vessels and disrupt the blood brain barrier (Polimeni and Prato, 2014). MMPs further enhance the TNF-α and cytokines secretion by macrophages and lymphocytes. In cerebral malaria, several cytokines; TNF-α, interleukins (IL-12, IL-6 and IL-10) are playing a critical role in disease development. Role of TNF-α in ICAM-1 over-expression on endothelial surface was studied in many laboratories. Elevated interleukins and chemokine level thought to play critical role in cerebral malaria. (Idro et al., 2010) The seizure during cerebral malaria may be due to the

kynureinic metabolites such as quinolinic and kynureinic acid. Quinolinic acid is a NMDA receptor agonist and excitotoxin which may have a role in neurological symptoms and complication (Jain et al., 2013). There are many unidentified factors involved in the development of cerebral malaria thus the neurological damage is still inconclusive.

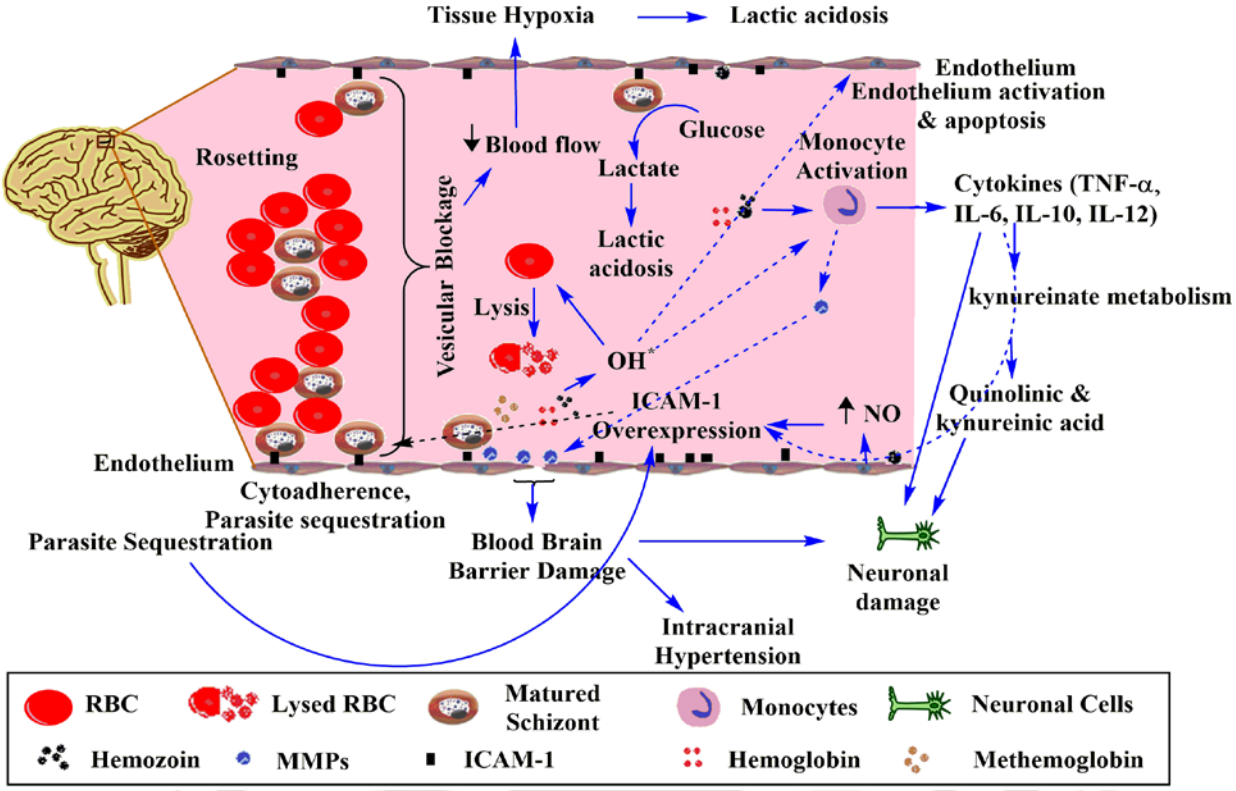


Figure 1.8: Summary of events responsible for pathology of cerebral malaria. Cerebral malaria results in untreated *P. falciparum* infected patients. Various events such as parasite sequestration, blood brain damages, neuronal damages, vesicular blockage can observe during cerebral malaria events. For further information see descriptions.

1.7.3 Splenomegaly: Abnormal enlargement of spleen is termed as splenomegaly and it is common during severe malaria (Zaki and Shanbag, 2011). Spleen has an important role in filtering the blood and removing aged RBCs, RBC derived products and bacteria (Mebius and Kraal, 2005). During malaria, uninfected RBCs and other blood cells undergo abnormal modification (including apoptotic marker expression) which reduces their life span. During malaria, immune complexes combine with activated complement C3b which can deposit CR1 (complement receptor 1) present on RBCs. Hemozoin also can enhance C3b binding on RBC CR1 receptor independently. This event can elicits splenic clearance of uninfected red blood cell and adds up work load on spleen leading to splenic infarction (Biryukov and Stoute, 2014). Red pulp macrophages recognize abnormal RBCs, products released from infected RBCs (Hb, MetHb, hemin and hemozoin) and they phagocytose them. The elevated level of cytokines, IgM levels during malaria also may have a role in splenomegaly but the links are not clear (Biryukov and Stoute, 2014; Buffet et al., 2011). The excessive turnover of RBCs (production and destruction) and toxic by-product released from

infected RBCs during malaria may lead to spleen dysfunction and splenomegaly. The schematic diagram to explain different biochemical processes leading to splenomegaly during malaria is given in Figure 1.9.

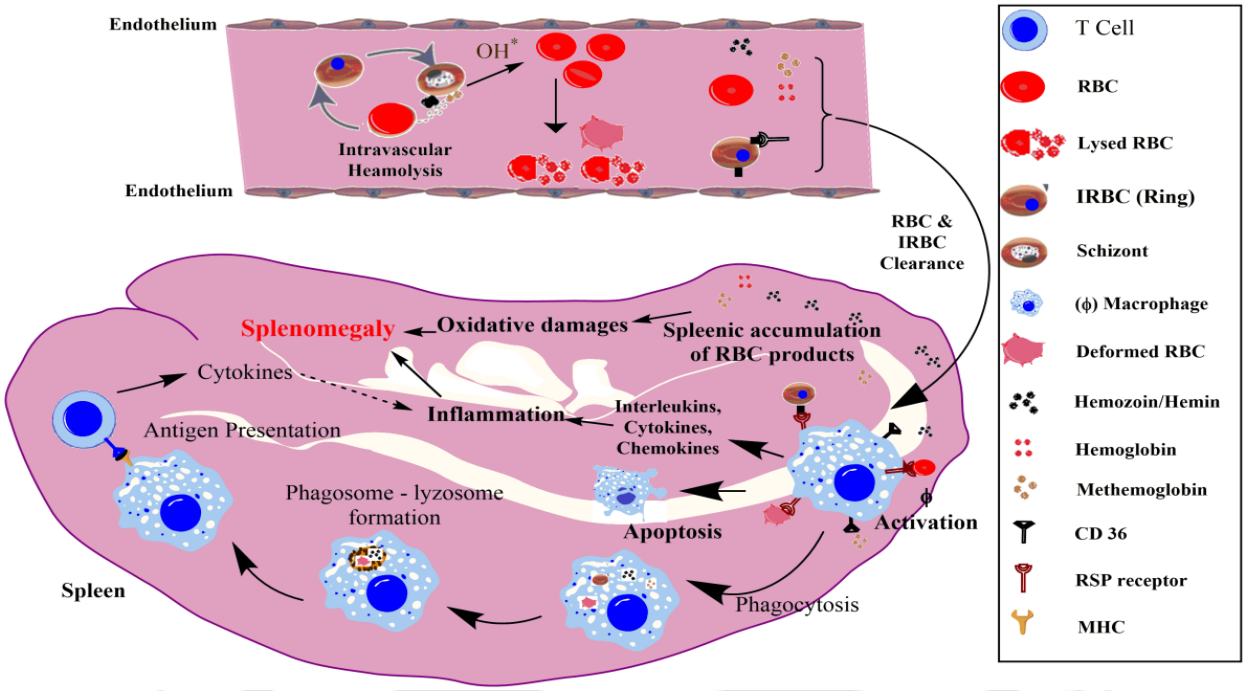


Figure 1.9: Pathophysiology of Splenomegaly. Enhanced hemolysis, RBC clearance, red pulp macrophage activation, inflammation and oxidative damages together lead to the splenomegaly in malaria patient.

1.7.4 Liver complication: About 2.5 to 62 % of malaria patients suffered by the liver problems and they developed the clinical jaundice. Liver dysfunction is not common in such cases but still it contributes into malaria mortality up-to 2 to 5 %. Parasite life cycle starts in the hepatocytes in human and during this stage, they modulate biochemical properties and increase stiffness of the infected hepatocytes (Eaton et al., 2012). Hepatocytic plasma membrane during sporozoite infection of the liver causes the secretion of the hepatocyte growth factor (HGF) and evidences indicate the HGF involvement in parasite infection into hepatocytes (Carrolo et al., 2003; Jin et al., 2004). Further, there is no remarkable observation on liver pathophysiology during pre-erythrocytic malaria. But hemolysis, congestion of hepatocytes, hepatocyte swelling, kupffer cell hyperplasia, deposition of hemozoin, steatosis, were observed in autopsy of dead malaria patients (Anand and Puri, 2005; Autino et al., 2012b; Kochar et al., 2003a; Kochar et al., 2003b). During erythrocytic cycle, infected red blood cells (IRBC) undergo lysis during parasite egress. Parasitic and IRBC derived products adhere on normal blood cells which also get lysed by the combined action of cytokines. The free Hb and hemin are being removed by liver after binding with haptoglobin and hemopexin respectively (Smith and McCulloh, 2015). Excess IRBC products oxidatively damage the cells present in microenvironment and lead them to apoptosis or lysis (Pamplona et al., 2009; Totino et al., 2010). RBC released products along with parasitic antigens amplify the immune

responses which lead into vascular dysfunction such as blockage, damage, IRBC sequestration, hemolytic product accumulation in liver. Accumulation of hemolytic products in liver, cause the severe damages to the hepatocyte through induction of apoptosis involving mitochondria dysfunction, DNA fragmentation and oxidation of cytoskeleton proteins (Guha et al., 2006). Hemozoin also increases the secretion of cytokines from the macrophages and lymphocytes. The elevated level of NF-κB and TNF-α in kupffer cell generates inflammatory response against hepatocytes during malaria. Hepcidin mediated iron delocalisation in hepatocytes also damage the hepatocytes (Spottiswoode et al., 2015). Other mechanisms underlying in liver damage during malaria is still not clear (Autino et al., 2012b; Viriyavejakul et al., 2014). The schematic diagram to explain different biochemical and molecular pathways leading to liver damage during malaria is given in Figure 1.10.

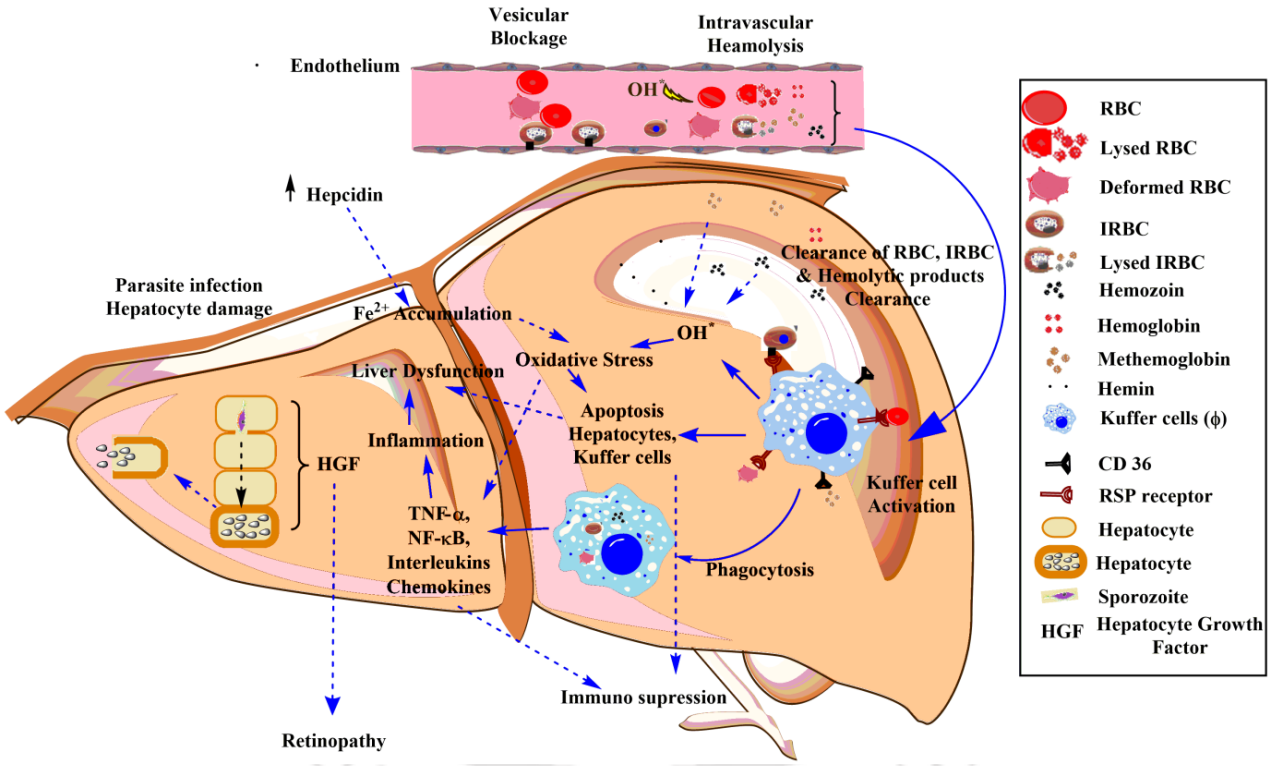


Figure 1.10: Pathophysiology of Liver dysfunction. Various degree of liver failure is common in malaria cases. Increased detoxification pathways, inflammation and oxidative stress mediated damages together involve in the liver damages during malaria.

1.7.5 Renal failure: Severe malaria develops acute kidney injury in around 40 % malaria patient. Proteinuria, hematuria, oedema, hypertension were observed during malaria, especially with *P. falciparum* infection. Kidneys meet high stress during malaria due to the high parasitemia. Parasites mediated lactic acidosis may overload the renal acid-base balance system. IRBC sequestration and RBC resetting in blood vessels of kidney cause hypoxia which affects the kidney cells activity. IRBC sequestration further allows the deposition of parasites and malaria related toxic products on endothelium lining of kidney. It results the immune reactions in the kidney and lead to the cell death at extreme condition. All together, they direct the kidney failure during

malaria (Ehrich and Eke, 2007). Intravascular hemolysis activates endothelial cells, alteration in hemodynamics through generation of oxidative stress, nitric oxide and TNF-α may lead to renal ischemia, acute tubular necrosis and acute interstitial nephritis (Nguansangiam et al., 2007). Nephritis during malaria showed involvement of INF-γ, IL-1α, IL-6 by altering pro-inflammatory and anti-inflammatory cytokines (Autino et al., 2012b; Ehrich and Eke, 2007; Sinniah et al., 1999).

1.7.6 Placental malaria: Placental malaria is another common complication observed in pregnant women infected with *P. falciparum* (Figure 1.11). Placental malaria leads to the miscarriage, poor birth weight, neurological sequale and birth defects in new born baby in severe cases. Placental malaria risk is higher in women with the age below 25, and onset of pregnancy such as primiparity (Magistrado et al., 2008). In placental malaria, phagocytic cells accumulate at intervillous space to phagocytose IRBC. TNF-α, IL-10 activate the macrophage to accumulate hemozoin, IRBC and induces leukocyte infiltration. It results in increase of thickness of trophoblast basement membrane to modulate the space between intervillous and perivillous. It results in reduction of nutrient and oxygen transport across placenta. The role of elevated C5a during pregnancy may contribute in placental malaria through dysregulated angiogenesis, release of chemokine and cytokine (Autino et al., 2012b). Placental hormone human chorionic gonadotropin seems to promote Malaria parasites

1.7.7 Malaria induced diabetes: Malaria mediated damages are not restricted to host organs but also modulating the host metabolism. Parasite infection mediated host metabolic modulations are possible in various conditions such as increased parasitic metabolism in host, altered the immune responses and hormonal dysregulation. Anaerobic metabolism of parasites is directly influencing on host blood lactic acid level. Parasitemia level also directly correlates with TNF-α, interleukins and other cytokines level. Hormonal dysregulation during malaria is unexplained yet but abnormal hormone level was observed during placental malaria (Megnekou et al., 2015). Elevated blood insulin level (hyperinsulinemia) during severe malaria was reported in various studies (Acquah et al., 2014; Eltahir et al., 2010). Recent study about type II diabetes relation with malaria indicated that the incidence of type II diabetes is increasing among malaria survivors in malaria endemic growth during early trimester of pregnancy (Rohrig et al., 1999). region (Acquah et al., 2014). In addition, type-II diabetes increases the risk of malaria infection (Danquah et al., 2010). Further human insulin effects in mosquito indicate that it suppresses the NF-κB level in mosquito to help the parasite survival in normal or starved mosquitoes (Pakpour et al., 2012b; Zhao et al., 2012). Interestingly, hyperinsulinemia often connected with hypoglycemia during malaria. During cerebral malaria, hyperinsulinemia in conjugation with hyperglycemia is causing more death instead of hypoglycemic condition alone (Giha et al., 2009; Tombe et al., 1993). These interesting observations need to explore the connection between interaction of insulin with parasites and its down-stream effects in disease development.

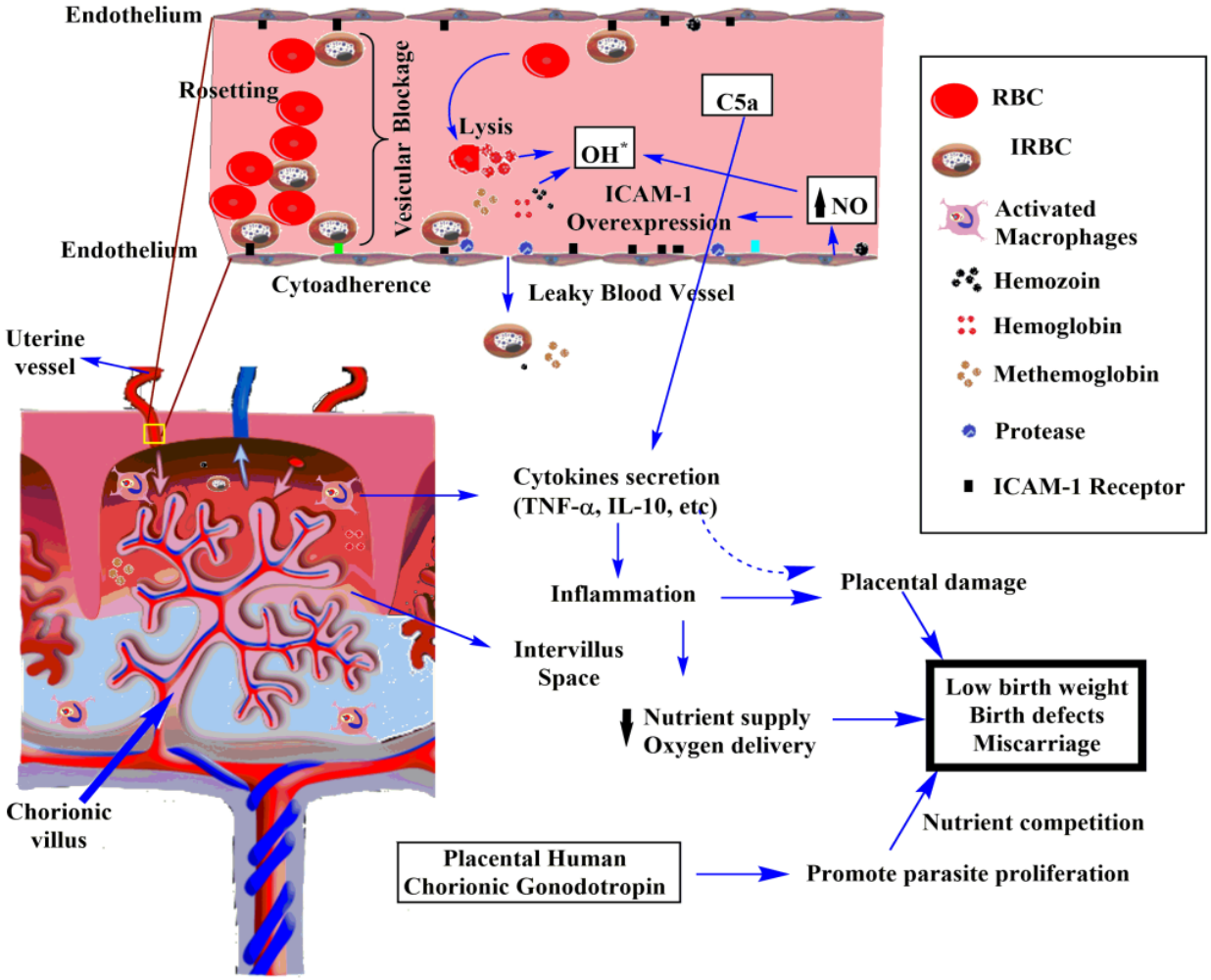


Figure 1.11: Pathophysiology of Placental malaria. Parasitic sequestration, macrophage mediated inflammatory reactions, accumulation toxin in intervillus space cummalative effects worsen the fetus condition in pregnant woman.

1.8 Factors involved in malaria associated complications

Malaria parasites infection in human causes multi-organ failure during severe malaria which associated with different physical, chemical and biological factors. In following sections, we discussed the effect of the various factors involvement in developing the severe malaria.

1.8.1 Environmental (physical) factors:

Temperature: Normal physiological human body temperature is around 98.4ºF. Infection, allergy and few metabolic diseases attributes the high temperature in which body temperature raise above the normal and such states called as fever. Characteristic malaria fever is cyclic in nature and reaches to the peak in every 3rd day (tertian fever) or 4th day (quarten fever) up to 106ºF depends on the *Plasmodium* species, i.e., *P. vivax* and *P. ovule*. *P. falciparum* does not show any distinct pattern of fever but continuously display high temperature. Malarial paroxysm fever can segregate into 3 phases as cold, high temperature and sweating phases (Figure 1.12). Initial cold phase retains ~15-60 min, and the body temperature fall below normal (upto 97.7ºF) with shivering which gives

intense cold felling to the patient. In second phase temperature level sharply increase ~106ºF and patient feel skin burning sense with skin dryness, headache, fatigue, nausea and myalgia. This stage lasts 2-6 hours. Finally in sweat phase, due to sweating, body temperature falls to normal temperature and this stage lasts for another 2-4 hours. After the third phase, patients' body temperature return to normal and the similar events results during next cycle. Temperature modulation is accompanied with TNF-α level (Karunaweera et al., 1992).

Fever is a major symptoms being observed during infections and it is considered as common host defence mechanism. Hyperthermia was in practice as a therapy for few infectious diseases such as syphilis, endocarditis and pneumococcal meningitis before antibiotic discovery (Roberts, 1979). As *P. falciparum* host defence, hyperthermia also cause adverse effects such as increased heart rate, basal metabolic rate, negative nitrogen balance, dehydration and albuminuria (Roberts, 1979). In vivo & in vitro experimental hyperthermic studies suggest that fever like condition, activates the macrophage and lead to secret the TNF-α level in the host (Lee et al., 2012), increase the rigidity of the infected RBCs (Marinkovic et al., 2009), increase the *P. falciparum* growth in RBC (Pavithra et al., 2004). Studies used temperature in between 38 to 40ºC were reported discrepant results but the studies used temperature above 40ºC were consistently reported the damages to the host cells.

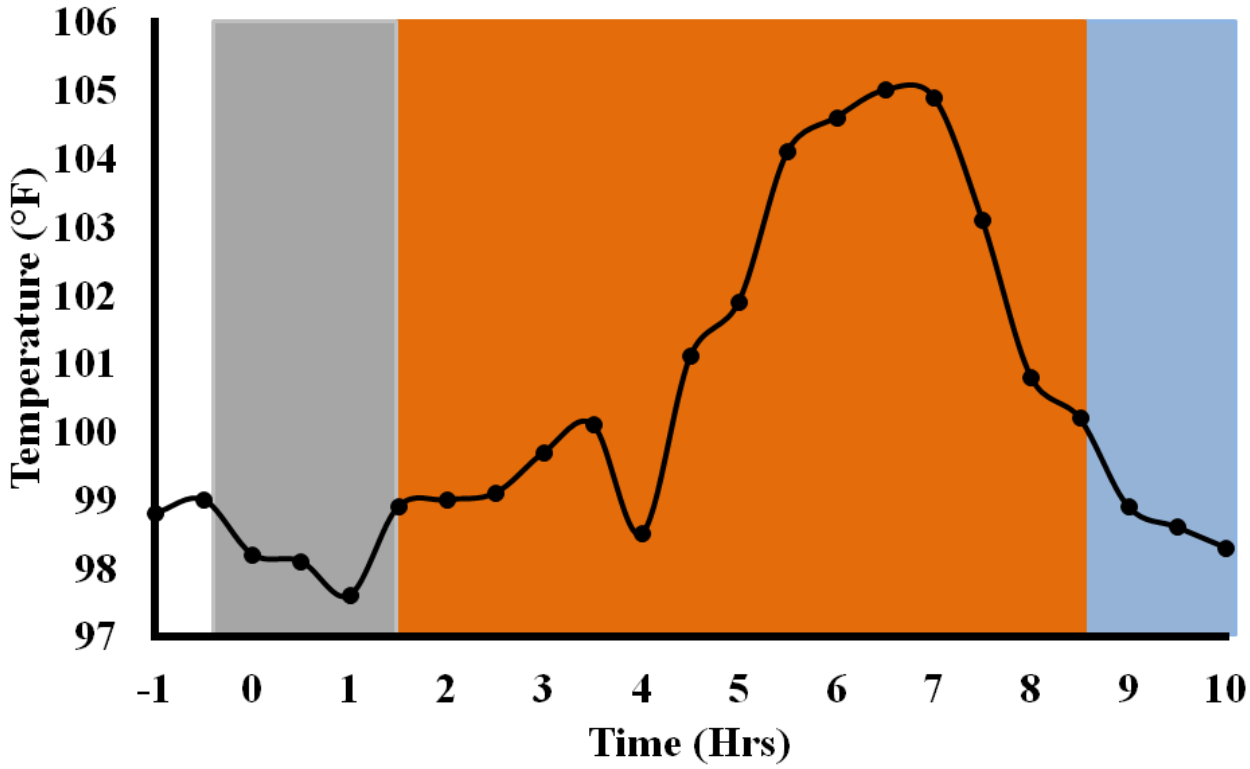


Figure 1.12: Classical paraoxymal fever pattern during malaria. Paroxymal fever symptoms start with the onset of chillness. This cold phase followed by high temperature phase where malaria patient feel extreme hot and in finally it ends with the sweat phase. Karunaweera et al., 1992, data was utilized to plot the curve.

pH: Changes in physiological pH (below 7.35) associated with various clinical complications such as respiratory difficulties and hypoxia which lead to the kidney failure, coma and death (Maciel and Park, 2009; Morimatsu et al., 2009). But, acidosis contribution in severe malaria pathophysiology is not investigated yet properly. During severe malaria, lactate level is >2 mmol/l and lactate level in patient is a predictor for death (Autino et al., 2012b). During malaria, capillary obstruction may lead the reduction of blood flow in capillaries which develops the hypoxia in tissues. It may cause the aerobic to anaerobic metabolic shift, which leads to the excess lactate production. But clinical studies could not able to find out the link between the microvasculature obstruction and oxygen delivery (Hanson et al., 2012). Excess glucose utilization by parasites also generates further rise of lactate in host (Mehta et al., 2005). Excessive lactate level impairs the pH-homeostatic systems in host (Koeppen, 2009). Apart from lactate, elevated level of various organic acids such as hydroxylphenyllactate, α-hydroxy butyric acid, β-hydroxy butyric acid in plasma and methylmalonate, ethylmalonate, α-ketoglutarate in urine was observed. Abnormally high hydroxyl-phenyllactate level was observed in patients who died by malaria and hydroxyl phenyllactate stronger prognostic factor for death than lactate. Higher level of lactate and other organic acids were observed in patient with severe malaria (Herdman et al., 2015).

Oxidative stress: Oxidative stress is one of the common factor for developing various diseases like diabetes, vascular complications, heart diseases, renal diseases and neurological diseases (Shah and Brownlee, 2016). Free hemin is very toxic in nature due to ability to develop the oxidative stress. Hemin contains Fe^{3+} which generate hydroxyl radical ($OH^{\bullet}$) with the contact to the hydrogen peroxide in aqueous environment through 'Fenton reaction' (Rodopulo, 1951). Parasitic metabolic activity also leads the development of the oxidative stress within the infected RBCs. Hydroxyl radicals oxidatively damage the cells present in their vicinity (Lum and Roebuck, 2001; Nuchsongsin et al., 2007a). Activation of nitric oxide synthase in endothelial cells and uninfected RBCs, lead the excessive synthesis of nitric oxide and further contribute into the oxidative stress in host. At the same time, oxidative stress also promotes the cell adhesion molecules such as ICAM1, E-selectin, etc on the endothelial cell surface which increase the chance of vascular blockage and cytoadherence (Lum and Roebuck, 2001; Piantadosi, 2008). Oxidative stress is suspected for the RBC changes. Red cell deformability and damage during malaria is multifactorial. Hemin-induced oxidative damage of the red cell membrane, alterations in the phospholipid bilayer and membrane linked cytoskeletal proteins (Nuchsongsin et al., 2007a). Thermally driven membrane fluctuations during febrile temperature can increase the cell rigidity (Islam et al., 2011). Nitric oxide (NO) also can affect the membrane rigidity and reduction in deformability of the red cells in during *falciparum* malaria.

1.8.2: Biological factors: Parasites and malaria associated toxic products, different host cells and their secretary products, and immunological factors are the different biological components which are involved in the development of severe malaria.

(A) Host cells: Human body made up of different kind of cells. They are involving in different functions such as body defence, sensors, digestion, respiration, etc. During malaria role of immune cells, RBCs and endothelial cells are studied extensively in context to malaria associated pathology. Here we have discussed few important cells which are involved in malaria pathology.

Red blood cells: Red blood cells are anucleated, circular biconcave shaped and involved in gaseous exchange from lung to different parts of body. Life cycle of malaria parasite partly spares in red blood cells during asexual stage and known as erythrocytic cycle (Figure 1.13). The infection of the red cells by malaria parasites, results in progressive modulation in structural, biochemical, and mechanical properties of RBC. Post infection, formation of knobs like structure on the surface of the IRBC plasma membrane helps parasitic sequestration through binding of IRBC to endothelial cells. Knob mediated IRBC endothelium cytoadherence and IRBC-URBC aggregation cause the vesicular blockage. IRBC derived products cause oxidative insults to the other cells, activates the macrophages to induce the cytokine secretions, suppression of hematopoiesis, release of microparticles, etc (Deshmukh and Trivedi, 2014; Nuchsongsin et al., 2007a). Vesicular blockage causes the severe complications such as cerebral malaria, splenomegaly, hepatic damage, nephritic syndrome, etc., during malaria. Vesicular blockage affects the blood flow rate and it lead to the oxygen depletion in tissues. Prolonged oxygen depletion severely causes tissue damage leads to the organ failure. Red blood cell derived microparticles (RMS) are the major microparticles produced during malaria (Nantakomol et al., 2011). RMS is up regulating the macrophage CD40 expression and increased TNF-α secretion (Couper et al., 2010). RMS surfaces are providing signal for C-reactive proteins to promote the coagulation in the blood vessels (Koshiar et al., 2014). RMS exported free heme to endothelial cells turn on vaso-coagulation during sickle cell hemolysis (Camus et al., 2015).

Platelets: Platelets are formed from megakaryocytic cytoplasm during hematopoiesis. Platelets are involved in blood coagulation. Platelets secrete platelet derived growth factors during blood coagulation and it is involved in wound healing. Accumulation of platelets was observed from Malawian paediatric cerebral malaria patient brain samples (Grau et al., 2003). Platelets have CD36 (GPIV) to recognize and bind IRBC as well as serve as a mediator for IRBC and endothelial cyto-adherence (Chapman et al., 2012; Mayor et al., 2011; Wassmer et al., 2004). In a mouse experimental malaria model, IRBC activates platelets and to secrete chemokine, platelet factor 4, which results in immune activation including T cell population decrement in brain (Srivastava et al., 2008). Platelets also involved in antigenic presentation process independently and activates the

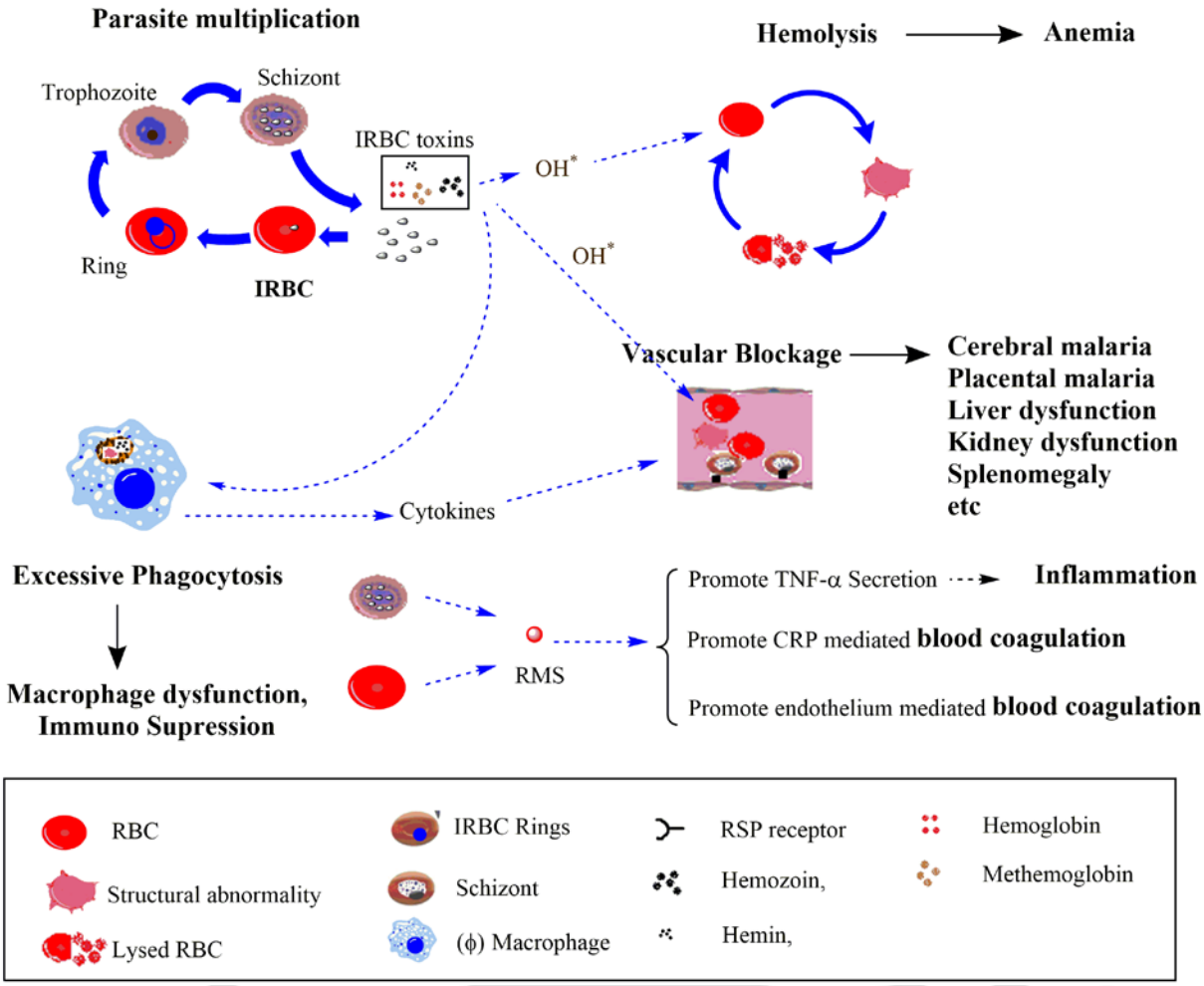


Figure 1.13: RBC role in severe malaria. RBCs involve in malaria pathology through hemolysis and RBC aggregation. RBC derived microparticles (RMS) cause inflammation, blood coagulation during malaria. Infected RBC released toxins entails macrophage as well as endothelium cell lines to cause the severe organ damages during malaria.

T cells which further may lead to the vascular diseases (Chapman et al., 2012). Platelet derived microparticles involve in malaria pathology in various ways. Platelet derived microparticles can binds with IRBC surfaces (depend on the *P. falciparum* EMP1 variant expression) which may provide the signal to immune cells for phagocytosis process, In addition it also support IRBC-RBC adhesion in the blood vessels. Platelet derived microparticles also can bind with brain endothelial cells receptor such as CD-31 and CD-36 and get internalize. It increases the endothelial cell and IRBC cytoadhesion which lead to the cells accumulation in brain vessels and results in cerebral malaria (Faille et al., 2009). Injection of platelet derived microparticles rich plasma from cerebral malaria mice into malaria developed mice resulted the microparticles lining on the brain endothelium surfaces. In healthy mice platelet derived microparticles were cleared quickly from blood (El-Assaad et al., 2014). Other roles of platelet derived microparticles are under investigation.

Neutrophils: Neutrophil population is around 40-75 % among white blood cells. The major function of the neutrophil is to provide the non-specific (innate) protection against invading

pathogen. During malaria, neutropenia is observed and the blood neutrophil count decrease abnormally. Hemozoin mediated bone marrow suppression may be responsible for the neutropenia. Neutrophils can recognize the *Plasmodium* sp. through TLR and they secrete the cytokines and chemokines to the plasma. Cytokines intensify the innate immune system against the pathogen (Leoratti et al., 2012). But in malaria condition neutrophil show less activity in cytokine secretion and chemotaxis process. Overall it results in impaired defence against pathogen and offers the secondary opportunistic infections (Diou et al., 2010; Lokken et al., 2014; Thompson et al., 2012; Were et al., 2011).

Antigen presenting cells (monocytes, macrophages, dendritic cells, etc): Macrophages, monocytes, dendritic and B lymphocyte are the professional antigen presenting cells. Monocytes are the group of white blood cells and involved in non-specific immunity. Macrophages and dendritic cells are the differentiated form of monocytes and they are present in tissues. At the moment of *Parasite* antigenic contact, specifically parasite DNA-protein complex or DNA-hemozoin complex, with TLR-9 present on dendritic cells activates the cytokine secretions (Perkins et al., 2011; Wu et al., 2010). Antigenic contact activates the maturation process in APCs and lead to the secretion of cytokines, TNF-α, interleukin-6 (IL6), interleukin-10 (IL-10), interleukin-12 (IL-12), interleukin-1β (IL-1β), etc (Al-Fadhli et al., 2014; Lyke et al., 2004; Takeuchi and Akira, 2010). Cytokines involve in different clinical complications such as high temperature, oxidative stress, apoptosis, inflammation, hormonal imbalance, glomerulonephritis, hypergammaglobulinemia, severe anemia, immune-suppression, hepatocyte damage, placental damage, cerebral malaria, etc (Erta et al., 2012; Grau et al., 1990; Huang et al., 2014). Anti-inflammatory role of IL-10 may help the host during cerebral malaria or hepatic damage. At the same time down regulation of TNF-α, IL-12, IFN-γ by IL-10 may deteriorate the host immunity against the parasite (Niikura et al., 2011). IL-10 and IL-12 are involved in Th1 maturation to secrete interferon-γ (INF-γ). Excessive secretion of INF-γ may cause the tissue damage through auto-inflammatory responses. Secretion of TNF-α positively correlated with severe malaria in patients. Elevated TNF-α level leads to the fever, apoptotic cell death, cachexia and inflammation. In presence of TNF-α, stimulation of macrophages lead to excessive production of free radicals thus damages the local environment homeostasis (Perkins et al., 2011). Altered RBCs are phagocytised by macrophages which lead to the severe anemia. Phagocytosis of macrophages towards parasitic antigens, infected RBC products and IRBC leads the accumulation of toxins within the cytoplasm which leads to the macrophage inactivation, immune-suppression, splenomegaly, etc. Toxic nature of IRBC by products develops the oxidative stress within the macrophage and cause the oxidative burst (Deshmukh and Trivedi, 2014). IL-10 mediated hepcidin secretion lead to the iron delocalization which affects the erythropoiesis and damages the

hepatocytes (Huang et al., 2014). Complement C5a also enhance the macrophage mediated cytokine secretion (Ansar et al., 2006; Autino et al., 2012b). The role of APC in malaria pathophysiology is shown in the Figure 1.14.

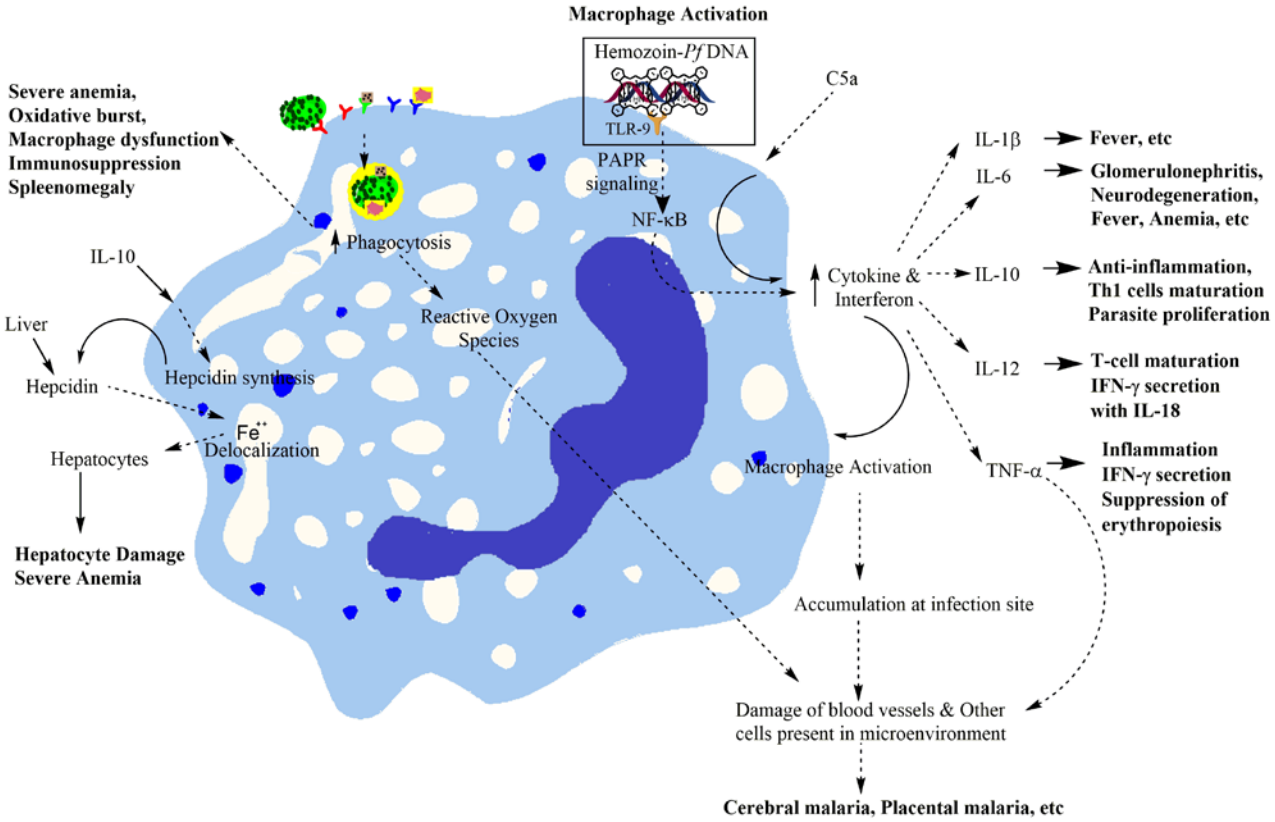


Figure 1.14: Monocytes and macrophage during malaria. During malaria, macrophage activation can cause the severe damage to brain and placenta through inflammation. Further it also primes severe anemia, immunosuppression, splenomegaly, etc. For further information see description.

Lymphocytes: Lymphocyte is the second major population (~ 30 %) among white blood cells. T-cells, B-cells and natural killer (NK) cells are the variants of the lymphocytes. T-cells are responsible for cell mediated immunity, B-cells and NK cells are responsible for antibody mediated cellular immunity and innate immunity respectively. Immune activation during malaria initiates with the contact of antigen or infected RBC to the dendritic cells or antigen presenting cells. Activation of dendritic cells further triggers the differentiation of T-cells (CD 8^+, CD 4^+) as well as B cells formation. CD 8^+ T-cells further proliferate into NK cells but excessive activity of T cell lead to the tissue damages in different organs through INF-γ. Activation of CD 4^+ lead to the maturation of Th1 and Th2 cell. The secretory products of Th cells are IFN-γ, IL-4 and IL-10. IL-4 may involve in the tissue inflammation along with INF-γ. The protective response of T-cells with INF-γ and TNF-α activates hepatocyte (Schmidt et al., 2011), modulate the macrophage activity in brain blood vessels to contribute into the cerebral malaria (Pai et al., 2014). IL-10 regulate the IL-12, which is known to enhance the TNF-α secretion to start the inflammation reaction during malaria.

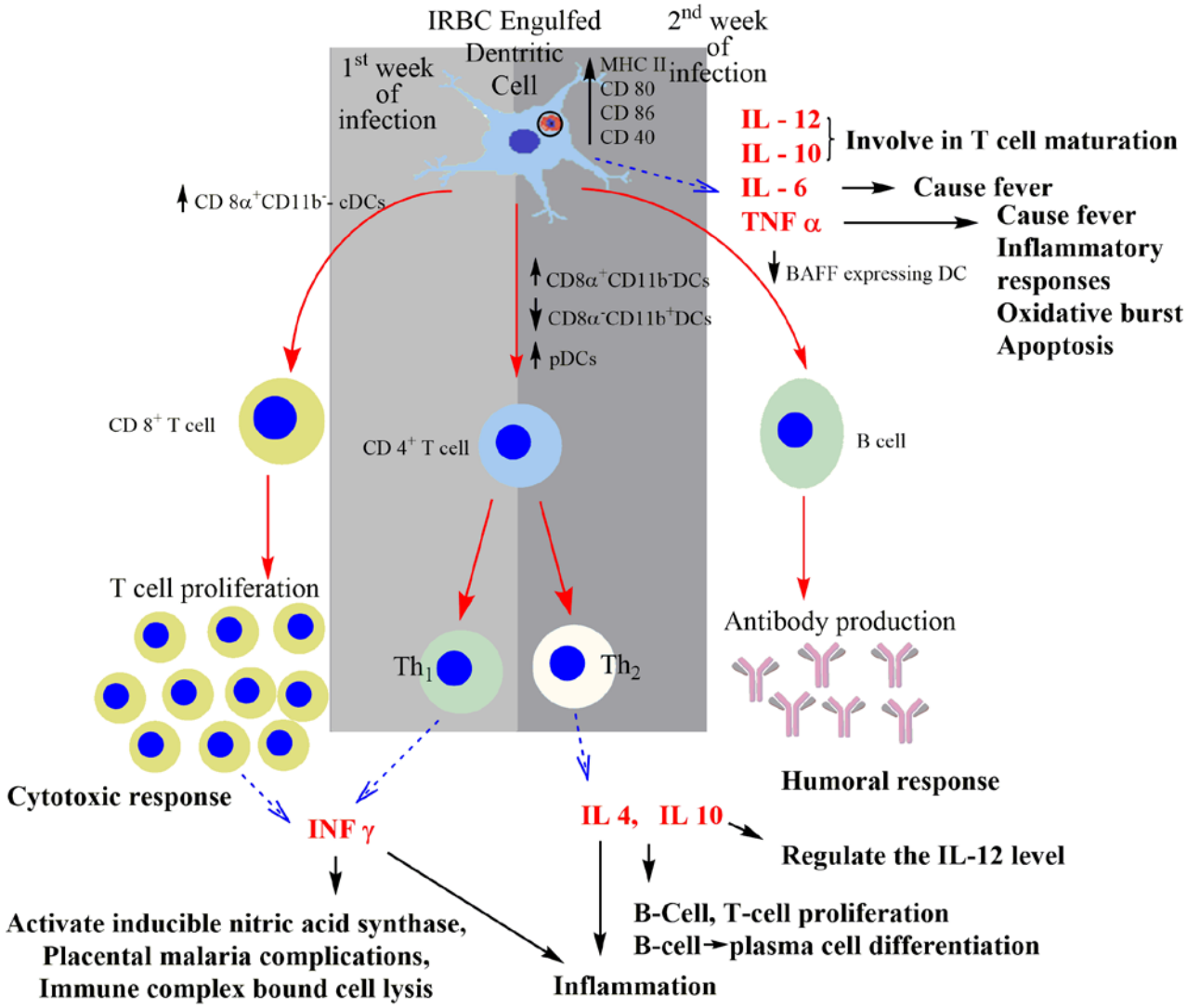


Figure 1.15: T cell activation and the consequences during malaria.

Endothelial cells: Endothelial cells are present as a layer to form blood vessels. The endothelium lining serves the selective entry of essential molecules such as nutrients, hormones, etc to the brain site. Endothelial cells are also involved in angiogenesis and anticoagulation process. Damage in endothelium usually causes leaky blood vessels which results tissue swelling, activation of thrombosis, and chronic inflammation. During malaria, Endothelial cell is activated by IRBC lysate products, immune cells, IRBCs, cytokines, etc (Howland et al., 2015; Razakandrainibe et al., 2012). Earlier, we have discussed how ROS and NO are generated by immune cells while processing antigen. ROS, NO and cytokines act on endothelial cells individually and activate the endothelial cells to express higher number of receptors such as ICAM-1, VCAM-1, CSA, CD36 etc., which provides the binding sites for IRBCs, immune cells (macrophages, T-cells, etc), platelets and their derived particles, etc on the endothelium surface (Lum and Roebuck, 2001; Piantadosi, 2008). Simultaneously, oxidative stress also dysfunction the endothelial cells through various pathways. Parasite derived GPI binding on endothelial receptors activates the MyD88 –

NFκB pathway to secrete the cytokines and chemokines. But the GPI binding also stimulate tyrosine kinase – PKC pathway leads to the apoptosis and cellular dysfunction. Together all these cellular events damage the endothelium and disturb the barrier function of endothelium. IRBC to endothelial cytoadherence leads the cytoskeleton changes and disturb the endothelial-endothelial cell junctions through Rho dependent or calcium dependent pathway activation of focal adhesion protein (FAP) results the leaky cell junctions (Lum and Roebuck, 2001). Moreover, expression of adhesion molecules (ICAM1, VCAM1, CSA, etc) also increase the parasite sequestration, which leads to the vascular blockage, reduced blood flow, development of hypoxia, disturbance in nutrient supply. Phagocytic cells, present in the blood, are the sources for different MMPs such as MMP9, MMP3 (Prato and Giribaldi, 2011). Elevated level of MMP-9 was observed during cerebral malaria which may involve in the disruption of endothelial matrix (Prato and Giribaldi, 2011). Involvement of MMPs, cytokines and IRBC together cause the damage of blood brain barrier and results the various complications such as tissue inflammation, edema, cerebral malaria, multi organ failure.

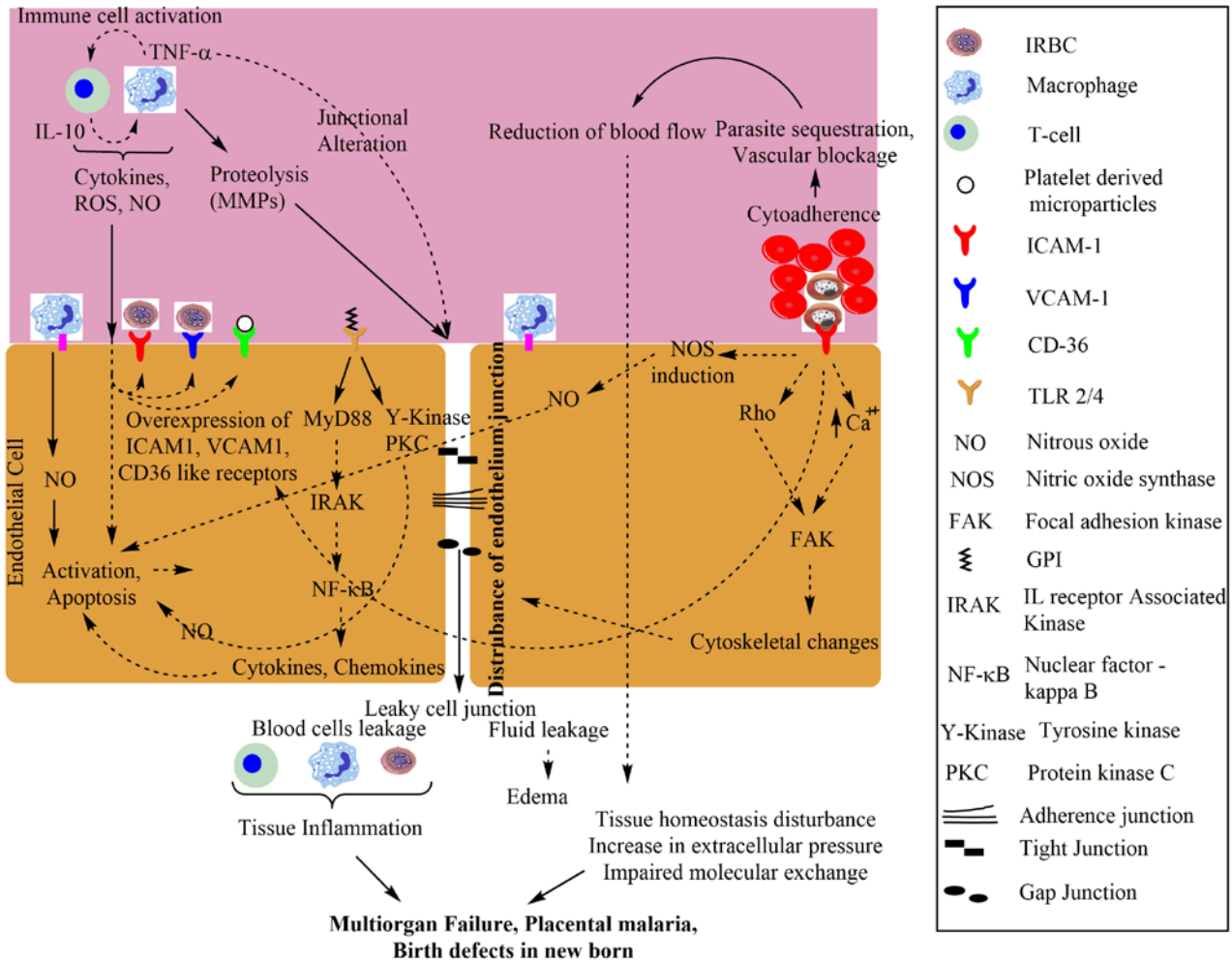


Figure 1.16: Endothelium dysfunction during severe malaria. Cytoadhesion of endothelial cell with macrophage or IRBC or platelete activate the endothelium which lead to the cytokine secretion, damage the blood brain barrier, inflammation etc. It leads to the cerebral malaria, placental malaria, etc. For further information see description.

(B) Host derived factors: The factors are released from host cells, as response against the parasitic infection and metabolic activities, are discussed briefly in host pathology contribution.

Cytokines: Cytokines are the small molecules (5-20 kDa) secreted from immune cells, it includes chemokines, lymphokines, monokines and interleukins. They play an important role in immune responses such as activation, differentiation, proliferation of immune cells, inflammation and erythropoiesis . Malaria infection causes development of inflammatory response due to secretion of several cytokines which act as either pro-infammatory (TNF-α, IL-1, IL-6, IL-18, RANTES, IFN-γ) or anti-inflammatory mediators (IL-10, IL-12) (Dinarello, 2000; Opal and DePalo, 2000; Tayal and Kalra, 2008).

Tumor necrosis factor alpha (TNF-α) is a cytokine and it is secreted by activated macrophages, neutrophils, eosinophils, CD4+ lymphocytes, NK cells in response to antigen. During malaria, monocyte in contact to antigen, induces the immune reaction by secreting the TNF-α. Hemozoin phagocytosis by monocytes and macrophages lead to the higher secretion of TNF-α (Santos-Martinez et al., 2008). TNF-α effects different cell types such as antigen presenting cells, T-cells, endothelial cells, erythropoietic cells, and causes NF-κB activation, stress linked MAPK activation and caspase-8 activation. Together, these effects result in tissue inflammation during severe cerebral malaria, apoptosis of cells in different organs especially liver and spleen, dysregulation of erythropoiesis in bone marrow. In addition, it causes elevated NO synthesis in endothelial cells, MMP secretion by monocytes and macrophages, and lymphocyte activation (Perkins et al., 2011).

Interleukins are another group of cytokines majorly secreted by T-lymphocytes, macrophages and monocytes. There are several studies demonstrated the crucial role of interleukin in host immune response and support host immunity to clear the infection. During malaria, IL-1β, IL-6, IL-10, IL-12 secrete along with other interleukins (Lyke et al., 2004). IL-1β is positively associated with disease severity and responsible for fever during parasitic infections (Chua et al., 2013). In malaria infected mice, IL-1 causes potent pyrogenic and inflammatory responses (Pascual et al., 2005). IL-6 role is protection from the infection (Chua et al., 2013). Also IL-6 elevates the body temperature to precipitate fever. Moreover through B-cells, IL-6 also causes the hypergammagluobulinemia, glomerulonephritis (immune complex disease) (Grau et al., 1990).

Elevation of IL-6 also affects the nerve cell differentiation, hypothalamus hormonal regulation, etc., which cause neurodegeneration, suppression lipid synthesis, and hormonal imbalance (Erta et al., 2012). IL-10 involves the anemia development through increased hepcidin secretion from macrophages (Huang et al., 2014). In response to infection, IL-12 stimulates the production of TNF-α, IFN-γ from lymphocytes (T and B-cells) and augments the type-I immune

response. IL-10 is an another important interleukin and it is essential for suppression of IL-12 production (Mosser and Karp, 1999). Reduction of IL-12 level during malaria can cause the dysregulation of erythropoiesis leading to the severe anemia. IL-4 secreted by Th_2 cell involve in B-cell differentiation to plasma cell and proliferation of lymphocytes (B and T-cells) (Perkins et al., 2011). IL-10 and IL-12 are involved in Th1 maturation to secrete interferon-γ (INF-γ). Excessive secretion of INF-γ may cause the tissue damage through auto-inflammatory responses. (Perkins et al., 2011). During malaria, IFN-γ secreted by NK cells and $CD4^+$ T-cells to destroy the IRBCs. IFN-γ secretion increases the cellular H_2O_2 production and lead to the death of the macrophages to contribute into the state of immuno-suppression. Moreover, enhancement of immune response against sequestered IRBCs in brain lead to the severe outcomes in cerebral malaria (King and Lamb, 2015).

Hepcidin: Hepcidin is a small iron regulating hormone, secret by kidney and liver, in response to low blood oxygen level. Elevated hepcidin levels were observed during malaria in response to the infection. During malaria, hepcidin blocks the ferroprotein to inhibit iron absorption in intestine. Moreover, hepcidin also delocalize the iron from macrophages to liver. The overall effect damages liver, develop the anemia by suppressed erythropoiesis but at the same time, it protects the host from super-infection (Spottiswoode et al., 2015).

Human chorionic gonadotropins (HCG): HCG is a placental hormone which helps the embryo development by interaction with leutinizing hormone receptor and up-regulating the progesterone by corpus leuteum. Under in-vitro incubation studies, HCG supplementation leads to the acceleration of malaria parasite growth (Pong et al., 2009; Rohrig et al., 1999).

Hepatocyte growth factor (HGF): Sporozoite infection during malaria, causes membrane rifts on several hepatocytes before forming a parasitoporous vacuole, lead to the secretion of hepatocyte growth factor. It plays an essential role in sporozoite infection by rearranging the cytoskeleton protein actin (Carrolo et al., 2003). In severe malaria, retinopathy is also observed with visual defect or loss of vision (Barrera et al., 2015). HGF exposure in mice causes retinal displacement and probably could explain retinopathy developed during malaria (Jin et al., 2004).

Insulin: Insulin controls various physiological processes in our body such as controlling glucose metabolism, cellular growth by increasing DNA synthesis and protein synthesis. Hyperinsulinemia was observed during malaria but the reason is not known yet. Elevated insulin level during malaria may possible due to TNF-α mediated effects on hepatocytes, myocytes and insulin receptor present on adipocyte. In addition, quinine induced hypoglycaemia and hyperinsulinemia conditions are being reported in malaria patients (Taylor and White, 2004). At the same time, hyperglycaemia and hyperinsulinemia was also observed in malaria patients (Binh et al., 1997; Eltahir et al., 2010). Interestingly, type II diabetic patients and malaria patients share several common clinical features;

hyper-insulinemia, renal failure, vascular diseases, retinal problem and immuno-suppression (Zaki and Shanbag, 2011). It hints that the insulin might be a major player in multi-organ failure during malaria. In recent studies, insulin found to suppresses the IL-10 production from T-cell through AKT/mTOR signalling pathway in regulatory T-cells which elevate secretion of IFN-γ (Han et al., 2014). Low level of IL-10 secretion may allow the uncontrolled secretion of TNF-α from macrophages. High IFN-γ/TNF-α level causes inflammatory reaction to exhaust the immune cells and result into the immuno-suppression (Chua et al., 2013). During Blood meal, mosquito ingest insulin from host and that activates the insulin/insulin like growth factor-1 signalling pathway in mosquito to suppress the NF-κB dependent immune response (Pakpour et al., 2012a; Surachetpong et al., 2011). Insulin supplementation to mosquito helps in parasite development and growth (Beier et al., 1994). But the in-vitro assessment of insulin effect on parasite growth in vertebrate host (liver or RBC stages) is not conclusive but hyperglycemic condition allows the parasites to proliferate faster (Humeida et al., 2011).

Matrix metalloproteases (MMP): Matrix metalloproteases are the metal (Zn^{++}) dependant proteases and cleaves the matrix proteins to help for growth and tissue remodelling. During malaria, hemozoin loaded macrophages and monocytes show high secretion of MMP-9 and MMP-1 secretion (Geurts et al., 2012; Khadjavi et al., 2014; Polimeni and Prato, 2014; Polimeni et al., 2013). MMP-9 cleaves, occludin, zonula occludens-1, claudin-1 and claudin-5 to disturb endothelial tight junction, and lead to the blood brain barrier damage. MMP also involve in the homeostasis regulation through tissue plasminogen activator and μPA. It may have a connection in thrombus formation in brain microcirculation (Prato and Giribaldi, 2011; Santos-Martinez et al., 2008).

(C) Infected RBC derived factors: Previously, we have discussed the host cells and their secretions which involved in the malaria pathophysiology. Here, we are discussing the role of parasite derived factors in severe malaria development.

Heme/Hemin: Heme is the prosthetic group of different proteins which involve in electron transport cycle, Gaseous exchange (O_2/CO_2), xenobiotic metabolism etc., In erythrocytic cycle, parasites degrades up-to 80% hemoglobin for their growth and nutrient requirement. It leads to release of heme and it start to accumulate inside the parasite food vacuole. Free heme moiety spontaneously oxidizes to hemin in aqueous environment. Hemin generates the hydroxyl radical through 'Fenton reaction' and induce oxidative stress in the host (Nuchsongsin et al., 2007b). The merozoite egress from IRBC also releases free heme/hemin into the blood. In cellular free environment hemin effectively generates reactive oxygen species ($OH^{\cdot}$). Free radicals damages RBCs, WBCs, platelets, endothelium cells in various pathways and cause the cellular death. Hemin also non-specifically binds with proteins and lipids which cause oxidation of proteins and lipids.

Hemin promotes cyto-adhesion of RBCs to endothelium and cause vesicular blockage. Hemin mediated RBC damages lead to the severe anemia. Hemin stimulation of macrophages cause inflammation and also leads to macrophage apoptosis. Cytotoxic effect of hemin on endothelium leads to endothelium dysfunction and develops the vascular complication, cerebral malaria, splenomegaly, etc (Deshmukh and Trivedi, 2014; Roumenina et al., 2016).

Hemozoin: Chemically, hemozoin is a dimer of hemin with different parasitic components such as lipids and peptides. During malaria, parasites polymerize the toxic hemin to less toxic hemozoin in order to avoid the oxidative stress. But, it also significantly generates hydroxyl radical and develops the oxidative stress in host. Oxidative stress leads to the various vascular complications such as endothelium dysfunction, vesicular occlusion, etc. Hemozoin with parasite DNA stimulates the host cell apoptosis. Hemozoin and hemin non-specifically bind cellular plasma membrane to cause the cellular damage and adhesion. Along with platelets, hemozoin also involved in cyto-adherence of IRBC to endothelial cells (Grau et al., 2003). Hemozoin also induce matrix metallo-protease secretion from the macrophage thus lead to the leaky endothelium (Masocha and Kristensson, 2012). Phagocytosis of hemozoin by neutrophils, monocytes, and macrophages cause alignment in host immunity which results in immuno-suppression. (Perkins et al., 2011). Excessive hemozoin uptake by macrophage leads to reduced secretion of TNF-α and nitric oxide (NO) by macrophages (Taramelli et al., 1995). Parasite DNA and hemozoin together induce TNF-α secretion from monocytes. TNF-α secretion attribute the immune activation and inflammatory responses. Hemozoin deposition in liver and spleen may cause the dysfunction of liver and spleen. Hemozoin also suppress the erythropoiesis in bone marrow.

Hemoglobin: Hemoglobin is the major protein present in the RBC to transport the gases. It is a tetramer, consists of two α and two β chains with heme as prosthetic group. Hemoglobin synthesis occurs in erythroblasts and degradation in reticulo-endothelial system (Chiabrando et al., 2014). During malaria, while spanning their life in RBCs, parasites degrade the hemoglobin (upto 70 – 80 %) and utilize the free amino acids for their growth(Rosenthal and Meshnick, 1996). On the other hand, parasite metabolism causes the development of oxidative stress inside the RBC which oxidize the hemoglobin-Fe^{2+} into methemoglobin-Fe^{3+} (Percario et al., 2012). Egress of parasites from the RBC releases the both hemoglobin and MetHb to the plasma. Extracellular hemoglobin is again oxidizing into MetHb spontaneously. Further oxidation of MetHb into ferryl hemoglobin (Fe^{4+}) generates the hydroxyl radicals which damages the normal cells. Hydroxy radicals generating activity of hemoglobin is known as pseudoperoxidase activity and Fe is playing a key role in this reaction (Schaer et al., 2013). Cyclo-oxygenase activity of Hb accelerates the prostaglandin synthetic pathway by oxidizing the plasma membrane of long chain unsaturated fatty acids. It spontaneously activates the inflammation in local environment (Zilletti et al., 1994).

Hemoglobin also plays an important role in nitric oxide homeostasis (Grubina et al., 2007). The growing evidences suggest that the accumulation of extracellular hemoglobin is a novel mechanism for various vascular complications during vascular hemolysis.

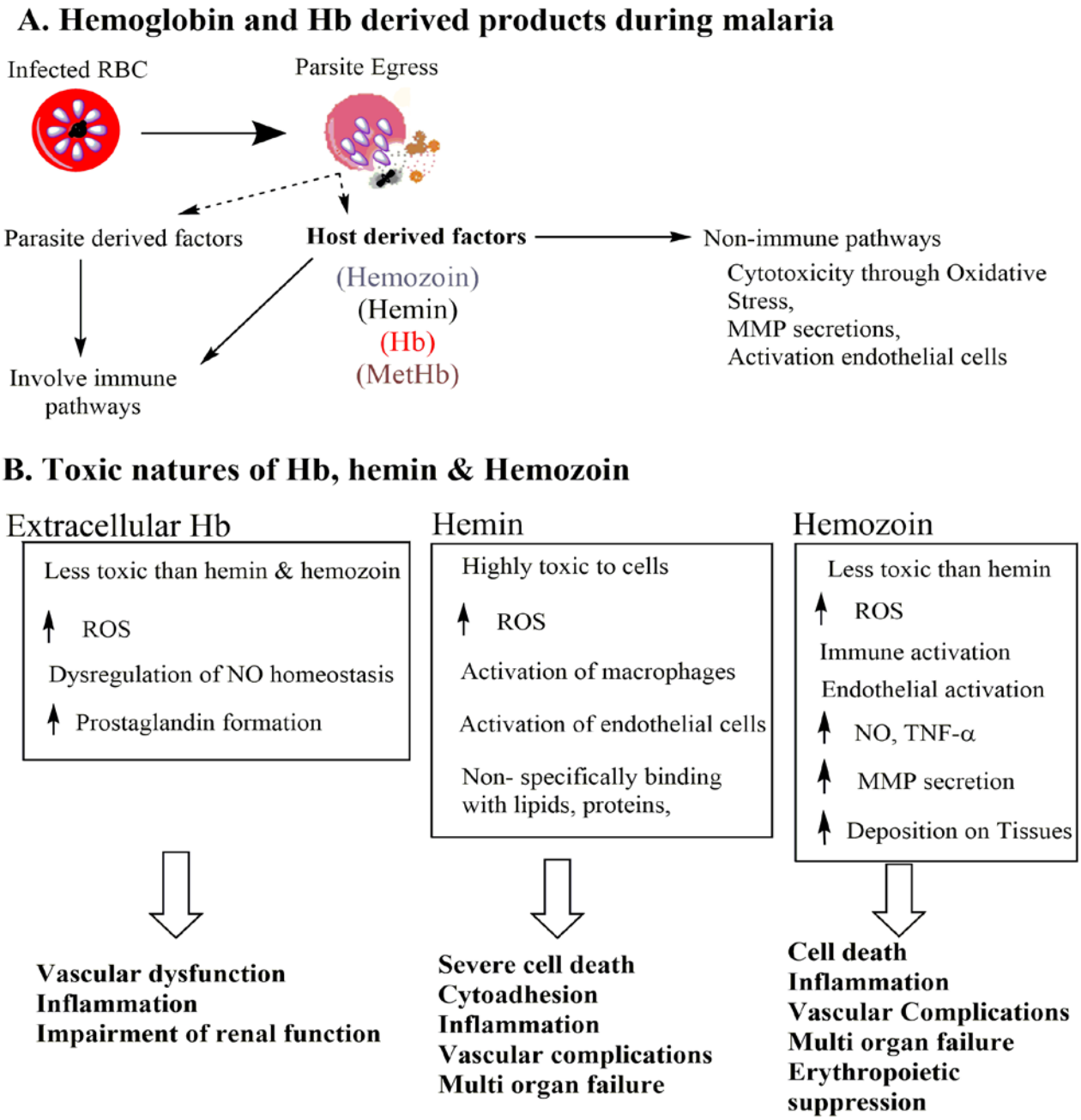


Figure 1.17 Toxic role of hemoglobin and and its' derivatives. Hemoglobin and hemoglobin derived products such as hemin, hemozoin, iron and methemoglobin can cause the cell death through various mechanisms during malaria. Here the toxic role of extracellular Hb, hemin and hemozoin were listed.

Methemoglobin: Oxidized form of Hb is known as MetHb. Increased level of MetHb in blood is known as methemoglobinemia. Normally in humans, ~ 1 % of Hb (0.15 g/dl) present as MetHb. MetHb related clinical complications can diagnose when the level is > 6 %. Anaesthetics like benzocain, environmental toxins such as nitrate salt containing water, heavy metals ingestion and

drugs are often developed the methemoglobinemia condition. In various diseases such as malaria, elevated level of MetHb (1 to 3.5 mg/ml = ~ 6 to 23 %) is often neglected for therapy. Accumulation of MetHb in RBC leads to the disturbance of gaseous transport thus cause the severe hypoxia in tissues. MetHb has potential to increase the IL-6 and IL-8 level in endothelial cells. Similarly, it can increase the receptors such as E-selectin on endothelial cells (Liu and Spolarics, 2003). MetHb ability to cause the oxidation of LDL may develop the artherosclerosis risk. But other effects of MetHb in humans are not studied well. But based on understanding different toxic effects of MetHb such as oxidative stress development, hemolysis, structural alteration, inflammation, vascular complications including endothelium activation can be predict (Umbreit, 2007).

Parasite glycosylphosphatidylinositols (GPI): It is one of the malarial toxin, released into the blood during parasite egress. During parasite egress, released GPI lead the TNF-α secretion from macrophage. It also exerts pyrexia and inflammatory responses along with hemozoin in malaria patient (C. S. Boutlis et al., 2011). GPI increase the synthesis of triglycerides in adipocytes. GPI elevates the NO level in plasma by stimulating the macrophages and endothelial cells. Stimulation of macrophages and lymphocytes also cause the inflammatory responses and immune suppression. Enodothelial stimulation lead to the over-expression of VCAM, ICAM-1 molecules on the endothelium surface and offers the cyto-adherence opportunity (Deshmukh and Trivedi, 2014).

(D) Anti-malarial drugs: In section 1.6.2, we have discussed the anti-malarial drugs and their mechanism of action. While working against the parasite, these drugs also cause the adverse side effects on patients and prolong their recovery especially in children and pregnant women.

Quinine: Quinine causes adverse effects known as cinchonism, including primarily tinnitus, nausea, headache, vertigo and blurred vision. In few cases vomiting, abdominal pain, diarrhoea, ototoxicity, hypoglycaemia and thrombocytopenia is also reported. Quinine increase insulin secretion and develop hypoglycaemia in severe malaria patient which can adversely affect the coma, tachyponia, and convulsion (Taylor and White, 2004; Team, 2000).

Chloroquine: Chloroquine administration causes headache, faintness, hazy vision, gastrointestinal upset, itching, de-pigmentation and photosensitivity. Long term administration of chloroquine rarely causes neuromyopathy, retinopathy and idiosyncratic reactions (Taylor and White, 2004). Chloroquine consumption during pregnancy may result into systemic lupus erythematosus or rheumatoid arthritis in new born babies (Klinger et al., 2001).

Artemisinin and their derivatives: Artemisinin is less toxic than quinine and chloroquinine. However adverse effects such as nausea, dizziness, fatigue and neurotoxicity can be seen in prolonged usage and over dosage.(Nontprasert et al., 1998; Price et al., 1999; Taylor and White, 2004).

Primaquine: Gastrointestinal toxicity such as abdominal pain or cramp, nausea, vomiting are the mild adverse effects seen in PQ administrated patients. Decreasing WBC counts, developing methemoglobinemia are common adverse effects in PQ uptake. PQ is highly intolerable for glucose 6 phosphate dehydrogenase deficient individual and in those cases PQ administration cause severe hemolysis. Thus, PQ is strictly prohibited in glucose 6 phosphate dehydrogenase deficient patients (Taylor and White, 2004).

1.9 Objectives

Involvement of different molecules in malaria pathology were discussed here, but still many more links are not covered completely. In this study, we have focused about the MetHb contribution in malaria pathology. MetHb toxicity was often ignored due to the pronounced effect of other molecules such as hemin, cytokines, etc. The mild increment in MetHb was observed during different clinical conditions including malaria, none of the report claims MetHb mediated toxicity in mammals. The parent molecule for the MetHb is Hb, which is considered as a toxic compound extracellularly due to its ROS generation potential. ROS is known to cause the cellular damages. With above background information about MetHb, we wanted to explore whether MetHb has potential to cause toxicity to RBC. If the MetHb is toxic, whether it will be involved in development of anemia or vascular complication? Similarly, anti-malarial administration also causing toxicity in the patient. In presence of antimalarial, what will be MetHb effect on host system? And, how can it be resolve? Keeping these questions in mind, we have framed following objectives.

- Understanding the role of extracellular methemoglobin catalyzes rapid RBC lysis to explain acute anemia during malaria.
- Exploring mechanistic details of RBC aggregation during malaria like microenvironment
- Role of methemoglobin contributes into primaquine toxicity and its molecular mechanism
- Understanding the molecular mechanism of NAC mediated protection in RBC and role of methemoglobin

1.10 Significance of the Work

Oxidative stress development by MetHb is often neglected during malaria. In our study, we established that the extracellular MetHb is a potential molecule to cause the RBC lysis and RBC aggregation. RBC lysis and RBC aggregation contribute the severe hemolytic anemia and vascular blockage during malaria. Primaquine is the only relapse agent for malaria but its' adverse effect is not studied well. In our study, we found the evidence that MetHb metabolize the PQ in presence of hydrogen peroxide and converts into carboxyprimaquine and desaminoprimaquine. It results RBC membrane damage and prone them for the lysis. N-Acetyl cysteine, an antioxidant, inhibits the MetHb and perishes the peroxidase activity which protects the RBCs from hemolysis and membrane damages.

Chapter 2. Extracellular Methemoglobin Catalyzes Rapid RBC Lysis to Explain Acute Anemia During Malaria*

2.1 Introduction

Red blood cell (RBC) is a biconcave disc shaped cell with an average diameter of 7.5μm. RBC is produced in bone marrow with an average life span of 120 days in humans. During this period, RBC need to do several cycles of gaseous exchange (CO_2/O_2), experience shear stress (passage through small blood vessels) and other physiological or non-physiological stresses. As a result, RBC inevitably aged out and express specific cell surface molecule to get cleared by circulating phagocytes (McQueen and McKenzie, 2004). The whole process of RBC destruction within reticulo-endothelial system doesn't cause inflammation (Condon et al., 2003). However during patho-physiological conditions such as malaria, dengue, sickle cell anemia, internal injury, an accelerated RBC lysis occurs, leading to the development of hemolytic anemia (Rother et al., 2005). During hemolysis, hemoglobin present inside RBC is released into the blood circulation. Hemoglobin in the external microenvironment is oxidized by molecular oxygen to form MetHb (Fe^{3+}) or oxidation by reactive oxygen species (ROS) into ferryl hemoglobin (Fe^{4+}=O^{2-}) (Balla et al., 1993; Wright et al., 1999). Ferryl hemoglobin is unstable and eventually gets converted into the MetHb through electron transfer. Besides direct oxidation of hemoglobin in cell free micro-environment, a number of extrinsic factors, such as, toxic drug molecules, metabolic by-products of pathogenic organisms, and pro-inflammatory cytokines causes imbalance of oxidation/reduction system of RBC (Kiefer and Snyder, 2000). As a result, a large amount of intracellular hemoglobin oxidized to form MetHb and induce RBC cytoskeleton rearrangement, membrane depolarization, increase in deformity and hemolysis (Matarrese et al., 2005; Mohanty et al., 2014; Totino et al., 2010). In most of the pathological situation, rate of RBC destruction is very high and underlying molecular events are not clear. Accelerated destruction of RBC correlates well with poor antioxidant protection within RBC and higher rate of hemoglobin conversion to MetHb (Kiefer and Snyder, 2000). According to a rough estimate, during malaria every infected RBC causes lysis of 10 or more uninfected RBC to exhibit hemolytic anemia (Gaudreault et al., 2015; Pamplona et al., 2009).

MetHb, a pro-oxidative molecule has potential to produce large amount of peroxide to accelerate oxidative-mediated tissue damage (Boretti et al., 2009; Goldman et al., 1998). Methemoglobinemia (presence of MetHb in serum) is known to produce functional and structural defects in RBCs (Buehler and D'Agnillo, 2010). During hemolysis, RBC present in the close

vicinity will be the first target of MetHb directly or free radicals derived from it. Hence we explored a nuclear fission model of RBC lysis, where molecules released from initial hemolysis destruct more RBCs and thereby amplify the lysis reaction. MetHb mediated activation of astrocytes showed elevated level of TNF-α, TLR-4 and IL-1β mRNA level and higher TNF-α secretion. MetHb could be another potentiator molecule for neurological sequlae during cerebral malaria (Gram et al., 2013). In this chapter, we have validated the nuclear fission model with released MetHb in amplification of lysis reactions that cause physiological and structural alterations in exposed normal RBC. Our data clearly supported the involvement of MetHb release to makes RBC cells vulnerable to osmotic shock and responsible for high level of hemolysis. It caused development of oxidative stress and a sharp ROS spike was responsible for structural defects in RBCs. Based on these results, we propose that MetHb released from RBC is responsible for enhanced hemolysis and severe outcome of pathologic disorders.

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2.2 Methods

2.2.1 Preparation of RBC from human blood: Human blood drew from healthy volunteers in EDTA containing tubes with informed consent. Whole blood was centrifuged at 1000 rpm for 10 min and plasma was removed. Wash the RBC pellet with PBS containing glucose (1 mg/ml) and cells from top layer containing WBCs were removed. This step was repeated 3 times and finally RBC pellet was re-suspended in glucose containing PBS at 5 % hematocrit. A thin smear was made on glass slide to monitor the purity of RBC preparations. In all of the experiments, we have used RBC preparations with <1% other blood cells.

2.2.2 Measurement of RBC osmotic fragility and hemolysis: RBC preparations (5% hematocrit) were treated with different concentration of MetHb (0 - 1000 µg) and after treatment, RBCs were separated from reaction mixture by centrifugation at 1000 rpm and supernatant was read at 540 nm in infinite M200 (Tecon, Austria) microtiter plate reader to measure hemolysis. RBC incubated in PBS was treated as control and 1% v/v triton-x-100 lysed RBC was considered as 100 % hemolysis. For osmotic fragility, RBCs isolated from reaction mixture were exposed to 100 mM NaCl solution for 30 min at room temperature. Cells were centrifuged again and supernatant was read at 540 nm in infinite M200 microtiter plate reader (Tecon, Austria). RBC incubated with PBS was treated as control. 1% v/v Triton x-100 lysed RBCs were considered as 100% hemolysis. For antioxidant treatment, RBC cells were treated with antioxidants, N-acetyl cysteine (5 mM) and Mannitol (2.5 % (w/v)) either before treating with MetHb or 1 hr. post treatment.

2.2.3 Flow cytometric analysis of ROS containing RBC population: RBCs were loaded with fluorescent probe 'DCFH-DA' (10 µM) for 1 hr. at 37°C in 5% CO_2 incubator. RBCs were washed 3 times with PBS to remove excess 'DCFH-DA' and treated with MetHb (700 µg) for 15 min at 37°C. After treatment all samples were kept on ice and analyzed in FACS Caliber (BD Biosciences) to detect ROS containing RBC population. In similar conditions, control cells were also analyzed.

2.2.4 Fluorescence microscopy of MetHb treated RBC: RBCs were loaded with fluorescent probe 'DCFH-DA' (10 µM) for 1 hr. at 37°C in 5% CO2 incubator and treated with MetHb. A thin smear was prepared and microscopy was performed on fluorescence microscope 80Ti (Nikon) under 100x oil immersion objective to observe ROS containing fluorescent RBC cells. Control cells were similarly processed and analysed. Files were opened in Adobe Photoshop 7.0 and gray levels were adjusted by using the auto level command with black and white clip set to 0 %. Images were cropped again and scaled for final display.

2.2.5 Measurement of oxidative stress within RBC by estimating lipid peroxidation level: Freshly purified RBCs were treated with MetHb (700 µg) in dextrose (1 mg/ml) containing PBS at 37°C for 0-2 hr. and immediately lysed with 1 % triton x 100 in cold environment (4°C). Small amount of RBC lysate (20 µl) was used to measure the protein level by Lowrys' method. 200 µl of lysate were mixed with equal volume of ice cold (4°C) trichloro acetic acid (TCA – 10 %) and left it for 15 min in ice flakes for protein precipitation. TCA-RBC lysate mixture was centrifuged at 3000 rpm for 15 min at 4°C and 300 µl of supernatant was heated with equal volume of thiobarbituric acid (0.67 %) in a boiling water bath for 10 min. Developed pink coloured solution was measured at 532 nm calorimetrically (infinite M200 microtiter plate reader - Tecon, Austria). Obtained values are matched with the standard curve and the final lipid peroxidation values were normalized with the total protein present in the lysate. MetHb untreated sample was used as a control and used to calculate the fold changes. (Trivedi et al., 2005a).

2.2.6 Measurement of oxidative stress within RBC by estimating protein carbonyl level: To measure the protein carbonyl level, till the MetHb exposed RBC lysate preparation process was followed as described in section 2.2.4. Then RBC lysate was aliquoted equally and labelled as blank as well as sample. Further both aliquotes were treated with equal volume of ice cold TCA (10%). After 15 min incubation, samples were centrifuged at 3000 rpm for 15 min at 4°C and obtained sample-pellet was incubated with 500 µl of dinitrophenylhydrazine (DNPH - 0.2 %) in 2N HCl for 2 hr. at 37°C. Simultaneously, blank-pellet was incubated with 2N HCl. All the samples were again treated with 55 µl of TCA (100 %) and centrifuged at 8000 rpm for 10 min at room temperature. Samples were washed thrice with ethanol:ethylacetate (1:1) mixture. Pelletes are dissolved in 600 µl of guanidine-HCl (6 M) in 20 mM sodium phosphate buffer (P^H 6.5) and measured at 370 nm calorimetrically (infinite M200 microtiter plate reader - Tecon, Austria).

Molar extinction coefficient value for the protein carbonyl is 21 mM at 370 nm which was used to calculate the protein carbonyl level. Blank values were used to subtract the corresponding samples protein carbonyl level. Values were recalculated according to the proteins level present in the samples and converted into folds as mentioned earlier in section 2.2.4 (Trivedi et al., 2005a).

2.2.7 Measurement of oxidative stress within RBC by estimating glutathione (GSH) level: To estimate the GSH level, MetHb exposed RBCs, as mentioned earlier section 2.2.4, were collected and lysed with ice cold 20 mM EDTA-sulphosalicyclic acid (0.6%). 20 µl of RBC lysate was used to measure the total protein level. 200 µl of RBC lysate was precipitated by incubating with the equal volume of ice cold TCA (10%). Samples were centrifuged at 5000 rpm for 5 min at 4°C and obtained supernatant (60 µl) was incubated with 120 µl of 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB–2.5 mM) in 0.4 M tris buffer (P^H-8.9) reagent for 5 min. Developed yellow colour compound was measured at 412 nm calorimetrically (infinite M200 microtiter plate reader - Tecon, Austria). Obtained values are matched with the standard curve and the final GSH values were normalized with the total protein present in the lysate. MetHb untreated sample was used as a control and used to calculate the fold changes. (Trivedi et al., 2005a).

2.2.8 Preparation of clotrimazole modified MetHb (CLT-MetHb): MetHb (10mg/ml), clotrimazole (20 mM) and hydrogen peroxide (1 mM) solutions were mixed together in a glass beaker in a ratio of (120:6:1) with constant shaking for 30 min at 25°C. The reaction volume was dialyzed against PBS (pH7.4) for 24 hrs with 4 changes. Concentration of clotrimazole modified MetHb (CLT-MetHb) was adjusted to 7 mg/ml and stored at -80°C in small aliquot for future use.

2.2.9 Measurement of MetHb peroxidase activity: MetHb peroxidase activity was measured by guaiacol oxidation as described previously (Trivedi et al., 2005b). In brief, guaiacol (20 mM) in sodium phosphate buffer (50 mM – P^H 7.2) mixed with 33.3 µg/ml of MetHb and incubated for 5 min with/without clotrimazole (33.3 µg/ml). Reaction was initiated by adding H_2O_2 (0.27 mM) and after 20 min images was acquired and cropped to display the peroxidise activity of MetHb with/without clotrimazole.

2.2.10 Measurement of ROS production in external microenvironment by MetHb: Measurement of ROS production activity of MetHb was performed as described earlier (Halliwell and Whiteman, 2004). In a 0.2 ml reaction RBCs and MetHb (700µg) were mixed together and as indicated after every time point, an aliquot (50 µl) from reaction mixture was taken out and RBCs were separated by centrifugation. Supernatant was added to the 950 µl DCFHDA (10 µM) and fluorescence spectra were recorded in Flumax-3 (λ_{ex} 488 nm and λ_{em}-530 nm). Fluorescence value at 530 nm was taken into account for calculating amount of ROS and PBS treated RBCs were used to calculate fold change in ROS level. Clotrimazole modified MetHb (CLT-MetHb) was analyzed under the identical conditions.

2.2.11 Statistical analysis: Statistical analysis was performed using student t-test (MICROSOFT EXCEL 2007) and p-value < 0.05 was considered significant.

2.3 Results

2.3.1 External MetHb exposure makes RBC susceptible for hemolysis: RBCs are very sensitive to osmotic stress and they maintain the osmotic balance by running energy driven proton pumps (Whittam and Ager, 1964). Under stress or oxidation-reduction imbalance, membrane polarity is compromised and RBC lysis occurs. To test whether MetHb can affect the ability of RBC to maintain osmotic balance, we exposed the RBC to different concentration of MetHb for 30 min at 37°C. Supernatant was collected into the glass tube and imaged with Nikon Coolpix (12 megapixel) camera for the hemolysis demonstration (Figure 2.1-C). Cells were recovered from reactions and osmotic fragility was assessed by exposing them to 100 mM NaCl (hypotonic) solution at room temp (RT). Similarly like hemolysis demonstration, 100 mM NaCl exposed RBCs supernatant was collected into the glass tube and imaged with Nikon Coolpix (12 megapixel) camera to demonstrate the RBC fragility (Figure 2.1-A). MetHb dose experiments performed in triplicate. All error bars indicate standard deviation (SD) and dependently causes RBCs susceptible for osmotic stress and at 1000 µg more than 50% RBCs were vulnerable to osmotic shock (Figure 2.1-B). Loss of ability

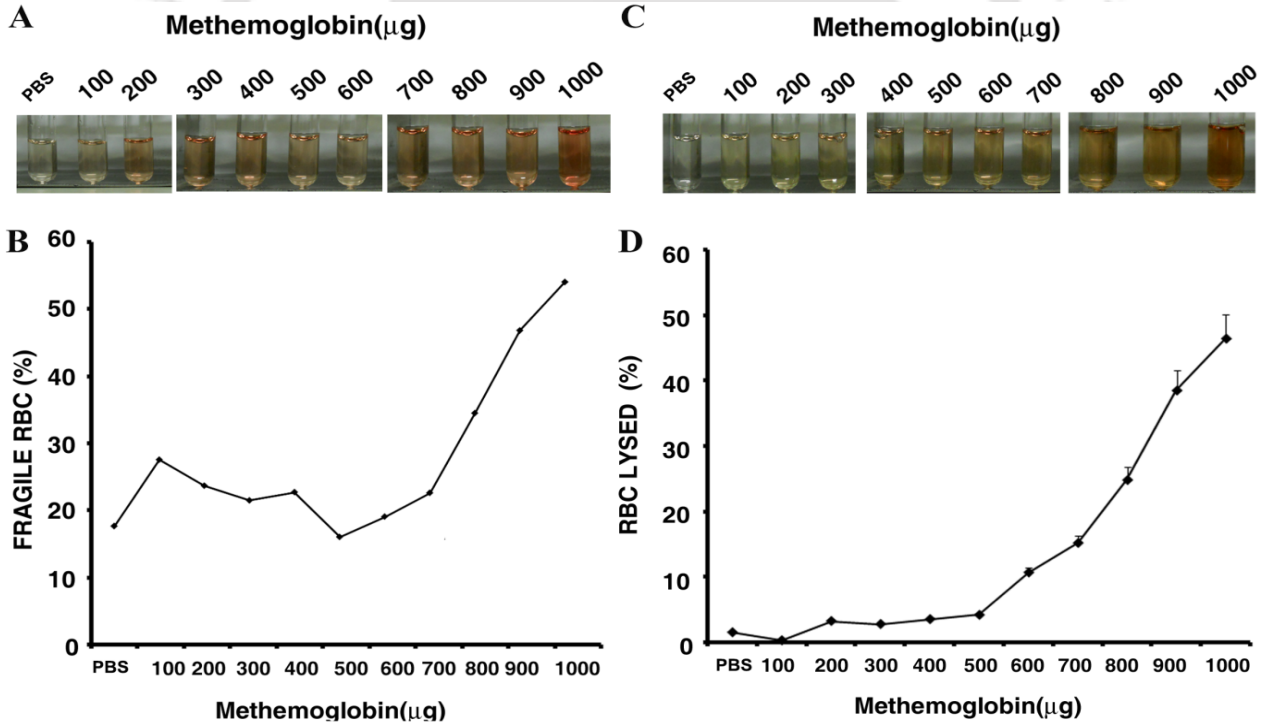

Figure 2.1: MetHb exposure causes RBC cells susceptible for hemolysis. Hematocrit (5 % RBCs in PBS) was prepared and exposed to different concentration of MetHb (0 - 1000 µg) for 30 min at 37°C and the supernatant was aliquoted into a test tube for photographic demonstration (A) osmotic fragility or (C) hemolysis as described in methods section. Another duplicate set of reactions were performed to measure the (B) osmotic fragility or (D) hemolysis as mentioned in methods section. Data was obtained from two independent and triplicate measurements. If not visible, error bar is smaller than symbols.

to maintain osmotic balance results into the RBC lysis. As expected, we found RBC lysis with increasing concentration of MetHb complementing osmotic fragility results (Figure 2.1-D). Together the data indicates that MetHb exposure reduces the ability of RBC to maintain osmotic balance and if MetHb exposure remains high it leads to cell lysis (hemolysis).

2.3.2: MetHb exposure causes oxidative stress within RBC: Most of the observed structural membrane abnormities in RBC are associated with hemoglobinopathy, small molecule toxicants or pathological diseases such as malaria (Arya et al., 1995; Hall et al., 1986; Mohandas and Evans, 1994; Staines et al., 2000). Disturbance in hemoglobin metabolism within RBC or pathogen mediated stress develops oxidative stress within RBC. Oxidative stress in turn remodels the RBC cytoskeleton, which leads to the development of membrane abnormalities (Bradberry, 2003). MetHb has potential to produce large amount of peroxide to accelerate oxidation mediated tissue damages (Boretti et al., 2009; Goldman et al., 1998). The extent of oxidative insult can be measured by multiple biochemical markers. A flow cytometric assay was performed to know population of RBCs with oxidative stress due to MetHb exposure (Figure 2.2-B). RBCs, loaded with fluorescent ROS probe 'DCFH-DA', were treated with MetHb (700 µg) for 15 min. Cells were washed with PBS three times and analyzed in flow cytometry. MetHb treated RBCs gives several fold high fluorescence compare to RBCs exposed to MetHb for 0 hrs and analyzed under identical conditions. Simultaneously analysis of fluorescence signal from MetHb treated RBC under fluorescent microscope was showed higher signals when compare to the control samples (Figure 2.2-A). Analysis of FACS curves indicated that MetHb exposure system can be assessed by biochemical parameters such as increase in lipid peroxidation or protein carbonyl levels and reduction in GSH levels. RBCs treated with MetHb (700 µg) were lysed with 1% v/v Triton x-100 and lipid peroxidation and protein carbonyl levels were measured. Within 15 min, lipid peroxidation level sharply raised 3-fold compared to control samples. Afterwards, it sharply scaled down to basal level (Figure 2.2-C). MetHb treated RBCs also exhibited similar response curve for protein carbonyl levels as well (Figure 2.2-D). MetHb treatment caused a sharp reduction in GSH levels within 15 min and then increased after wards to reach levels comparable to control cells (Figure 2.2-E).

2.3.3 MetHb mediated initial ROS spike prime the RBCs to exhibit membrane defects: ROS pattern within RBC had two phases, a sharp increasing phase (spike) and then gradual increasing phase (accumulation) at later time point. We performed an inverse experiment of removing the ROS by antioxidant treatment to probe spike (1st hour) or accumulation part (2nd hour) of ROS curve. MetHb treated and untreated RBCs were incubated with two natural antioxidant molecules, N-acetyl cysteine (NAC) and mannitol either from beginning (to remove spike) or after 1hr. (to remove accumulation). Both treatments significantly reduced the ROS level within MetHb treated

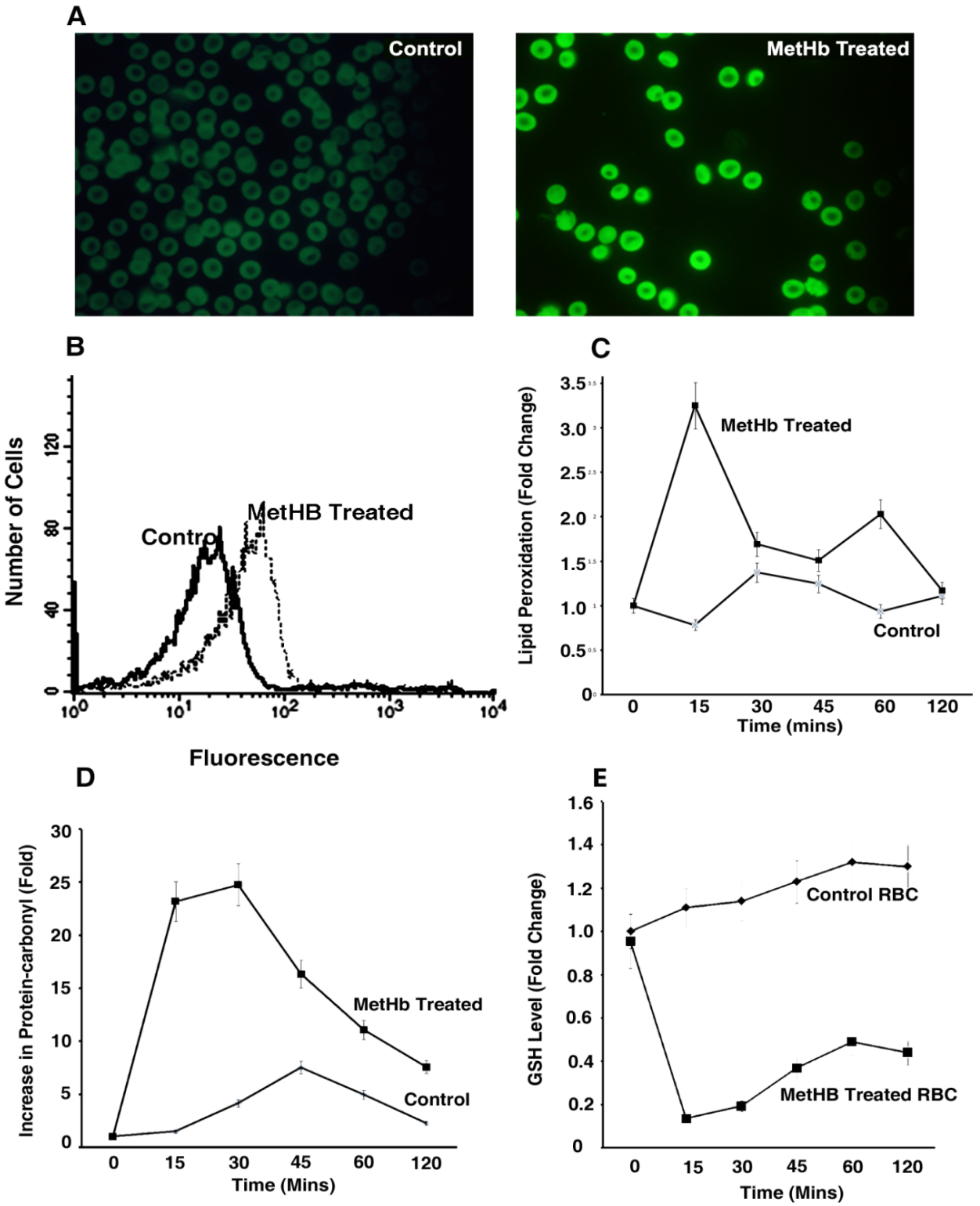

Figure 2.2 MetHb exposure causes development of oxidative stress within RBC Cells. (A&B) RBCs loaded with fluorescent probe 'DCFH-DA' (10 μM) for 1 hr at 37°C, exposed to MetHb causes RBC population with high ROS level. (A) A small aliquot of cells used were taken to prepare thin smear and observed with fluorescence microscope Nikon 80Ti equipped with DS-Fi1-U-2 CCD camera. (B) MetHb exposed DCFH-DA loaded RBCs further showed significant increase of ROS inside the cell during Flow cytometry analysis. (C) lipid peroxidation, (D) Protein carbonyl level and (E) decrease in GSH level. RBC cells treated with glucose containing PBS serve as control. Data were from three (n=3) independent experiments performed in triplicate. All error bars indicate standard deviation (SD) and were calculated from triplicate measurements. If not visible, error is smaller than symbols.

RBC cells (Figure 2.3-A). Antioxidant treatment during initial spike phase inhibited the MetHb mediated membrane fragility and hemolysis comparable to control level, where as antioxidant treatment after 1hr. had no or very little inhibitory effects on MetHb mediated osmotic fragility and hemolysis (Figure 2.3-B & C). The antioxidant treatment experiment clearly indicates MetHb mediated ROS spike primed the RBCs to exhibit osmotic fragility and ultimately caused hemolysis.

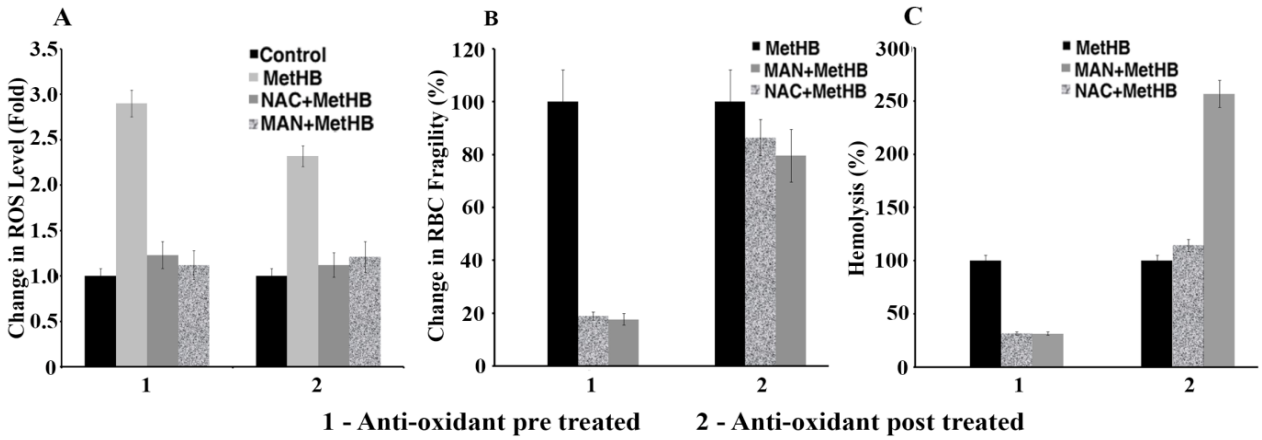

Figure 2.3 MetHb mediated ROS spike is responsible for observed RBC osmotic fragility and hemolysis. Two natural antioxidants, N-acetyl cysteine (NAC) and mannitol were used to probe the role of oxidative stress in MetHb treated RBC with osmotic fragility and hemolysis. (A) Both antioxidants were treated to RBC 15 min before the addition of MetHb in '1' and antioxidants were treated after one hr. exposure of MetHb in '2'. Both treatments were efficient to abolish ROS spike or accumulation phase. ROS levels were determined using fluorescent probe, 'DCFH-DA' as described in method section. RBC cells treated as in (A) and used to determine (B) Osmotic fragility or (C) hemolysis. Data were from four independent experiments (n=4) performed in triplicate. All error bars indicate standard deviation (SD) and were calculated from triplicate measurements**.**

2.3.4 MetHb associated peroxidase activity causes RBC defects: MetHb has a very low level of intrinsic pseudo-peroxidase activity which can have the potential to produce free radicals in the micro-environment (Reeder et al., 2004). But a recent report suggests that peroxidase activity associated with MetHb attenuate H_2O_2 induced oxidative stress (Widmer et al., 2010). To probe the role of MetHb peroxidase activity with observed RBC defects, we tested many peroxidase inhibitors using guaiacol oxidation assay. Clotrimazole mediated dose dependent inhibition of *P. falciparum* hemo-peroxidase was reported earlier and the mode of inhibition is suicidal in nature (Trivedi et al., 2005a). We tested the effect of CLT on MetHb in guaiacol oxidation assay. To our surprise, CLT irreversibly inhibits MetHb associated peroxidase activity, even after its removal by dialysis (Figure 2.4-A, Inset). Exploiting this feature we prepared a CLT modified inactive MetHb (CLT-MetHb) as given in method section. CLT-MetHb didn't produce significant ROS where as MetHb causes high ROS in the micro-environment (Figure 2.4-A). In comparison, CLT-MetHb (inactive MetHb) was inefficient to cause osmotic fragility and hemolysis compared to MetHb (Figure 2.4-B & C). CLT-MetHb even at very high concentration (~2000 µg) for 2 hrs also failed to

cause any osmotic fragility or hemolysis (data not shown). Together our data strongly suggested that peroxidase activity of MetHb is responsible for observed RBC defects and hemolysis.

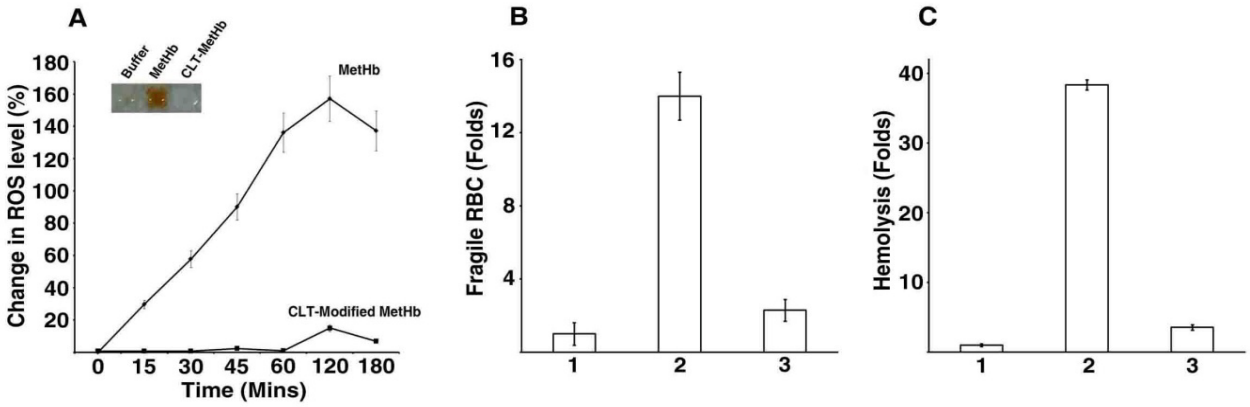

Figure 2.4 Pseudo-peroxidase activity of MetHb is responsible for ROS accumulation within microenvironment. (A) MetHb pseudo-peroxidase activity causes production of reactive oxygen species in the microenvironment. CLT-MetHb was inactive in a guaiacol oxidation assay (Inset, A). CLT-MetHb was inefficient in causing production of ROS within micro-environment as compare to unmodified MetHb. All values were expressed as fold change in comparison to samples at time 0. RBCs were treated with PBS, MetHb or CLT-MetHb (1=PBS, 2=MetHb or 3=CLT-MetHb) and **(B)** Osmotic fragility or (**C)** hemolysis were measured as described in method section. All error bars indicate standard deviation (SD) and were calculated from triplicate measurements. If not visible, error is smaller than symbols.

2.4 Discussion

Toxic substances (external or metabolic derived by-products) appear in blood during infection or therapy, toxin or drug may injure RBC in a variety of ways which results hemolysis. A number of different models have been proposed in the past to explain hemolysis with much emphasis on steps of RBC lysis (Beck and Saari, 1977; Bokori-Brown et al., 2016; Brumen and Heinrich, 1984; Hoshen et al., 2000; Wagner et al., 2003). Earlier biochemical model proposed that RBC lysis is a two step process, initial “sensitization” and then actual “lysis”. Both step are catalyzed by two distinct molecules, hence hemolysis model believes that molecules causes severe damage to RBC membrane fragility (sensitization) but needs additional help to facilitate the efficient lysis (hemolysis) (Beck and Saari, 1977; Bokori-Brown et al., 2016; Brumen and Heinrich, 1984; Wagner et al., 2003). Conversion of Hb to MetHb within RBC triggers the development of oxidative stress and causes structural membrane defects (sensitization). An additional physical damage (via passing through thin vessels) to cells causes hemolysis (Lisovskaia et al., 1994). In this chapter, we explored a unique role of MetHb playing a master role to catalyze both the steps of hemolysis. Based on our results, we proposed a “nuclear fission model” of RBC lysis (Figure 2.5).

In the proposed model, initial RBC lysis causes release of Hb or MetHb into the blood or local microenvironment. MetHb due to its pseudo-peroxidase activity causes production of large

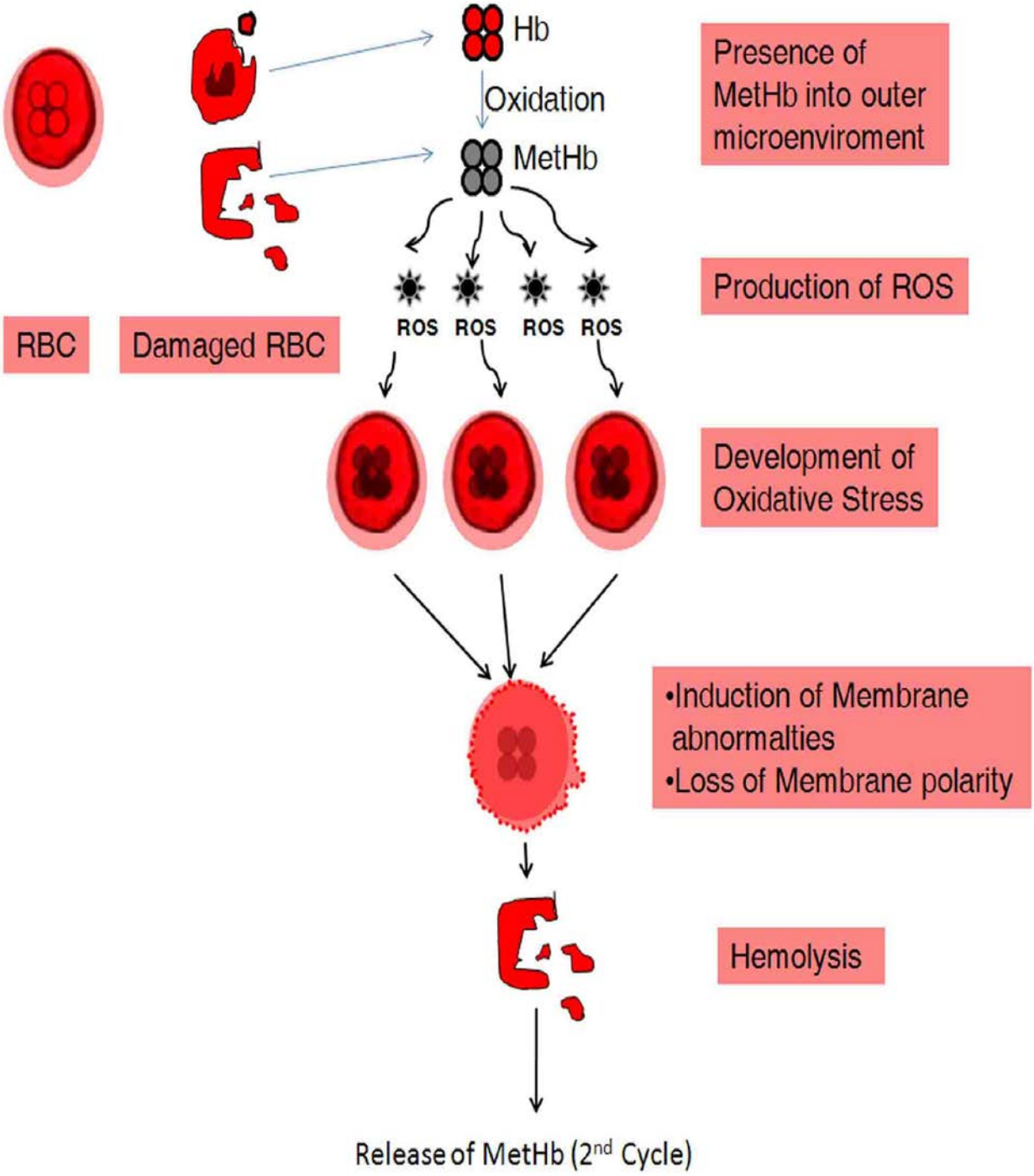

Figure 2.5 Proposed Nuclear Fission Model of RBC damage by MetHb. A probable model of MetHb mediated RBC destruction is shown, mimicking "nuclear fission model" where molecules released from first RBC catalyze further RBC destruction and amplifying initial lysis signal. A detailed description about model is given in text.

amount of reactive oxygen species (ROS) outside (sensitization). External ROS disturbs interior oxidation/reduction balance of RBC to develop oxidative stress. Oxidative stress induces cytoskeleton changes (Protein carbonyl formation) which results into structural membrane defects. Loss of proton gradient inhibits ATP production and RBC could not be able to run energy driven

proton pump to maintain osmotic balance. RBC cells at this stage are vulnerable for immune-mediated elimination and hemolysis. RBC cells lysis releases Hb/MetHb to start another cycle to amplify RBC damage. Hence according to the proposed nuclear fission model, MetHb mediated ROS production is a critical step to amplify the initial lysis signal. Development of oxidative stress and down-stream events prime the RBC to get lysed. Infusion of hemoglobin solution in healthy volunteers as well as variety of acquired and iatrogenic hemolytic disorders suggest links between hemolysis and hemoglobinemia (Lamy et al., 2000; Przybelski et al., 1999; Rifkind et al., 2014; Sloan et al., 1999) interior oxidation/reduction balance of RBC to develop oxidative stress. Oxidative stress induces cytoskeleton changes (protein carbonyl formation) which results into structural membrane defects. Loss of proton gradient inhibits ATP production and RBC couldn't be able to run energy driven proton pump to maintain osmotic balance. RBC cells at this stage are vulnerable for immune-mediated elimination and hemolysis. RBC cells lysis releases Hb/MetHb to start another cycle to amplify RBC damage. Hence according to the proposed nuclear fission model, MetHb mediated ROS production is a critical step to amplify the initial lysis signal. Development of oxidative stress and down-stream events prime the RBC to get lysed. Infusion of hemoglobin solution in healthy volunteers as well as variety of acquired and iatrogenic hemolytic disorders suggest links between hemolysis and hemoglobinemia (Lamy et al., 2000; Przybelski et al., 1999; Rifkind et al., 2014; Sloan et al., 1999).

During malaria, accelerated RBC destruction attributes to induction of apoptosis like process in uninfected RBC and development of oxidative stress (Totino et al., 2010). Oxidative stress developed during 1st hour (a sharp ROS spike by 15min) of MetHb treatment was found to be responsible for observed loss of membrane fragility and hemolysis (Figure 2.3). Recent studies indicate a cyto-protective antioxidant like properties of hemoglobin to reduce H_2O_2 mediated toxic effects in endothelial cells (Widmer et al., 2010). Compare to classical heme dependent peroxidases, radical originates on MetHb but leaks out into microenvironment (Reeder et al., 2004). Hence, the chemistry of hemo-protein in the aqueous system is complex and final biological effects depend on the presence of other substances (free radicals) in the microenvironment.

Hemolysis is a critical parameter to decide the quality of preserved blood. During a period of 42 days of storage, free hemoglobin/MetHb level reaches to 200 mg/dL (Berra et al., 2014; Hogman et al., 1991). According to the American association of blood banks (AABB) blood or blood products with 1% hemolysis is safe for transfusion, if it provides 75% RBCs in next 24 hours (Scott et al., 2005). It is interesting to note that in our study we have used the MetHb at a concentration of free MetHb in the preserved blood units in blood bank. In the current chapter we clearly highlighted the probable mechanism for the observed destruction of remaining 25% RBC cells within 24 hours transfusion of stored blood. In an isolated system MetHb exposure mediated

induction of loss in osmotic fragility and hemolysis is never been studied. But under in-vivo conditions, contribution of molecules from other blood cells cannot be ruled out.

2.5 Summary

MetHb is associated with oxidative stress. During oxidative stress condition such as malaria, MetHb level increases significantly and it further enhances anemia during malaria through membrane compromization and RBC lysis. MetHb (MetHb) exposure dose dependently makes RBCs susceptible for osmotic stress and causes hemolysis. MetHb mediated oxidative stress in RBC correlated well with osmotic fragility and hemolysis. Interestingly, a reactive oxygen species (ROS) spike at 15 min was responsible for observed effects in RBC cells. Two natural antioxidants N-acetyl cysteine (NAC) and mannitol protected the RBC from MetHb-mediated defects, which clearly indicated involvement of oxidative stress in the process. MetHb due to its pseudo-peroxidase activity produces ROS in the external microenvironment. Therefore, classical peroxidase inhibitors were tested to probe peroxidase activity mediated ROS production with defects in RBCs. Clotrimazole (CLT), which irreversibly inactivates the MetHb (CLT-MetHb) and abolishes peroxidase activity, did not produce significant ROS outside RBC and was inefficient to cause osmotic fragility and hemolysis. Hence, following a nuclear fission mechanism, MetHb released from ruptured RBC produces significant ROS in external microenvironment to make RBC membrane leaky and enhanced hemolysis.

MetHb mediated defects in RBCs such as protein carbonyl formation may lead cytoskeleton disturbances and cause the RBC shape alteration. Shape altered RBCs are rapidly been removed by splenic macrophages which may contribute into spleenomegaly. MetHb effect on immune and endothelial cells can be study further to understand the malaria pathophysiology.

Chapter 3. Extracellular Methemoglobin Primes Red Blood Cell Aggregation in Malaria like microenvironment*

3.1. Introduction

Malaria caused by *Plasmodium falciparum* has a high mortality rate due to anemia, fever, multiple organ damage, renal failure, liver dysfunction and neurological disorders (MacPherson et al., 1985; Pasvol et al., 1982; Seilmaier et al., 2014; Weatherall and Abdalla, 1982; Weatherall et al., 1982). A number of clinical, biochemical and immunological studies tried to explain patho-physiological mechanisms during malaria but our understanding is still limited and underlying molecular events are not clear (Aley et al., 1987; Chotivanich et al., 2002a; Chotivanich et al., 2002b; Jacobs et al., 1987; Oo et al., 1987; Wassmer et al., 2015). The main process responsible for tissue damage and dysfunction is mechanical blockage of blood vessels. Vesicular obstruction is caused by infected RBC adherence to the endothelial lining of blood vessels and aggregation of uninfected RBC (MacPherson et al., 1985; Wassmer et al., 2015). RBC aggregation during malaria is a complex process governed by adhesion ligand on infected RBC cells, receptor expression on uninfected RBC, coagulation factors, platelet mediated agglutination and plasma proteins (Chien et al., 1975; Chotivanich et al., 2004; Chotivanich et al., 2000; Dondorp et al., 2000; Kumaravel and Singh, 1995; Mayor et al.; Tietjen et al., 1975). The sticky forces between infected and un-infected RBCs slow down microcirculatory flow but molecular mechanism and master player are not known.

A number of factors such as changes in shear stress, protein - protein interactions and erythrocyte membrane charge alteration regulate the RBC aggregate formation (Chien et al., 1975; Jayavanth and Park, 2007; Rao et al.). Aggregation induced by an ‘aggregant’ to form clumps of cells (aggregate), resistant to physical forces. During malaria oxidative insult makes RBC membrane sticky to bind infected RBC cells expressing PfEMP-1 to form rosettes and aggregates (Dondorp et al., 2004). In addition, infected RBCs express phosphatidyl serine (PS) to mimic aged or senescent RBCs and been cleared from circulation by macrophages (Giribaldi et al., 2001; Schwarzer et al., 1992). Toxic by-products released from infected RBCs during parasite life-cycle develop pro-coagulant microenvironment. Hemin released from infected RBC reduces the RBC deformality (RBC-D), an important property of normal RBC to pass through the small capillaries and avoid splenic clearance (de Back et al., 2014; Nuchsongsin et al., 2007). Both infected and normal RBC lysis releases large amount of hemoglobin in the blood. Hemoglobin released from ruptured RBC is oxidized by molecular oxygen to form MetHb. Extracellular MetHb further

accelerates the RBC lysis to contribute into elevated levels of MetHb and hemoglobin degradation products. MetHb exposure to the endothelial cells produces a number of inflammatory markers and cellular adhesion molecules to promote endothelial cell-RBC adhesion (Liu and Spolarics, 2003; Pamplona et al., 2009; Silva et al., 2009). Here, we have discussed the potential role of MetHb present in infected serum to cause RBC aggregation and its mechanism. The results present in this chapter indicate that pseudo-peroxidase activity associated with MetHb causes production of free radicals to induce oxidative stress within RBC. Oxidative stress within RBC causes phosphatidyl serine (PS) externalization to generate sticky patches to initiate aggregation. Hence, current study highlights that the MetHb induces RBC aggregation through membrane modification by oxidative stress mediated PS externalization.

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3.2. Methods

3.2.1 Preparation of RBC from Human blood: RBCs were purified from fresh blood as mentioned in the chapter 2, section 2.2.1, page 39.

3.2.2 Preparation of malaria culture supernatant: *P. falciparum* 3D7 was cultured in RPMI-1640 medium supplemented with antibiotics and albumax II as described previously (Trager and Jensen, 1976). In brief, *P. falciparum* 3D7 strain was cultured in O^+ve RBCs using RPMI-1640 complete media which contains 25 mM HEPES, 4 mg/ml glucose, 0.2 % sodium bicarbonate, 0.5 % albumax, 0.5 % hypoxanthine, 40 µg/ml gentamycin and 25 µg/ml amphotericin B, at 37°C in 5 % CO_2. RBCs were separated from parasite culture by centrifugation (with slow acceleration to avoid RBC lysis) at 350 g and culture supernatant was collected. Supernatant was preserved at -80ºC for future use.

3.2.3 Measurement of MetHb level: MetHb level was measured in malaria culture supernatant as described earlier (Rodkey et al., 1979). In brief, 20 µl of parasite culture supernatant was mixed with 3 ml of KCN (200 mg/l in 10 mM Tris (hydroxymethyl) aminomethane) diluent. Samples were incubated for 5 min and absorbance of the mixture (A1) was recorded at 420 nm. Further 10 mg of $Na_2S_2O_4$ was added to this mixture and absorbance of the mixture (A2) was recorded after 15 min incubation at 432 nm. MetHb level was calculated by using the equations 3.1, 3.2 & 3.3:

$$A_1 = [\varepsilon_{420}^{COHb}(1-x) + \varepsilon_{420}^{CNMetHb}\, x] * l * c \qquad \text{..... Eq. 3.1}$$

$$A_2 = \varepsilon_{420}^{COHb}\, x * l * c \qquad \text{..... Eq. 3.2}$$

$$x = \frac{(A_2 - A_1) * \varepsilon_{420}^{COHb}}{A_2 * (\varepsilon_{420}^{COHb} - \varepsilon_{420}^{CNMetHb})} \qquad \text{..... Eq. 3.3}$$

x – fraction of total Hb present as CNMetHb; l – light path; c – concentration;

$\varepsilon_{420}^{COHb}=1.970*10^5 mol^{-1}cm^{-1}$; $\varepsilon_{420}^{Hb}=0.988*10^5 mol^{-1}cm^{-1}$; $\varepsilon_{420}^{CNMetHb}=1.104*10^5 mol^{-1}cm^{-1}$.

3.2.4 Measurement of RBC Aggregation: RBC aggregation was measured with few modification based on protocol given by (Fusman et al., 2000). Freshly prepared RBCs (5 % hematocrit) were treated with different concentration of MetHb (0 - 1000 µg) and incubated for 1 hr. at 37ºC. RBCs were fixed with glutaraldehyde (0.5 % v/v) and a thin smear was prepared on a glass slide. RBC smears were stained with Leishmans' eosin methylene blue. To determine the RBC aggregation different microscopic fields (100x-oil immersion) were chosen randomly to count the aggregated RBCs. Further aggregated RBCs were grouped based on number of RBC in an aggregate such as (2 - 4 grouped, 5 and above grouped). Aggregation percentage was calculated by using the following equation 3.4:

$$\text{RBC Aggregation } (\%) = \left(\frac{\text{Total number of aggregated cells x 100}}{\text{Total number of RBCs per 100x microscopic field}}\right) \quad \text{..... Eq. 3.4}$$

3.2.5 Ammonium sulfate fractionation of malaria culture supernatant: The fractionation strategy is given in Figure 3.1-B. The malaria culture supernatant was clarified by passing through 0.22 µm filter to remove cellular debris and particulate matter. The clarified supernatant was subjected to 65% ammonium sulfate saturation, centrifuged at 15,000 RPM to separate pellet (P1) and supernatant. The resulting supernatant was further fractionated with 85% ammonium sulfate saturation, centrifuged at 15,000 RPM to separate pellet (P2) and supernatant (S2). Pellet fractions were re-suspended in PBS and all the fractions were dialyzed against PBS with 3 changes to remove ammonium sulfate. The dialyzed fractions were checked for their ability to induce RBC aggregation as described. MetHb concentration in each fraction was determined as described previously (Rodkey et al., 1979).

3.2.6 Preparation of MetHb deprived culture supernatant: Human Hb antibody (Santacruz) was coupled on CN-Br activated sepharose column following manufacturer instructions. Filtered sterile malaria culture supernatant was loaded onto the anti-Hb column at 0.5 ml/min flow rate and flow-through was collected in a separate tube at 4°C. In a parallel setup, a non-specific primary control antibody is coupled on CN-Br activated column and malaria culture supernatant was passed. Flow-through fractions from both columns were used to determine MetHb level and their ability to cause RBC aggregation.

3.2.7 Flow cytometric determination of the ROS level in RBC: Intracellular ROS in RBC was measured as described in chapter 2, section 2.2.3, page 39.

3.2.8 Preparation of clotrimazole modified MetHb (CLT-MetHb): CLT-MetHb was prepared as described in the chapter 2, section 2.2.8, page 41.

3.2.9 Determination of Phosphatidyl serine (PS) level on RBC surface: RBC surface phosphatidyl serine (PS) level was determined as described previously (Sola et al., 2009). For PS localization on RBC aggregates, a small aliquot of RBCs were used to prepare a thin smear on a glass slide and observed with fluorescence microscope 80Ti (Nikon) under 40x objective. Control cells were similarly processed and analyzed. Files were opened in Adobe Photoshop 7.0 and gray levels were adjusted by using the auto level command with black and white clip set to 0 %. The images were cropped again and scaled for final display.

3.2.10 Analysis of RBC aggregates in scanning electron microscope: RBC hematocrit (5%) treated with MetHb (200 µg) for 60 min at 37ºC and samples for scanning electron microscope (SEM) was prepared as mentioned earlier with minor modification (Hortola, 1992). In brief, MetHb treated or untreated cells were washed with PBS and fixed in 1% glutaraldehyde solution for 24 hr. at 4ºC. Cells were washed twice with 0.1 M phosphate buffer and were dehydrated with graded ethanol from 50 % to 100 % in a vacuum environment. Samples were coated with gold film in a Polaron sputter coater. A total of 10 fields were identified randomly and observed with field emission scanning electron microscope sigma (Carl zeiss). The instrumental setting such as EHT, width and signal were 7 kV, 3.6 mm and inLens respectively.

3.2.11 Statistical analysis: Statistical analysis was performed using the student t-test (MICROSOFT EXCEL-2007) and a p-value < 0.05 was considered significant.

3.3. Results

3.3.1 MetHb is an aggregant present in *Plasmodium falciparum* culture supernatant: The amount of MetHb in control culture media and *P. falciparum* culture supernatant was 0.08 ± 0.021 mg/ml and 0.18 ± 0.02 mg/ml respectively. *P. falciparum* culture supernatant causes RBC aggregation and rouleau formation in a microscopic RBC aggregation assay (Figure 3.1-A). The culture supernatant obtained from the uninfected RBCs incubated in complete media (RPMI 1640 supplemented with antibiotics and human serum) has very little RBC aggregation activity (Figure 3.1-A). Human serum used to culture parasite and RBCs used in aggregation assays are compatible (from the same individual or with similar blood group) to exclude the contribution of serum components in promoting non-specific RBC aggregation. The parasite culture supernatant is a mixture of serum components, parasite derived proteins, heme, hemoglobin degradation (α-chain and β-chain) and MetHb (James et al., 1991; Pichyangkul et al., 1994). Extensive dialysis of parasite culture supernatant against PBS with a 12.5 KDa cut off membrane removes small molecular weight molecules and salt present in parasite culture supernatant. It reduces the aggregation activity of parasite culture supernatant by ~16.25% (Table 3.1). This is probably by the contribution of small molecular weight factors, heme present in the parasite culture supernatant. An

ammonium sulfate fractionation of dialyzed culture supernatant was done as outlined in Figure 3.1-B. RBC aggregation activity and amount of MetHb present in each fraction is determined as given in section 3.2.4 & 3.2.3 respectively. An ammonium sulfate fractionation of *P. falciparum* culture supernatant gives different fractions (P1, P2 or final supernatant S2) with varying amount of MetHb. The total RBC aggregation of the pellet fractions P1 & P2 was 43.23% and 52.67 %

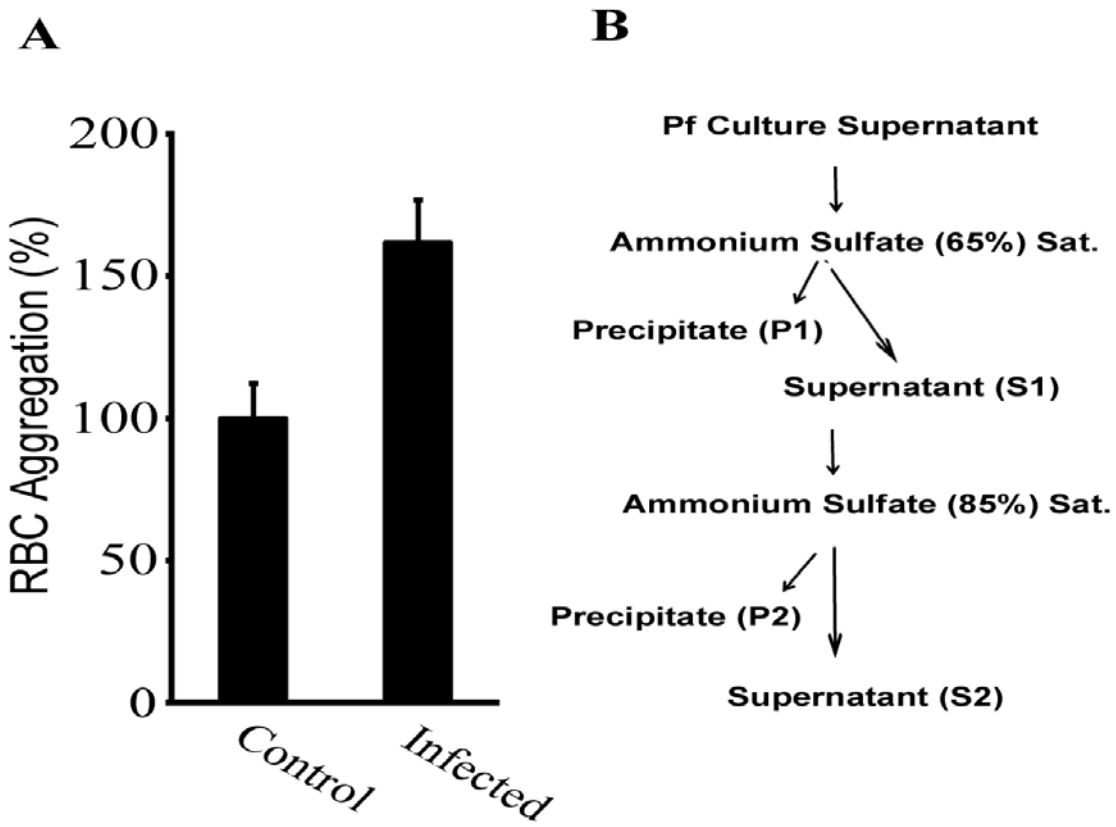

Figure 3.1 **Malaria culture supernatant causes RBC aggregation.** (A) RBCs (~5 %) exposed to control media (RPMI 1640 containing 1 % antibiotics, 0.5 % human serum), *P. falciparum* culture supernatant to induce RBC aggregation. RBCs (blood groups used) and human serum in culturing *P. falciparum* were from the same source and compatible with each other. RBC aggregation by control media and culture supernatant was statistically significant ($p<0.01$). (B) Ammonium sulfate fractionation strategy of malaria culture supernatant.

respectively in comparison to total activity (100 %) present in culture supernatant where as supernatant fraction (S2) contains no RBC aggregation activity (Table 3.1). The level of MetHb and RBC aggregation activity in different fractions indicates the probable role of MetHb in RBC aggregation during malaria. An identical ammonium sulfate fractionation strategy was performed with the supernatant from control culture obtained from the uninfected RBCs incubated in complete media (RPMI 1640 supplemented with antibiotics and human serum) to rule out contamination of MetHb from RBC lysis during the procedure. No significant RBC aggregation activity was found in control purification (Data not shown). To further confirm with a specific tool, *P. falciparum* culture supernatant was passed through an affinity column with anti-Hb antibodies coupled to sepharose beads, to remove MetHb specifically from the culture supernatant. A control

affinity purification with anti-IgG control antibodies coupled to sepharose beads was performed to exclude non-specific antibody effects. The MetHb level in flow through from anti-Hb is significantly low as compare to flow through from control affinity purification (Table 3.1). Interestingly, immuno-depletion of MetHb from malaria culture supernatant drastically reduces the RBC aggregation (to ~3%) where as flow through from control affinity purification with anti-IgG antibodies has similar degree of aggregation (~80%) as crude *P. falciparum* culture supernatant. A 20% decrease in activity might be due to partial denaturation of MetHb or removal of other small aggregant such as heme or hemozoin.

Table 3.1 RBC aggregation activity of different fractions from parasite culture supernatant.

S.No.	Conditions	Total MetHb (%)	RBC Aggregation Activity (%) ± SD
1	Parasite culture supernatant[#]	100.00 ± 8.31	100.0 ± 5.63
2	Parasite culture supernatant (Dialyzed)	96.33 ± 4.71	83.75 ± 3.45
3	Pellet 1 (P1)	38.88 ± 3.94	43. 23 ± 5.61
4	Pellet 2 (P2)	41.55 ± 7.81	52.67 ± 3.43
5	Supernatant (S2)	02.66 ± 1.23	0 ± 1.67
	Immuno-depletion of methemoglobin from parasite culture supernatant		
6	Flow Through (anti-Hb antibody)	3.14 ± 0.31	3.12 ± 1.67
7	Flow Through (anti-IgG antibody)	93.12 ± 3.12	78.51 ± 3.18

The parasite culture supernatant was subjected to ammonium sulfate fractionation as outlined in Figure 3.1-B and all the fractions were dialyzed against PBS with 3 changes to remove ammonium sulfate. The dialyzed fractions were checked for their ability to induce RBC aggregation and level of MetHb as described in material and method section. [#]The level of MetHb in parasite culture supernatant is 0.18 ± 0.02 mg/ml (100 %) and used to express MetHb for other fractions. In another experiment, MetHb was immuno-depleted from parasite culture supernatant by passing through an affinity column of anti-Hb antibodies coupled to CN-Br activated sepharose beads. A control affinity purification with anti-IgG control antibodies coupled to CN-Br activated sepharose beads was performed to monitor non-specific antibody effects.

To further understand the potential of other pro-oxidant molecules present in *P. falciparum* culture supernatant, RBCs were exposed to heme, heme-BSA (to mimic degraded hemoglobin or peptide associated heme), MetHb and RBC aggregation was measured as described in section 3.2.4, Page 53. As expected, heme or heme-BSA induces RBC aggregation but free heme is more potent than heme-BSA complex (Figure 3.2-A). Hence, a combination of generic salt fractionation and subtractive approach gives clear indication of MetHb as a factor present in *P. falciparum* culture supernatant responsible for RBC aggregation. An extensive study might help to unravel the other other factors present in the parasite culture supernatant and their relative contribution. High concentration specifically induces formation of middle order (5-9 cells per aggregate) or high order aggregate (more than 10 cells per aggregate). RBCs exposed to PBS under identical conditions

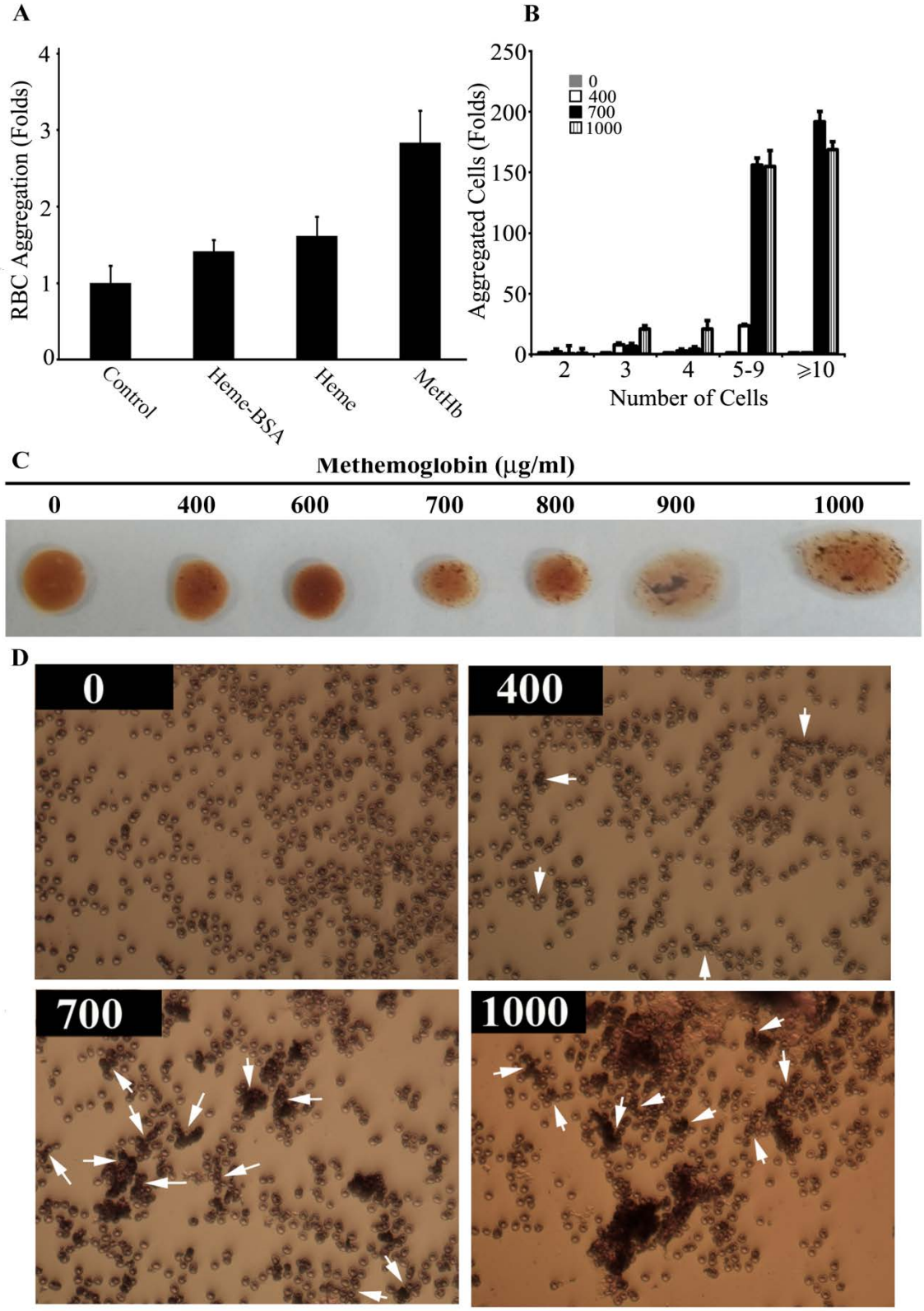

Figure 3.2 Methemoglobin primes the RBC aggregation dose dependently. (A) MetHb leads the RBC aggregation but not heme. (B) MetHb dose dependently causes high degree of RBC aggregates. (C) Pictorial demonstration of MetHb mediated RBC aggregation in solution which is visible with naked eyes. (D) Microscopical observation of MetHb treated RBCs. Images were collected using 40x objective lens and Nikon high resolution camera. RBC aggregates were marked with arrows.

exhibit a very low level of aggregates (Figure 3.2-B). RBC aggregates in the presence of high MetHb concentration (>0.4 mg/ml) were showing branching and de-branching to give the complex

nature of the aggregates (Figure 3.2-D). RBC treated with PBS exhibits a homogenous red colored clear reaction mixture with no visible RBC clumps where as RBC treated with increasing concentration of MetHb (0-1 mg/ml) shows precipitated material containing RBC aggregates (Figure 3.2-C). The complex RBC aggregates are more potent in blocking blood vessels and capillaries (MacPherson et al., 1985).

3.3.2 MetHb mediated oxidative insult causes RBC aggregation: RBCs exposed to parasite culture supernatant causes development of ROS level compared to control RBCs (Figure 3.3-A). To explore the role of ROS development as a mechanism behind RBC aggregation, ROS level and RBC aggregation were measured in MetHb (0.7 mg/ml) treated RBCs. RBC treated to MetHb exhibits high ROS compared to PBS treated cells (Figure 3.3-B). Both parameters were correlated well (r^2=0.92, n=4) especially in the initial high ROS phase (spike) where cells exhibit a sharp rise in the aggregation (Figure 3.3-C). To further confirm the role of ROS in the process of aggregation,

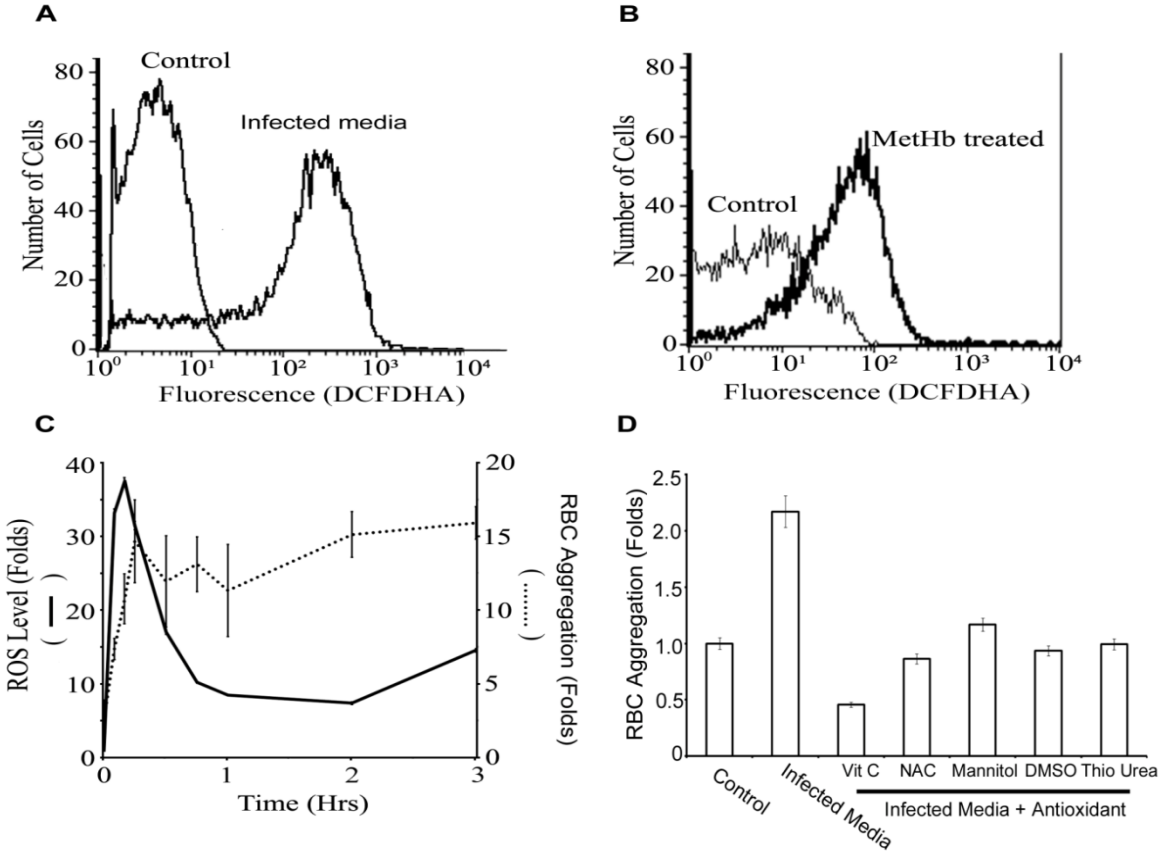

Figure 3.3 MetHb induces oxidative stress within RBC and development of oxidative stress correlates with RBC aggregation. (A) RBCs exposed to parasite culture was experienced very high ROS production inside the RBC when compare to control. (B) RBCs were treated with MetHb was also developed high oxidative stress inside the cell.(C) MetHb treated RBC population ROS level was correlated well with RBC aggregation (r^2=0.92). RBCs exposed to PBS (control) were used to calculate fold change. All error bars indicate standard deviation (SD) and were calculated from triplicate measurements (n=4). A 2-tail paired student t-test between control and MetHb gives p=0.000748. (D) Antioxidant pre exposure reducing the RBC aggregation effectively. Experiments were performed in triplicate and values were expressed as fold change in RBC aggregation ± SD.

RBCs was pre-incubated with antioxidant or free radical scavenger to remove ROS spike and treated with MetHb, RBC aggregation and ROS level were measured as described in the method section. Vitamin C, N-acetyl cysteine, thiourea are antioxidant molecules used to reduce cellular oxidative stress by scavenging oxygen centered radicals such as hydroxyl radicals, superoxide radicals (Lipinski, 2011). DMSO, mannitol is mainly being used as a radical scavenger in several studies to reduce the level of free radicals (Birinyi-Strachan et al., 2005; Rosenblum and El-Sabban, 1982). Antioxidant/free radical scavenger treatment abolishes MetHb mediated RBC aggregation (Table 3.2). Interestingly, they were more active against MetHb mediated higher order RBC aggregates (5-10 RBCs in each aggregate) than lower order aggregates (Table 3.2). Even DMSO and mannitol, less potent against RBC aggregation were potent to inhibit high order RBC aggregation ($P<0.0001$). High order aggregate is mainly responsible for blood vessel blockage and associated with the development of patho-physiology of MetHb toxicity.

Table 3.2 Effect of different antioxidant on MetHb mediated RBC aggregation.

S.No.	Conditions	RBC Aggregation ± SD	Degree of Aggregation ± SD	
			RBCs >2	RBCs > 5
1	Methemoglobin (MetHb)	43.7 ± 5.9	19.48 ± 7.3	9.61 ± 3.4
2	CLT-Methemoglobin[#]	25.2 ± 7.9	35.45 ± 7.3	4.54 ± 7.3
3	MetHb + Vitamin C	24.9 ± 2.4	12.13 ± 5.6	0.00± 1.2
4	MetHb + N-Acetyl cysteine	22.8 ± 3.1	16.31 ± 4.3	0.37 ± 0.25
5	MetHb + Thiourea	29.8 ± 3.9	13.62 ± 3.6	2.21 ± 1.2
6	MetHb + DMSO	37.4 ± 8.3	20.04 ± 4.2	4.76 ± 3.21
7	MetHb + Mannitol	44.7 ± 4.5	19.41 ± 3.2	1.20 ± 2.13
8	RBC + Vitamin C	26.9 ± 5.3	0 ± 2.2	0 ± 1.42
9	RBC + N-Acetyl cysteine	27.9 ± 5.9	0 ± 1.2	0 ± 2.16
10	RBC + Thiourea	29.6 ± 3.4	0 ± 2.3	0 ± 3.12
11	RBC + DMSO	35.0 ± 2.1	12.48 ± 3.4	0.92 ± 2.42
12	RBC + Mannitol	31.1 ± 1.8	5.36 ± 1.7	1.20 ± 3.18

RBCs were pre-incubated with Vitamin C (60 µM), N-Acetyl cysteine (5 mM), Thiourea (0.5 %), Dimethyl sulphoxide (0.2 %), mannitol (5 %) for 30 min at 37°C and treated with MetHb (0.8 mg/ml) for 15 min and RBC aggregation was measured as described in method section. RBC aggregation in PBS treated sample was considered as control (30.5 ± 3.2). Experiments were performed in triplicate and values were expressed as % RBC aggregation ± standard deviation (SD). [#]CLT-MetHb is a clotrimazole modified MetHb (inactive MetHb) where clotrimazole irreversibly binds to the heme part of MetHb and abolishes its peroxidase activity. A 2-tail paired student t-test indicates the RBC aggregation was statistically significant with $p<0.001$(control Vs MetHb), $p<0.0001$ (MetHb Vs antioxidants).

In addition, antioxidants or free radical scavengers are blocking parasite culture supernatant mediated RBC aggregation as well (Figure 3.2-D). More-interesting, the inhibition was specific towards high order aggregation and doesn't affect aggregation induced by control culture supernatant (Figure 3.2-D). RBCs treated with antioxidant and radical scavengers used in current study doesn't cause any RBC aggregation especially high order aggregates (Table 3.2).

3.3.3 Pseudo-peroxidase activity of MetHb makes RBC membrane sticky through PS externalization: MetHb has ability to generate reactive oxygen species (ROS) in the microenvironment through a low level of peroxidase activity (Wright et al., 1999). Probing the role of peroxidase activity of MetHb associated with RBC aggregation, we constructed a clotrimazole modified MetHb (inactive MetHb) where clotrimazole irreversibly binds to the MetHb didn't produce significant ROS in the microenvironment whereas MetHb produced ROS in a dose dependent manner (Data not shown). CLT-MetHb was not robust to cause RBC aggregation (Figure 3.4-A) but still had the ability to induce lower degree RBC aggregates (2 or 3 cells per aggregates) but higher order aggregates (>5 cells per aggregate) were absent (Table 3.2). During eryptosis, RBCs express high levels of Phosphatidyl serine (PS) on their plasma membrane (Moxon et al., 2011). PS externalization is associated with RBC aggregation in obese patients (Sola et al., 2009). To explore such a possibility we measured the expression of PS on the RBC membrane using a PS specific fluorescent legend, Annexin V-FITC. RBCs were exposed to MetHb, CLT-MetHb or PBS for 15 min and stained with Annexin V-FITC for 30 min on ice in dark and analyzed in FACS Caliber (BD Biosciences). RBC exposed to PBS serve as control and exhibits very low level of fluorescence. MetHb treated RBCs show more than 60% population with fluorescent Annexin V-FITC staining (Figure 3.4-B). In contrast, CLT-MetHb treated RBCs

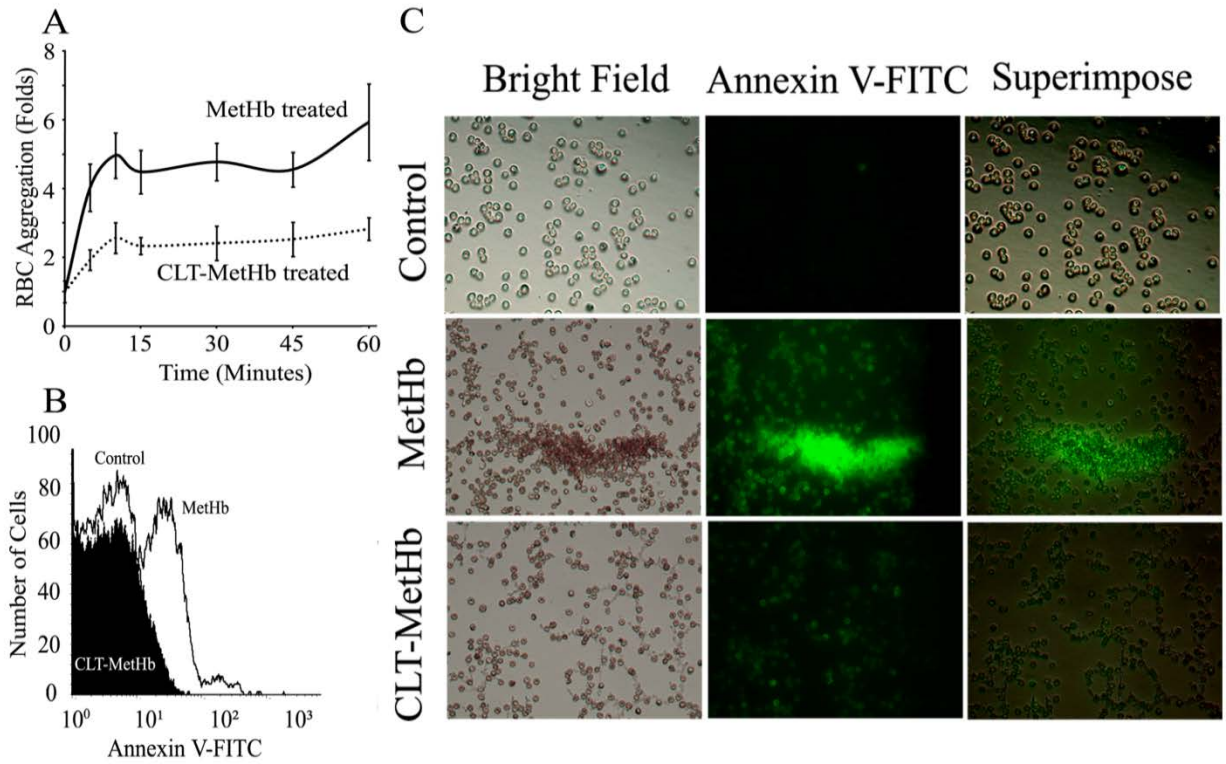

Figure 3.4: MetHb associated peroxidase activity causes phosphatidyl Serine (PS) externalization and RBC aggregation. RBC cells exposed to either MetHb or CLT-MetHb (inactive MetHb) and (A) RBC aggregation, (B) expression of PS on the membrane and (C) localization of PS on RBC aggregates was performed. All error bars indicate standard deviation (SD) and were calculated from triplicate measurements. All error bars indicate standard deviation (SD) and were calculated from triplicate measurements.

exhibit a low level of PS staining compared to control cells. In localization study, MetHb treated RBCs give high intense PS staining of plasma membrane, predominantly present in high order RBC aggregates (Figure 3.4-C, MetHb).Highly fluorescent PS positive RBCs present in low order aggregates was surrounded by PS negative low fluorescent RBCs. It is speculative that high PS expressing RBCs might be a nucleating aggregation process. RBCs exposed to PBS or CLT-MetHb didn't show any significant PS staining on their membrane but these sample exhibits lower level aggregates and rouleaux formation (Figure 3.4-C, CLT-MetHb or PBS).

Phosphatidyl serine (PS) expression of outer RBC membrane induces a negative charge on the plasma membrane. To testify the role of charged patches on the outer membrane in aggregation, RBC aggregates from MetHb exposed samples were observed in a FESEM microscope. A low resolution (1000x) observation of RBC aggregates indicates high order aggregates confirming our previous optical microscopic study (Figure 3.5). RBC aggregates at high resolution (12000x) exhibits RBC-RBC clumping involves descrete attachment points on adjacent RBC membrane (Figure 3.5, Inset). In an aggregate, RBCs are sharing a large surface area with each other but are intangeled with the specific attachment points between them.

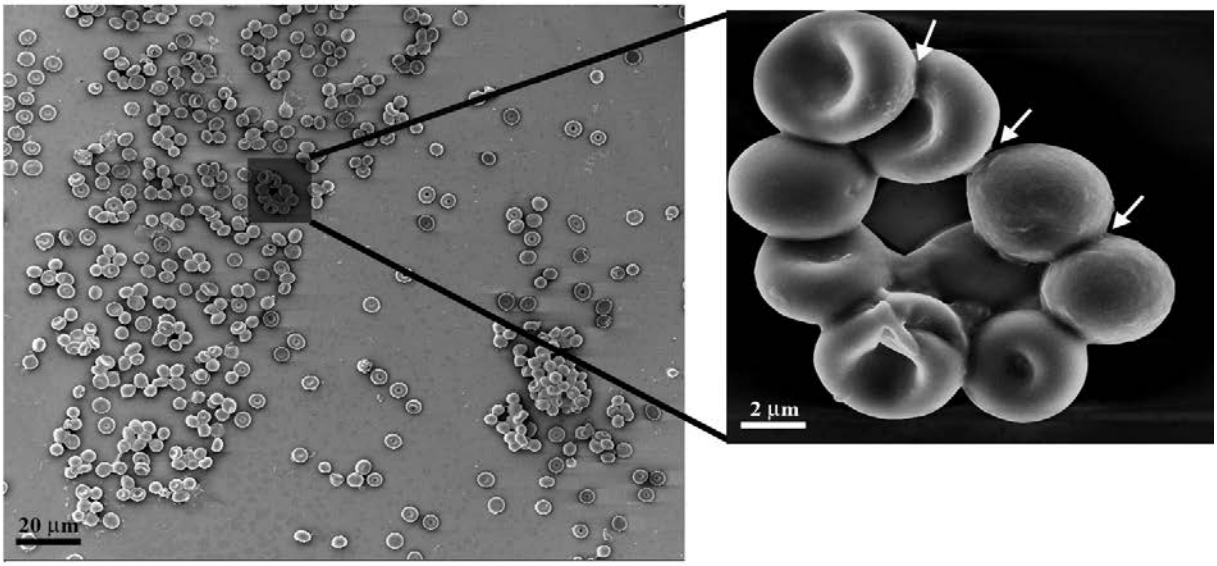

Figure 3.5: Methemoglobin causes RBC membrane sticky which favours firm aggregation. MetHb exposed RBCs thin smears were observed in field emission scanning electron microscopy (magnification; x1000, scale bar=20 µm) to investigate aggregate property. Zoom view of an aggregate from same field magnified further (magnification; x12000, scale bar=2 µm), which showed a defined pattern involving attachment points on MetHb exposed RBC membrane and the RBC attachment points were indicated with arrows.

To further validate role of patches on RBC membrane due to PS externalization, MetHb mediated RBC aggregation experiment was performed at different pH (acidic and basic side), calculated the change in RBC aggregation from neutral pH 7. RBCs were treated with MetHb (700 µg) for 60 min in buffered saline solution of different pH (4-10) and RBC aggregation was measured as described in the method section. RBCs suspended in each pH solution without MetHb

served as control for particular pH and used to calculate percentage change. RBC aggregation with MetHb at pH 7.0 was 28.3% compared to untreated control as 14 %. A 15-20% decrease in aggregation was observed in both acidic and basic pH solutions in comparison to aggregation at neutral pH (Figure 3.6). The variation in RBC aggregation in basic or acidic pH from neutral pH was statistically significant with p <0.02. It clearly indicates involvement of plasma membrane modification (changes in polarity or development of charge) with RBC aggregation.

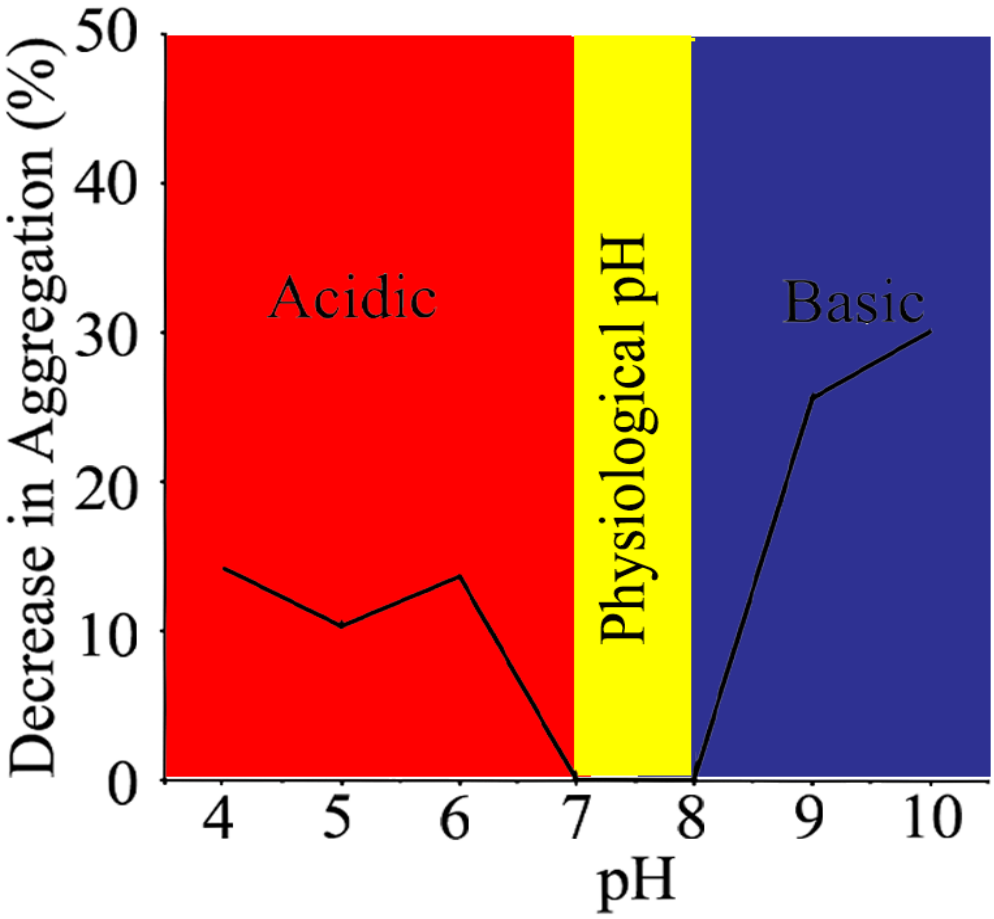

Figure 3.6: Effect of pH on MetHb induced RBC aggregation. A decrease in RBC aggregation was observed on both sides of neutral pH (acidic/ basic) was statistically significant (p<0.02).

3.4 Discussion

RBC aggregation is a physiological event required for maintaining normal homeostasis of the body. RBCs have the tendency to collide each other within a blood vessel during flow to form lower level aggregates containing 2 or 3 cells but these aggregates are very weak and disintegrate when passes through high shear stress. Under patho-physiological conditions, reaction or factors favoring RBC aggregation results into formation of big RBC aggregates, responsible for vesicular blockage and tissue damage (Autino et al., 2012; Dondorp et al., 2000). In the current objective, we have been exploring the MetHb present in *P. falciparum* supernatant as a factor to cause RBC aggregation. A partial purification of parasite culture supernatant indicates correlation of MetHb amount present in different fractions with RBC aggregation (Figure 3.1) but possibility of different

active factor in similar proportions cannot be rule. In an in-vitro RBC aggregation assay, MetHb causes dose dependent RBC aggregation in a serum free microenvironment (Figure 3.2). It preferentially induces formation of higher order RBC aggregates (5-10 cells per aggregate) and these effects were linked to the development of oxidative stress within RBC (Figures 3.3 & 3.4). Treating the RBCs with antioxidant protects the RBCs to form aggregates, specifically high order aggregates (Table 3.2). Interestingly, lower order aggregates were mostly present in PBS treated RBCs and mimics formation of bigger RBC aggregates but it does not affect physiological RBC aggregation. Antioxidant therapy with N-acetyl cysteine (NAC) in cerebral malaria reduces inflammation at the tissue injury site to delay the disease progression and helps faster recovery but in other reports NAC treatment is ineffective (Charunwatthana et al., 2009; Fitri et al., 2011; Watt et al., 2002). It implies that NAC dosing probably be a critical factor to decide the final outcomes. As NAC treatment doesn't interfere with the regular anti-malarial chemotherapy and can be proposed as an adjuvant therapy during malaria (Nuchsongsin et al., 2007). In this chapter, we highlighted another facet of the potential beneficial role of antioxidants as an adjuvant therapy during cerebral malaria.

MetHb contains heme as prosthetic group with an iron (Fe^{3+}). Due to which it can be able to catalyze peroxidation reactions to oxidize aromatic and halide substrates (Trivedi et al., 2005). Unlike classical peroxidases, MetHb is not well equipped to handle single electron generated on hemin, as a result electron leaks out into the microenvironment and produces free radicals (Widmer et al.). But the nature of electron donors present in the micro-environment and its exchange mechanism with electron from MetHb is not known and it is beyond the scope of the current study. A close relationship was found between peroxidase activity, ROS accumulation in the external microenvironment and RBC aggregation (Figure 3.3). RBCs are well equipped with the powerful antioxidant system and it is difficult to develop oxidative stress within RBC but studies indicate that during RBC aging MetHb mediated ROS production causes development of oxidative stress (Chiu and Liu, 1997). Oxidative stress in RBC causes membrane protein modification resulting in development of charge on the outer membrane. In addition, phosphatidyl serine (PS) externalization provides hydrophobic patches on the plasma membrane. This makes RBCs more likely to aggregate by making membrane sticky (Figure 3.4 & 3.5). Sensitivity of RBC aggregation to the different pH indicate a direct relationship between charge/membrane polarity with aggregation process (Figure 3.6). Two different models, bridging model and depletion model are proposed to explain RBC aggregation (Jayavanth and Park, 2007). Preliminary results indicate that MetHb induced RBC aggregation follows bridging model and a PS externalization might play a role to disrupt desegregation forces and initiate the aggregation reactions. But we have not performed rigorous validation of models or role of PS externalization with the RBC aggregation

and precise mechanistic details of the process might be multi-factorial in nature and beyond the scope of the current study.

Presence of MetHb in the blood is known to contribute malaria associated patho-physiological conditions but Methemoglobinemia is associated with other disease conditions too. A number of such conditions exhibit RBC aggregation but still it is not true for all of the patho-physiological conditions with high MetHb level (Dondorp et al., 2000; Tripette et al., 2009; Wiewiora et al., 2007). In addition, obese patient exhibits a high level of RBC aggregation but don't develop a high level of MetHb (Sola et al., 2009). RBC aggregation is a multi factorial event and that's made conditions even more complicated to explore. The flow of blood or shear stress can be one such factor to decide whether RBC will form stable aggregates or RBC aggregates will form but disintegrate afterwards. A detailed study is required to test the physiological relevance of other factors required for RBC aggregation and which is beyond the scope of the current study.

In this objective, we discovered a novel pathway of MetHb mediated RBC aggregation in malaria infected blood. Antioxidant treatment abolishes the MetHb mediated high order RBC aggregate formation and gives new insight into their role as adjuvant therapy. Presence of MetHb in the blood is known to contribute malaria associated patho-physiological conditions but further studies are required to test the physiological relevance of MetHb mediated RBC aggregation with severity of cerebral malaria and tissue damage.

3.5 Summary

Toxic byproducts from infected RBC cause rheological alteration and RBC aggregation. Malaria culture supernatant contains a significant high concentration of MetHb as an aggregant to exhibit RBC aggregation. Ammonium sulfate fractionation and immuno-depletion of culture supernatant further confirms the MetHb as a major aggregant present in parasite culture supernatant. In-vitro treatment of RBC with MetHb induces irreversible high order RBC aggregates, resistant to the shear stress and physical forces. MetHb mediated ROS accumulation in the external microenvironment to develop oxidative stress close to RBC membrane seems to be responsible for initiating and forming high order RBC aggregates through PS externalization. Removal of oxidative stress through antioxidant treatment abolishes the MetHb mediated high order RBC aggregate formation. In conclusion, we discovered a novel pathway of MetHb mediated RBC aggregation and its potential role in patho-physiological effects during malaria. As an aggregation promoter, MetHb role in RBC-endothelial cyto-adhesion, macrophage-endothelial cyto-adhesion can be study in future to understand vascular blockage during severe malaria.

Chapter 4. Methemoglobin contributes into primaquine toxicity through single electron oxidation and modification*

4.1 Introduction

Malaria, a tropical disease caused by *Plasmodium* species and it leads to 200,000 deaths every year. The disease in human starts with the bite of anopheles mosquito and it replicates in host liver and Red blood cells (Sachs and Malaney, 2002). In addition, parasite resides within the hepatocytes under dormant stages (hypnozoites) and causes disease on a preset time scale, a process known as relapses. Reappearance of disease on a frequent basis (monthly or quarterly) without reinfection causes an immense burden on host machinery to contribute into the observed deaths and be responsible for an additional level of complication to treat malaria (White, 2011). Chloroquine or artimisinin are the drugs to target blood stages of parasite where as PQ is the only drug available for clearing hypnozoites from hepatocytes (Baird and Hoffman, 2004; Sinclair et al., 2012). Primaquinine dosing is more important for patients with *P.vivax* to avoid potential relapses within weeks or even in months (Anthony et al., 2012). But prolonged treatment is known to cause methemoglobinemia, hemolysis, abdominal cramps and patho-physiological symptoms are more severe in the patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency (Baird, 2012; Taylor and White, 2004). G6PD is involved in regeneration of NADPH to support lipid biosynthesis, redox reactions and maintenance of intracellular glutathione (GSH) level (Stanton, 2012). The G6PD deficient RBCs accumulate reactive oxygen species (ROS) and makes RBCs prone to further oxidative damage (Beutler et al., 2007). PQ is oxidatively metabolized to carboxy PQ (CPrq), 6-methoxy 8-aminoquinoline (MAQ), hydroxylated derivatives (Avula et al., 2013; Frischer et al., 1991; Mihaly et al., 1984). Redox reaction of the PQ metabolites with cellular and antioxidant enzymes disturb oxidative balance to exhibits toxicological effects, such as hemolysis (Fletcher et al., 1988). Interaction of PQ oxidative metabolites with the RBC follow different mechanisms to cause hemolysis. 6-methoxy-8-hydroxylaminoquinoline (MAQ) causes oxidative damage to membrane lipids where as 5-hydroxyprimaquine (5-HPQ) causes injury to the cytoskeleton (Bowman et al., 2005a; Bowman et al., 2005b; Bowman et al., 2004). 5 hydroxy primaquine can able to withdraw an electron to the environment easily and this nature favours the hemoglobin to MetHb conversion (Liu et al., 2013). MetHb further cause RBC lysis through oxidative stress generation (Chapter 2 & 3). But still PQ metabolism and molecular assembly involved in the generation of oxidative metabolites are not conclusive.

Two major metabolic pathways have been presumed after an oral dose of PQ. The first pathway produces carboxy-primaquine, the major non-toxic metabolites detected in plasma (Frischer et al., 1991). The 2nd pathway involves production of oxidative metabolites responsible for PQ associated cyto-toxicity and hemolysis (Croft, 2001). In-vitro incubation of PQ with liver microsomal fraction or mitochondrial lysate indicates that microsomal enzymes preferentially process PQ to 5-hydroxy primaquinine where as mitochondrial fraction processed it to carboxyprimaquine. Co-incubation of PQ with recombinant cytochrome P450 isoform generates metabolites with potentials to cause MetHb formation and hemotoxicity (Ganesan et al., 2009). Production of reactive oxygen radicals, generation of MetHb and depletion of GSH is associated with hemotoxicity of PQ and PQ oxidative metabolites (Ganesan et al., 2012).

During malaria lysis of infected RBC releases hemoglobin, hemin, hemozoin and other toxic metabolites into the serum (Anstey et al., 1996). The released hemoglobin is readily being oxidized to form ferryl hemoglobin and MetHb (Reeder, 2010). The level of MetHb in the clinical studies and post antimalarial chemotherapy varies from 1-3.5 mg/ml and it depends on factors such as host age, sex, other clinical conditions, parasite strain, genetic background etc (Anstey et al., 1996; Carmona-Fonseca et al., 2009) In chapter 2, we have examined the role of extracellular MetHb in catalyzing the accelerated destruction of RBC. Extracellular MetHb disturb internal oxido-reductase balance of RBC by generating a ROS spike utilizing its pseudoperoxidase activity . In this chapter, we explored the role of extracellular MetHb in modifying PQ to oxidative metabolites (hydroxyl or methoxy derivatives) or PQ associated free radicals and enhancing hematotoxic effect of the parent molecule. Our result present in current chapter indicates that MetHb accepts PQ as a substrate and oxidizes it through a single electron transfer mechanism. The oxidation product is a hydroxyl and desamino PQ derivative as evidenced by LC-MS/MS analysis of the reaction mixture. PQ derivatives have potential to disturb antioxidant potentials of RBC by inhibiting antioxidant enzymes and generating hydroxyl radicals to develop oxidative stress. The PQ peroxidation product interacts with RBC and disturbs the RBC membrane structure as evident from SEM analysis. Hence, extracellular MetHb incites the PQ for hemolytic activity and can be a novel mechanism associated with PQ hemotoxicity.

* The content of present chapter is published as "**Methemoglobin incites primaquine toxicity through single electron oxidation and modification**, **S N Balaji** and Vishal Trivedi*, J Basic Clin Physiol Pharmacol. 2013;24(2):105-14".

4.2 Methods

4.2.1 Optical Spectral studies: Optical spectra were recorded in a total volume of 1ml containing 1.55 µM MetHb in 100 mM Tris pH 7.2 in a Cary 100 Bio UV Visible spectrophotometer-Varian, Australia at 25 °C.

4.2.2 Binding of PQ to MetHb: Optical difference spectra were recorded in a total volume of 1 ml containing MetHb in 100 mM Tris-HCl buffer, pH 7.2 in a Cary 100 Bio UV Visible spectrophotometer-Varian at 37ºC with quartz cells of 1 cm light- path. Binding of PQ to MetHb-H_2O_2 complex was monitored at different concentrations (22-133 µM). The equilibrium dissociation constant (K_D) for complex formation was calculated by using equation 4.1.

$$1/\Delta A = (K_D/\Delta A_\alpha)\ 1/S + 1/\Delta A_\alpha \quad \ldots\ldots \text{ Eq. 4.1}$$

where K_D is the dissociation constant of the MetHb-PQ complex, S is the concentration of PQ, ΔA is the observed absorption change at a particular wavelength, and ΔA_α is the absorption change at a saturating concentration of the ligand.

4.2.3 Identification and characterization of PQ peroxidation product: PQ (1.5 mM) was incubated in the reaction mixture containing MetHb (6.2 µM), and H_2O_2 (100 µM) in phosphate buffer pH 7.0 for 1h at 37ºC. The MetHb was removed from reaction mixture by 5 % TCA precipitation and PQ oxidation products were analyzed in ultra-performace liquid chromatography attached to mass spectrophotometer as described (Dongre et al., 2008) in Waters Q-Tof Premier & Aquity UPLC.

4.2.4 Hematocrit preparation: 5 % RBC hematocrit was prepared as described earlier in chapter 2, section 2.2.2., Page no. 39.

4.2.5 Oxidative stress measurements: RBCs treated with Pq peroxidation product (Pq_{ox}) for (0-30mins) at 37ºC and ROS level, lipid peroxidation and protein carbonyl level was measured as described earlier in Chapter 2, section 2.2.6, Page no. 40, section 2.2.7, Page no. 41 & section 2.2.8, Page no. 41.

4.2.6 Superoxide Dismutase (SOD) Assay: RBC hematocrit (5 %) treated with PQ peroxidation product (PQ_{ox}) for 30 min at 37ºC and activity of superoxide dismutase (SOD) was measured as described earlier (Monk et al., 1987). In brief, RBCs were lysed with 200 µl distilled water and interfering Hb was removed by solvent fractionation using chloroform:ethanol (1:1) mixture. The Hb free lysate was added to the assay mixture containing NBT (50 µM), methionine (3 mM), and EDTA (0.1 mM) in 0.1 M phosphate buffer system at pH 7.4. Riboflavin added to the reaction mixture (4 µM) last and production of superoxide was initiated by exposing the reactions to the UV illumination light for 30 min at room temp. The absorption was measured at 585 nm in spectromax $M2^e$ (Molecular devices) and used to calculate SOD activity. RBCs treated with buffer considered as 100 % activity and used to calculate % inhibitions with different treatments.

4.2.7 Glutathione-s-transferase (GST) Assay: RBC hematocrit (5 %) treated with PQ peroxidation product (Pq_{ox}) for 30 min at 37°C and activity of Glutathione-s-transferase (GST) was measured as described earlier (Habig et al., 1974). In brief, 50 µl RBC lysate was added to the 950 µl phosphate buffer (0.1 M, pH 7.4) containing 1 mM 1-chloro 2,4 dinitro benzene (CDNB) and GSH (1 mM). The progress of enzymatic conversion was monitored at 340 nm for 5 min with a 1 min interval in

spectramax M2^{e} (Molecular Devices). RBCs treated with buffer considered as 100 % activity and used to calculate % inhibitions in different treatments.

4.2.8 Catalase (CAT) Assay: RBC hematocrit (5 %) treated with PQ peroxidation product (PQ_{ox}) for 30 min at 37ºC and activity of catalase (CAT) was measured as described earlier (Beers and Sizer, 1952). In brief, 100 µl of RBC lysate was added to the 900 µl 50 mM phosphate buffer containing hydrogen peroxide (15 mM) and progress of enzymatic conversion of H_2O_2 to H_2O was monitored at 240 nm at 25°C for 30s with 1s interval. RBCs treated with buffer considered as 100 % activity and used to calculate % inhibitions in different treatments.

4.2.9 SEM Sample preparation: RBC hematocrit (5%) treated with PQ peroxidation product (PQ_{ox}) for 30 min at 37ºC and samples for scanning electron microscope (SEM) was prepared as mentioned in chapter 3, section 3.2.10, page no. XX. A total of 10 fields were identified randomly and observed with LEO 1430VP Scanning Electron Microscope. The instrumental setting such as EHT, width and signal were 10 kV, 18 mm and SE1 respectively.

4.2.10 Measurement of MetHb level: MetHb level was measured in PQ peroxidation product exposed RBCs as described earlier in chapter 3, section 3.2.4, page no. 51.

4.2.11 Hemolysis and osmotic shock measurement: RBC hematocrit (5 %) treated with PQ oxidation product (PQ_{ox}) for 30 min at 37ºC and osmotic shock and hemolytic assay was performed as described earlier in chapter 2, section 2.2.3, page no. 41. RBCs incubated with buffer is used as a control whereas 1 % (v/v) triton x-100 treated samples were considered as 100 % hemolysis.

4.2.12 Statistical Analysis: Statistical analysis was performed using the student t-test (MICROSOFT EXCEL 2007) and Anova module of Sigma plot 11 (Systat Software, Inc., USA). A p-value < 0.05 was considered significant.

4.3 Results

4.3.1 Metehemoglobin accepts PQ as a substrate: Binding of a substrate(s) to MetHb is essential for the successful oxidation of PQ to PQ_{ox}. It binds to the MetHb as evidenced in the decrease of absorbance at 405 nm in a difference spectroscopic studies with a binding constant of 6.4 µM (Figure 4.1-A). Peroxidase have potentials to oxidize various aromatic and halide substrates through a single electron transfer mechanism involving two or three stage transitions. An optical spectral study was performed to assess the ability of MetHb to accept and oxidize PQ as substrate. In the presence of H_2O_2 MetHb form compound II with a spectral shift from 405 nm (Figure 4.1-B, spectrum a) to 415 nm (Figure 4.1-B, spectrum b). Addition of Pq causes a spectral shift back to 408 nm with single electron transfer to PQ for oxidation (Figure 4.1-B, spectrum c). Hence, figure 4.1 support the notion that Methb has a well defined binding pocket to support binding of PQ, accept it as a substrate and oxidizes it following single e^{-} oxidation mechanism.

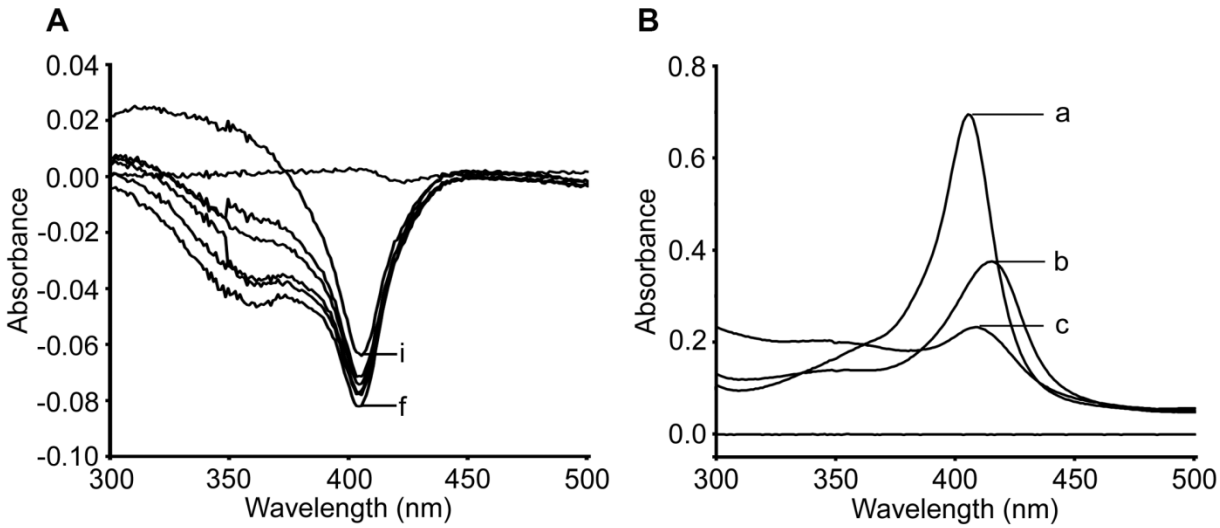

Figure 4.1: Methemoglobin has potentials to bind and oxidize PQ. (A) PQ binds to MetHb. Optical difference spectra of PQ with MetHb (i, initial scan at 22 µM; f, final scan at 133 µM). The plot of 1/ΔAbsorbance (nm) versus 1/[PQ] was used to calculate the K_D. (B) MetHb oxidizes PQ following single electron oxidation mechanism. In a total volume of 1ml containing 100 mM Tris-HCl buffer, pH 7.2, soret spectrum of (a) native MetHb (1.5 µM), (b) a+ H_2O_2 (140 µM); (c) b+PQ (22 µM) were recorded.

4.3.2 Characterization of PQ oxidation products: To further understand MetHb mediated potential PQ modifications, PQ (1.5 mM) was incubated with MetHb (6.2 µM), and H_2O_2 (100 µM) in reaction buffer (0.1 mM phosphate buffer pH 7.0 containing 1 mM DTPA) and oxidized PQ was isolated from the reaction mixture for ultra-performace liquid chromatography attached to mass spectrophotometer as described (Dongre et al., 2008). The developed method is sensitive to detect impurities and the chemical modification of PQ. The mass spectroscopy analysis of UPLC eluent indicates a chemical modification of PQ. The spectrum contains unmodified molecular PQ peak with m/z 260.43 ($C_{15}H_{21}N_3O$), hydroxylated PQ with m/z 300.53 ($C_{15}H_{21}N_3O_2$+Na+H) and desamino PQ peak with m/z 243.42 ($C_{15}H_{19}N_2O$) (Figure 4.2-C). There are other prominent peaks present in the spectrum but those peaks are not specific to peroxidation reaction catalyzed by MetHb and are present in the spectrum from PQ incubated in buffer alone (Figure 4.2-A) or PQ isolated from reactions without MetHb (Figure 4.2-B). MetHb in aqueous environment generates high level of hydroxyl radicals (OH^*) due to fenton reaction catalyzed by protein released iron (Reeder, 2010). But EDTA (1 mM), a known chelator was added to the reaction buffer to exclude the contribution of trace metal ions from MetHb on the appearance of PQ peroxidative metabolites peaks in spectrum.

4.3.3 Extracellular MetHb potentiates the Pq mediated methemoglobin generation in RBC: The yield of PQ peroxidation products is less than 0.5 % and a concentration of 7.5 µM PQ_{ox} is formed in the reaction mixture. Treatment of human erythrocytes in vitro with either PQ or MetHb for 30 min at 37°C causes a significant increase in MetHb formation in the RBC as mixture compare to PBS treated cells (Table 4.1). A robust increase in the formation of MetHb was noticed

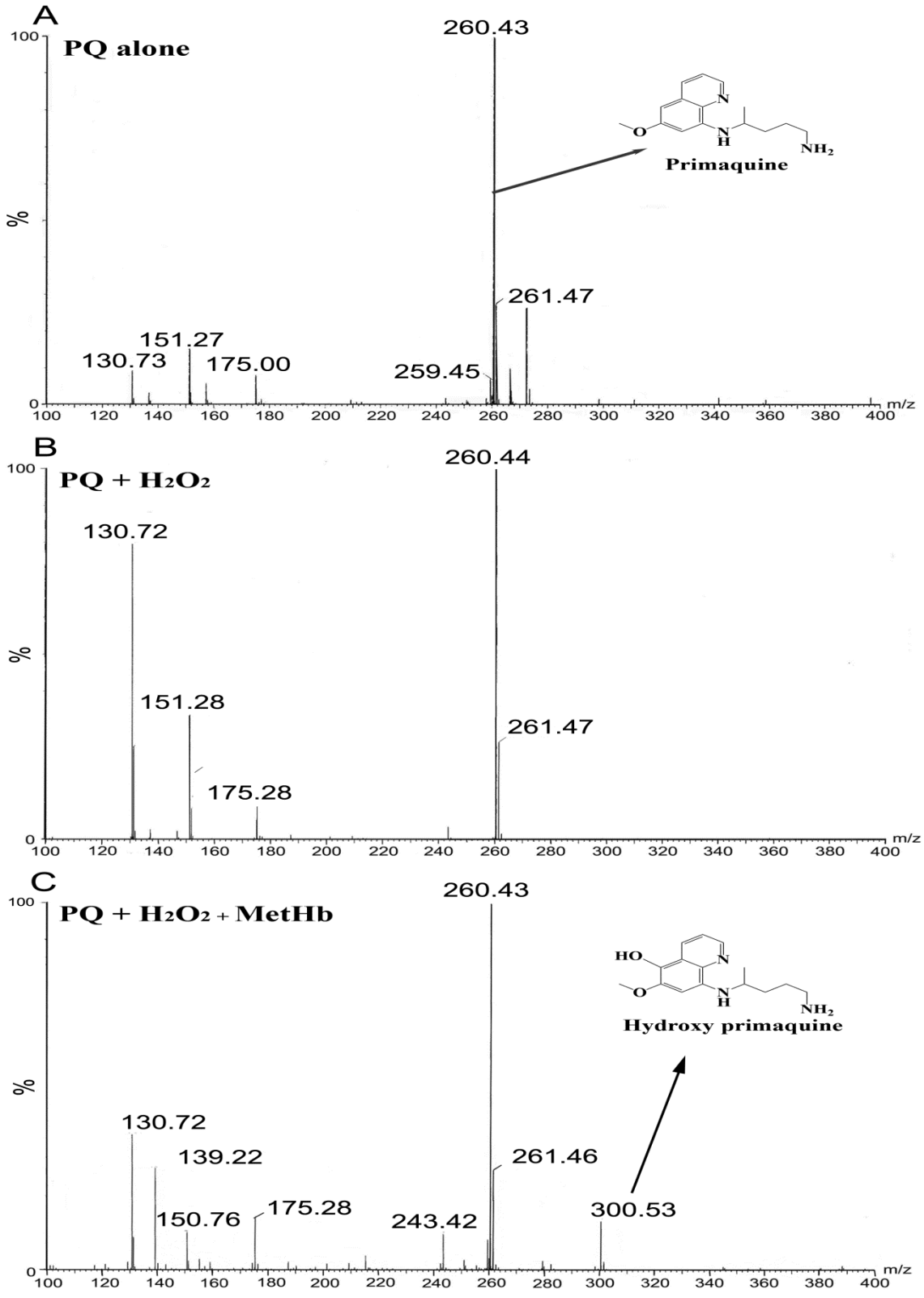

Figure 4.2: Primaquine incubated with MetHb generates PQ peroxidative metabolites. PQ (1.5 mM) was incubated into the reaction mixture containing either H_2O_2 (100 µM) or MetHb (6.2 µM) with H_2O_2 (100 µM) in phosphate buffer pH 7.0 for 1h at 37°C. The ESI - mass spectrum of the (A) PQ (B) PQ from a reaction mixture containing H_2O_2 (100 µM) or (C) PQ from a reaction containing MetHb (6.2 µM), and H_2O_2 (100 µM) in phosphate buffer pH 7.0.

when the erythrocytes were treated with PQ in the presence of MetHb and H_2O_2 (Table 4.1).The PQ induced MetHb formation were almost 2 folds high in the presence of MetHb and H_2O_2 as compared

to that observed with the parent drug molecule (P value: PQ vs PQ_{ox}, 0.001). The observation indicates direct involvement of PQ peroxidation by the MetHb to produce hemotoxic product(s).

Table 4.1: MetHb potentiates primaquine mediated MetHb generation.

Conditions	MetHb Amount (mg/ml)
PBS	**1.7 ± 0.3**
PQ	**7.7 ± 0.39**
MetHb	**7.2 ± 0.38**
H_2O_2	**2.8 ± 0.2**
MetHb + PQ + H_2O_2	**16.7 ± 0.9**

RBCs were treated with MetHb generated PQ for 30 min at 37°C and intracellular MetHb level was measured in RBC lysate as described in 'Methods'. RBCs treated with individual reaction constituents, MetHb, H_2O_2, PQ to test the effect of reactants on the conversion of hemoglobin to MetHb.

4.3.4 Primaquine peroxidation products hamper antioxidant potentials of RBC to cause oxidative stress: RBC antioxidant potential is determined by efficiency of antioxidant enzymes to recycle reducing equivalents and scavenging of free radicals with other small molecule antioxidants such as GSH (Parcell, 2002). PQ oxidative metabolites are known to severely affect RBC antioxidant potentials to causes generation of MetHb, development of oxidative stress and RBC lysis (Ganesan et al., 2009). RBCs treated with PQ isolated from different reaction mixture as given in Table 4.2 and activity of superoxide dismutase (SOD), glutathione S transferease (GST) and catalase (CAT) in RBC lysate was measured as described in methods. RBCs treated with a PQ peroxidation product causes 37 %, 25 %, 51 % inhibition of SOD, GST and catalase activity respectively (Table 4.2). The

Table 4.2. Primaquine peroxidative metabolites is involved in inhibition of RBC antioxidant enzymes.

Conditions	SOD (% Inhibition)	GST (% Inhibition)	CAT (% Inhibition)
Buffer Only	0.0 ± 0.3	0.0 ± 0.7	0.0 ± 2.2
MetHb	2.5 ± 1.4	1.4 ± 3.89	7.2 ± 1.2
H_2O_2	7.1 ± 1.7	1.8 ± 1.0	1.7 ± 5.8
PQ	5.6 ± 2.7	5.1 ± 2.9	3.5 ± 5.1
MetHb + PQ + H2O2	36.8 ± 3.2	25.2 ± 1.0	51.2 ± 0.9
PBN + MetHb + PQ + H2O2	8.3 ± 2.1	9.0 ± 0.9	13.9 ± 1.5

PQ was incubated with MetHb and H_2O_2 for 60 min at 37°C with intermittent shaking and modified PQ was isolated as described in method section. RBCs were treated with isolated modified PQ for 30 min at 37ºC and activity of superoxide dismutase (SOD), glutathione S-transferase (GST) and catalase (CAT) was measured from RBC lysate as described in method section. Activity of antioxidant enzymes from RBCs treated with buffer for 30 min at 37ºC was considered as 100 % and used to calculate % inhibition in enzymatic activity. RBCs treated with individual reaction constituents (MetHb, H_2O_2, PQ) to rule out the effect of contamination of remained reactants on the activity of antioxidant enzymes. To probe the role of PQ peroxidation reaction to form oxidative metabolites, PQ was incubated with MetHb and H_2O_2 in the presence of spin trap, PBN (5 mM) for 30 min at 37°C with intermittent shaking and PQ was isolated as described in method section.

RBCs incubated with PQ or other reactant individually cause a low level of inhibition of antioxidant enzymes. Inhibition of antioxidant enzymes will disturb the oxidation/reduction balance of cell and causes development of oxidative stress (Kiefer and Snyder, 2000). As expected, RBCs treated with PQ from complete reaction causes a time dependent increase in ROS level with a sharp peak at 15 min (Figure 4.3-A). Intracellular ROS in turn causes membrane lipid peroxidation and oxidation of cytosolic proteins. An increase in lipid peroxidation and protein carbonyl level was observed with a peak at 15 min (Figure 4.3-B & C). RBCs treated with PQ incubated in reaction buffer causes a very mild increase in ROS level, lipid peroxidation and protein carbonyl level (Figure 4.3). As GST activity is severely affected by PQ oxidative metabolites, we expect a decrease in the GSH pool of the cell but due to technical difficulty(cross reactivity of PQ with reagents to give high background), we failed to measure reduced glutathione (GSH) level in exposed RBCs.

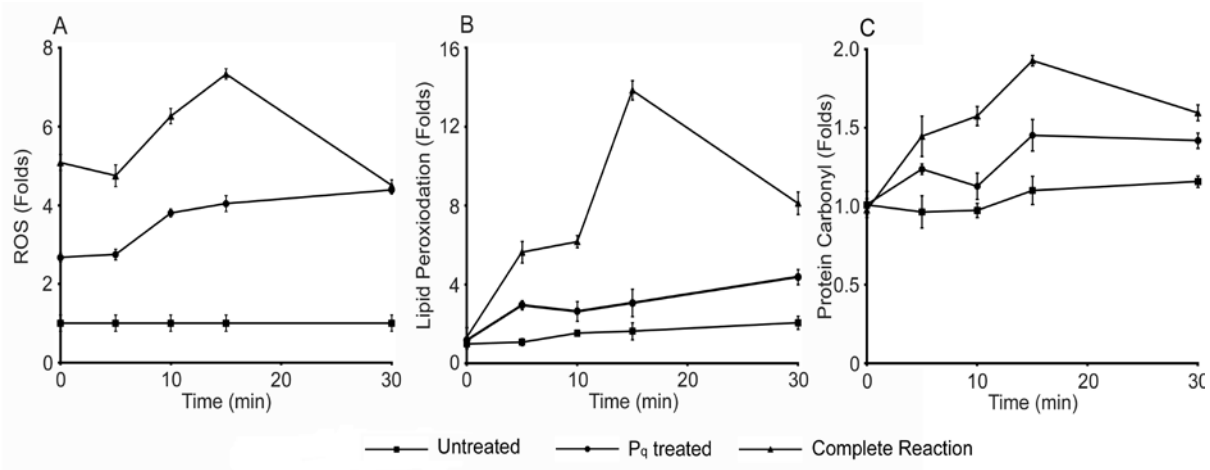

Figure 4.3: Methemoglobin generated PQ peroxidative metabolites causes development of oxidative stress in RBCs. RBCs treated with MetHb generated Pq oxidative metabolite causes (A) increase in ROS (B) lipid peroxidation (C) protein carbonyl levels. RBCs treated with Pq isolated from the reaction mixture containing MetHb (6.2 µM), and H_2O_2 (100 µM) in phosphate buffer pH 7.0 for 0-30 min at 37°C. Post treatment, ROS, lipid peroxidation and protein carbonyl level were measured as described in material and methods. RBCs treated with phosphate buffer was used to calculate fold change in oxidative stress indices. Data are the mean ±SD of four independent experiments (n=4) with triplicate measurement. The pairwise results were analyzed with Anova & student t-test and it was statistically significant with $P < 0.001$.

4.3.5 Primaquine peroxidative metabolites alter RBC membrane morphology. Exposure of RBCs to MetHb generated PQ peroxidative metabolites causes severe damage to the RBC membrane morphology and size as evident from scanning electron microscopic analysis. The RBCs exposed to PQ peroxidative metaboilites generated in the complete reaction lost the biconcave shape morphology and become spherical (sphereocytes), increase in diameter and injury to the cytoskeleton supporting membrane structure (Figure 4.4). Results revealed a high degree of disturbance of RBC membrane integrity with visible holes, damages to membrane protein. The RBCs exposed to PQ incubated in reaction buffer shows biconcave morphology with no visible sign of drug toxicity.

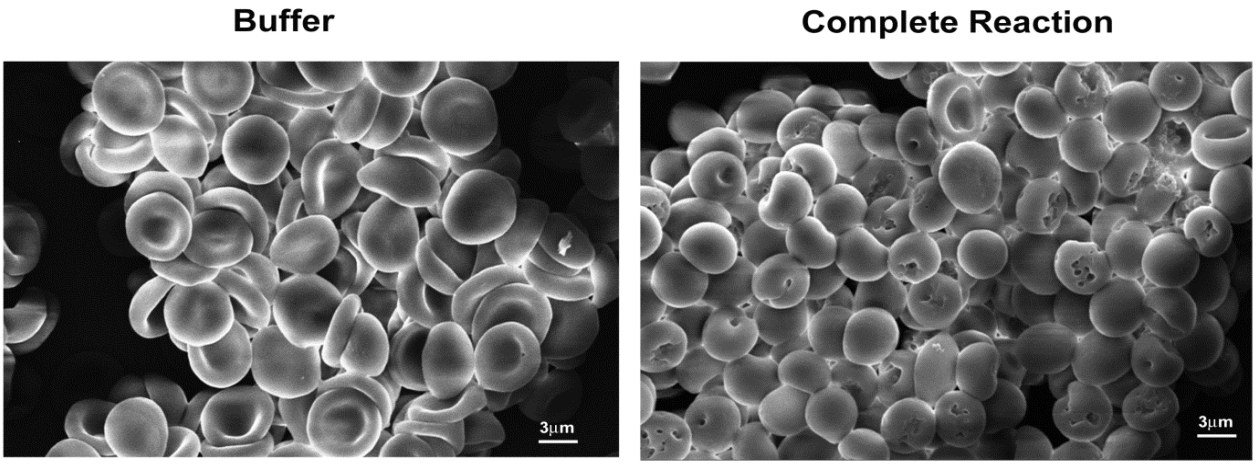

Figure 4.4: Pq peroxidative metabolite exposure disturbs the RBC membrane structure. RBCs were treated with PQ from buffer or Acomplete reaction for 30 min and images from a total of 10 different fields in both samples were captured with LEO 1430VP scanning electron microscope. The instrumental setting EHT, width and signal were 10 KV, 18 mm and SE1 respectively. A representative image of PQ from buffer (magnification; x2500) or complete reaction (magnification; x2500) is given.

4.3.6 Methemoglobin mediated primaquine peroxidation aggravate its hemolytic potentials: PQ resuspended in reaction buffer has potential to cause osmotic stress in RBC cells (Figure 4.5-A) and has very little ability to induce hemolysis (Figure 4.5-B). PQ isolated from a reaction mixture containing H_2O_2 or MetHb separately doesn't help the parent drug to exhibit additional hemolytic activity (Figure 4.5-B). But PQ isolated from a reaction mixture containing MetHb, H_2O_2 causes a

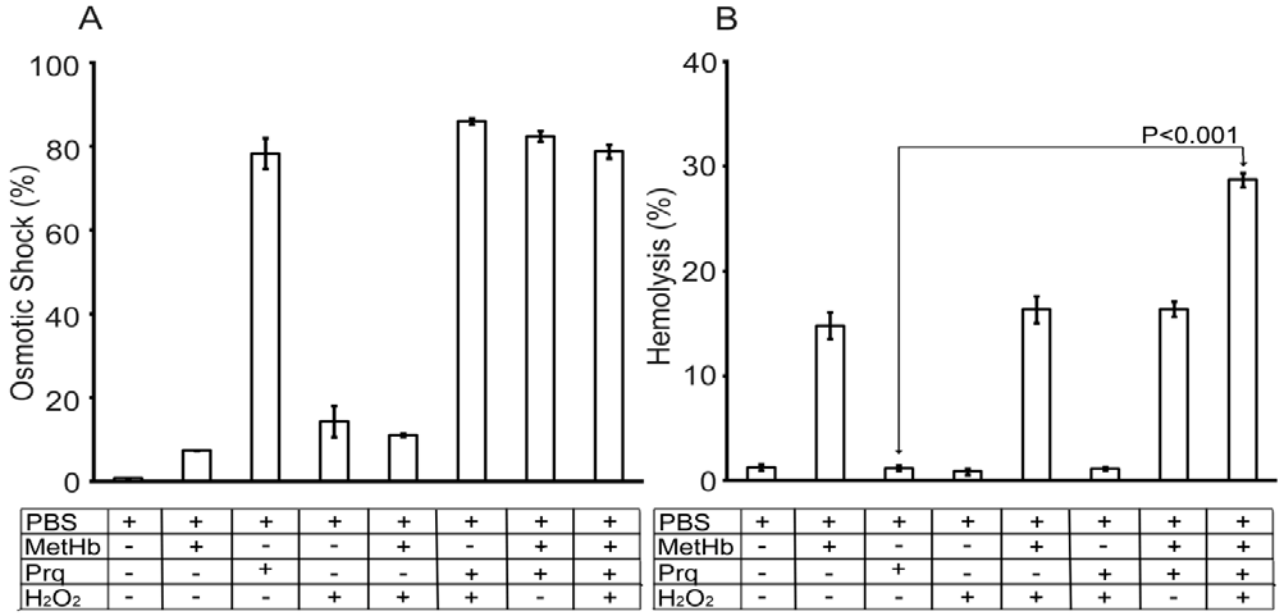

Figure 4.5 PQ peroxidative metabolites are hemolytic in nature. PQ (1.5 mM) was incubated into the reaction mixture containing H_2O_2 (100 µM) or MetHb (6.2 µM), and H_2O_2 (100 µM) in phosphate buffer pH 7.0 for 1 hr at 37°C. RBC hematocrit (5 %) prepared in PBS was exposed to PQ isolated from different reaction mixture as indicated for 30 min at 37°C and **(A)** Osmotic fragility or **(B)** hemolysis was measured as described in the material and method section. Data are the mean ± SD of three independent experiments (n=3) with triplicate measurement. The pairwise results of PQ alone and PQ+MetHb+H_2O_2 were analyzed with Anova & student t-test and it was statistically significant with P <0.001.

1.5 fold enhanced hemolysis compared to individual effects of these compounds separately (Figure 4.5-B). The hemolysis was statistically significant (P value: PQ vs PQ_{ox}, 0.001). As noted before, these effects are due to MetHb mediated PQ oxidation and generation of oxidative metabolites. The result suggests that MetHb in the presence of H_2O_2 oxidizes the PQ to stimulate molecular property of the PQ to lyse the RBCs more efficiently. In addition to PQ oxidative metabolites, molecular interaction of MetHb with PQ and H_2O_2 may give rise to hydroxide radical ($^{*}OH$), superoxide radical (O_2^{*-}) and other single electron species. To probe the role of particular free radical species in MetHb mediated PQ hemolytic potential, we incubated the RBCs with different antioxidant molecules (to probe the role of oxidants) or with spin trap TEMPO, PBN (to probe the role of single electron species). Antioxidants scavenging hydorxy radical, superoxide radical and hydrogen peroxide has very little or no effect on MetHb mediated PQ potentiation where as spin trap TEMPO, PBN pre-treatment abrogated the PQ hemolytic potentiation (Table 4.3). It indicates the probable role in the process. To further understand the role of spin-trap in protecting RBCs, we measured the activity of antioxidant enzymes. Surprisingly spin-trap reverses the PQ oxidative metabolites mediated inhibition of antioxidant enzymes and protects the enzymes (Table 4.2). It could be due to scavenging potentials of spin-trap to restore oxidative imbalance or a shielding effect around antioxidant enzymes to protect it from being getting inactivated to improve the cells ability to maintain oxidative balance.

Table 4.3: Effect of antioxidants and spin traps on MetHb mediated Primaquine hemolytic potentiation.

S.No.	Conditions	Hemolysis (%)
1	MetHb + PQ + H_2O_2	28.72 ± 0.67
2	MetHb + PQ + H_2O_2+Vit C	28.7 ± 0.2
3	MetHb + PQ + H_2O_2 + NAC	27.7 ± 1.43
4	MetHb + PQ + H_2O_2 + Mannitol	27.0 ± 1.7
5	MetHb + PQ + H_2O_2 + DMSO	25.8 ± 2.1
6	MetHb + PQ + H_2O_2 + Thiourea	26.1 ± 0.9
7	MetHb + PQ + H_2O_2 + Quercetin	26.1 ± 2.6
8	MetHb + PQ + H_2O_2 + GSH	23.2 ± 2.4
9	MetHb + PQ + H_2O_2 + TEMPO	11.1 ± 1.1
10	MetHb + PQ + H_2O_2 + PBN	16.9 ± 1.0

RBCs treated with PQ peroxidation product for 30 min in the absence or presence of vit C (40 µM), NAC (5 mM), mannitol (2.5 %), DMSO (0.5 %), thiourea (0.5 %), quercetin (0.5 %), GSH (4 mM), TEMPO (10 mM), PBN (5 mM) at 37°C and hemolysis was measured as described in method section. Data are the mean ± SD of two independent experiments (n=2) with triplicate measurement.

4.4 Discussion

Primaquine metabolism and mechanism of hemotoxicity are not clear. The series of studies indicate an active involvement of liver microsomal enzymes in accepting and converting PQ into the different oxidative metabolites. Primaquinine is oxidatively metabolized to carboxy primaquine

(CPrq), 6 methoxy 8 aminoquinoline (MAQ), hydroxylated derivatives (Avula et al., 2013; Croft, 2001; Frischer et al., 1991; Mihaly et al., 1984). The oxidative metabolites are highly reactive in nature and have potential to cause oxidative insult to the RBC by MetHb generation, ROS accumulation (Ganesan et al., 2009; Liu et al., 2013). The proportions of hemotoxic PQ oxidative metabolites are minor as compared to non-toxic carboxy primaquine. In addition, PQ oxidative metabolites have a short lifespan (Strother et al., 1981) and it is not clear if reactivation is required near RBC surface to exhibit hemotoxicity under in-vivo conditions.

Primaquine hemotoxicity to RBCs is associated with MetHb generation and reduction in antioxidant potentials of RBCs to maintain oxidation/reduction balance (Bowman et al., 2004). MetHb is central to disturbance of oxidation/reduction balance of RBCs and preformed MetHb level contributes significantly into the drug mediated hemotoxicity (Carson et al., 1981). Beagle Dog RBCs with an inherent deficiency to control the level of MetHb are more susceptible to the PQ toxicity. In a normal human, PQ metabolites cause ~30% MetHb formation compared to dog RBC with ~60% MetHb formation (Lee et al., 1981). Although previous studies provide indirect support for involvement of MetHb but the current study is the first direct evidence of MetHb with PQ hemotoxicity. MetHb has a well-defined binding site for PQ and it accepts PQ as substrate and oxidizes it through single electron oxidation (Figure 4.1). PQ oxidation products are hydroxylated or desamino derivatives of PQ (Figure 4.2). The determination of the exact chemical nature of derivatives is on its way as the yield of hydroxylated or desamino PQ derivative is very low (~0. 5%) and scaling up the reaction with more amounts of the enzyme/H_2O_2 gives nonspecific conversion of PQ as well as chemical degradation of the parent drug (Carson et al., 1981). PQ oxidation product cause oxidative insults to RBCs, inhibit antioxidant enzymes and enhances hemolytic potentials of the quiescent parent drug. In an earlier study PQ was found to inhibit cytochrome P450 class enzyme CYP-2D6 and underlying mechanism involves utilization of PQ as a substrate by CYP-2D6 (Li et al., 2003). PQ oxidation by cytochrome P450 class enzyme CYP-2D6 is responsible for inhibition and a similar mechanism behind the inhibition of RBC antioxidant enzymes cannot be ruled out. The exact mechanism of PQ oxidation products mediated inhibition of RBC antioxidant enzymes to disturb oxidation/reduction might be interesting to control the toxic hemolytic effects of the drug. Oxidative metabolites of PQ interact with RBC following different mechanism to cause hemolysis (Bowman et al., 2005a; Bowman et al., 2005b; Bowman et al., 2004). 6-methoxy-8-hydroxylaminoquinoline (MAQ) causes oxidative damage to membrane lipids where as 5-hydroxyprimaquine (5-HPQ) causes injury to the cytoskeleton (Bowman et al., 2005a). A SEM analysis of RBCs exposed to PQ peroxidation products give direct evidences of injury to the RBC cytoskeleton and plasma membrane proteins with visible holes (Figure 4.5). This is the first structural study to explain the PQ activation (either by oxidation as in the case of cytochrome P450 or peroxidation as in the case of MetHb) as a crucial event for observed perturbance in RBC membrane structure. It is difficult to conclude which

molecular event is primary; generation of PQ oxidation products and their direct interaction to the RBC membrane or disturbance of RBC metabolism to cause oxidative damage to cellular components and membrane lipids. Exposure of the infected RBC to PQ results in swelling of parasite mitochondria and these effects are more pronounced in schizont than free form merozoite (Beaudoin and Aikawa, 1968). Malaria parasites depend on hemoglobin metabolism for supply of essential amino acids to support the growth of the parasite. The level of MetHb is highest in the trophozoite and schizont stages whereas merozoite is a free form of the parasite with low MetHb level (Kamchonwongpaisan et al., 1997). Although no direct correlation has been established so far but MetHb generation might have an indirect role in PQ activity toward the parasite as well.

Oxidative insult to RBCs by drug or other chemical agents result in generation of MetHb and hemolysis. However, methemoglobinemia doesn't necessarily leads to the hemolysis (Hall et al., 1986). Hemolysis is a two-step process; initial sensitization by severe damage to RBC membrane to compromise membrane fragility and then actual hemolysis by subsequent stress either by another agent or physical forces such as shear stress (Brumen and Heinrich, 1984; Wagner et al., 2003). A molecule may have potential to execute the both events or another molecule is required to execute 2^{nd} step for robust hemolysis. PQ alone has potential to sensitize the RBC membrane to make it osmotically fragile but lacks ability to catalyze hemolysis (Figure 4.5-A). Where as PQ oxidation/peroxidation generates molecular species with potentials to exhibit severe hemolysis (Figure 4.5-B). A detail structure-function study is required to understand the molecular changes in PQ structure and hemolytic potentials. Our study highlights the importance of MetHb in enhancing hemotoxic potentials of PQ through utilizing its peroxidase activity and a MetHb specific inhibitor may be explored to control hemotoxicity to expand the therapeutic potentials of PQ.

4.5 Conclusions and future prospectus

The MetHb- H_2O_2 system transforms quiescent parent drug molecule to highly reactive oxidative form to exhibits severe hemolysis. MetHb-H_2O_2 mediated PQ hemolytic potentiation is sensitive to spin trap indicate the role of PQ***** radical or other single e-species in the process. MetHb stimulates the molecular property of PQ and peroxidase inhibitors can be explored to control drug associated toxicity.

Chapter 5. Suicidal inactivation of methemoglobin by generation of thiyl radical explains NAC mediated protection in RBC*

5.1 Introduction

Malaria caused by *P. falciparum* is associated with anemia, fever, multiple organ damage, renal failure, liver dysfunction and neurological disorders (Garcia-Garcia et al., 2012; MacPherson et al., 1985; Pasvol et al., 1982; Singer and Ataga, 2008). Reactive oxygen species (ROS) production and development of oxidative stress are crucial for disease pathology during malaria (Fibach and Rachmilewitz, 2008; Stocker et al., 1985). Pro-oxidant molecules released from parasitized RBCs (PRBC) or reactivity of immune cells toward PRBCs, mainly associated with development of oxidative stress (Schofield and Grau, 2005). Oxidative stress induces structural changes in RBC to activate immune cells and structurally altered RBCs tend to enhance vesicular blockage, cyto-adherence to promote cerebral malaria pathology (Dondorp et al., 2000; Pamplona et al., 2009). N-acetyl L-cysteine (NAC), a thiol (-SH) antioxidant, commonly used as a mucolytic agent to clear the respiratory tract blockages and recently used for adjuvant therapies such as paracetamol toxicity in liver cells, etc (Fitri et al., 2011; Kelly, 1998; Ziment, 1988). It improves antioxidant potentials of RBCs to give protections against oxidative stress induced hemolysis (Mazor et al., 1996). N-acetyl-l-cysteine, tested in a series of in-vitro drug toxicity studies or clinical studies to monitor malaria patient recovery during chemotherapy (Arreesrisom et al., 2007; Charunwatthana et al., 2009; Treeprasertsuk et al., 2003; Watt et al., 2002). Hemin released from an infected RBC decreased the RBC deformability, a cellular property required for RBC splenic clearance. NAC improves RBC deformability through reduction of hemin mediated RBC membrane oxidation and replenishing GSH reserves (Nuchsongsin et al., 2007). A number of critical parameters monitored to assess potentials of NAC as an adjuvant therapy during artesunate therapy in patients, but outcomes indicate that it made no significant improvement in lactate clearance times, mortality, coma recovery times or red cell rigidity (Charunwatthana et al., 2009).

Cyto-protective properties of NAC are due to direct interaction with a single electron containing free radical species, trapping toxic substances, inhibition of apoptotic and other inflammatory pathways or indirectly supports the synthesis of glutathione (GSH), a cellular antioxidant molecule (Sheffner et al., 1966; Stocker et al., 1985). The antioxidant potential of a cell determined by the level of GSH within the cytosol and maintained by the GSSG reduction through enzymatic activity of glutathione reductase (GR), non-enzymatic reduction by other small molecular weight antioxidants or de-novo synthesis of GSH (Parcell, 2002). NAC mediated free radical

scavenging is due to interaction of a free thiol group (-SH) to electrophilic ROS species (Ates et al., 2008; Bolli et al., 1989; Gaunt et al., 1981; Macone et al., 2011; Takashima et al., 2012). The kinetic measurement indicates a rapid interaction of NAC with hypochlorous acid (HOCl) and hydroxyl radical (OH^*), slowly with H_2O_2 and no interaction with superoxide radicals (O^*) (Aruoma et al., 1989; Bolli et al., 1989). NCB-20 cells treated with H_2O_2 found to reduce oxidative stress mediated NF-κB activation through the generation of NAC free radical (Kwak et al., 1995). The formation of NAC thiyl radicals within the cells or in a cell free system seems to be essential for biological activity of the molecule, but the cellular machinery for the NAC activation is not known (Harman et al., 1986; Sagrista et al., 2002; Subramanian et al., 2011).

The earlier biochemical model proposed that RBC lysis is a two step process, initial "sensitization" and then actual "lysis" (Beck and Saari, 1977). Conversion of hemoglobin to MetHb within RBC triggers the development of oxidative stress (sensitization) and causes structural membrane defects. Generation of MetHb mostly associated with the amplification of initial lysis and is responsible for enhanced hemolysis during malaria. The hemoglobin in the external microenvironment oxidized by molecular oxygen to form MetHb [Fe^{3+}] or oxidation by reactive oxygen species (ROS) into ferryl hemoglobin ($Fe^{[IV]}$=O). MetHb mediated ROS spike through the utilization of peroxidase activity is responsible for sensitization of RBC membrane to exhibit enhanced hemolysis (Trivedi et al., 2005c). In chapter 2, we have shown that NAC can be able to protect RBC from hemolysis only if it can be allowed to remove initial ROS spike in RBC cytosol. Sulfur containing compounds such as cysteine, GSH, methionine and NAC found to inhibit classical peroxidases following a non-competitive and mixed type inhibition mechanism (Gutierrez-Correa and Stoppani, 1999). MetHb exhibits a pseudo-peroxidase activity in the presence of H_2O_2 to oxidize organic and halide substrates (Reeder et al., 2004). In this chapter, we are showed that only two sulfur containing compounds, namely GSH and NAC inhibit MetHb peroxidase activity and give protection in the hemolysis. We explored the mechanistic details of NAC mediated inhibition of pseudoperoxidase activity of pro-oxidant MetHb with the accelerated destruction of RBCs. A number of evidences presented to show that NAC inhibits the MetHb peroxidase activity following a pseudo first order kinetics. MetHb-H_2O_2 active complex oxidizes NAC to form NAC free radicals (NAC^*) and NAC^* radical attacks on the heme of MetHb to form an irreversible heme-NAC complex. Furthermore, we have shown that formation of NAC^* is important for NAC binding to MetHb and provides protection against oxidative stress induced hemolysis. The studies thus provide additional insight into antioxidant mechanisms of NAC during oxidative stress induced hemolysis.

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5.2 Methods

5.2.1 Preparation of RBC from Human blood: Fresh blood collected in EDTA containing tubes and 5 % hematocrit was prepared in glucose containing PBS as described in chapter 2, section 2.2.2, page no. 39. In all of the experiments, RBC preparations contain less than 1 % other blood cells as monitored by blood smear preparation.

5.2.2 Measurement of RBC hemolysis: RBC preparations (5% hematocrit) treated with MetHb (800 µg, 15 min) or sodium nitrite (1 mg/ml, 2 hr.) in the presence and absence of NAC (0-7.5 mM) and hemolysis was measured as described in chapter 2, section 2.2.3, page no. 39. In the spin trap protection experiments, TEMPO, PBN was added to the RBCs prior to NAC and MetHb.

5.2.3 Assay of MetHb (peroxidise) activity in RBC: RBCs treated with sodium nitrite (1 mg/ml) in the presence or absence of different concentrations of NAC (0 - 2.5 mM) for a period of 2 hr. The RBCs washed twice with phosphate buffered saline (PBS) and MetHb was partially purified from control and NAC treated RBCs as described (Sugita et al., 1971). Peroxidase activity was measured from control, or NAC treated RBC lysate by following guaiacol oxidation as described previously in chapter 2, section 2.2.9, page no. 41.

5.2.4 Inhibition of MetHb by NAC: All kinetic measurements were made in infinite M 200 microtiter plate reader at 37±1ºC. To determine the peroxidase activity, guaiacol oxidation was monitored as described previously in a 0.3 ml assay volume (Trivedi et al., 2005b). The rate of inhibition of MetHb by NAC was measured by incubation of MetHb (2 µM) in the presence of H_2O_2 (100 µM) and NAC (0.04 - 0.4 mM) in a final volume of 20 µl containing 100 mM Tris-HCl buffer, pH 7.2. At different time intervals after addition of the inhibitor and H_2O_2, the incubation mixture transferred to 96 well plate containing 0.28 ml of assay mixture. During the course of the study of substrate or spin trap protection against inhibition, electron donor/spin trap were added to the incubation mixture containing the enzyme before the addition of NAC and H_2O_2.

5.2.5 Binding of NAC to MetHb: Optical spectra were recorded in a total volume of 1 ml containing MetHb in 100 mM Tris-HCl buffer, pH 7.2 in a Cary 100 UV Visible spectrophotometer (Varian, Australia) at 37±1°C with quartz cells of 1 cm light-path. Binding of NAC to native MetHb or MetHb-H_2O_2 complex was monitored at different concentrations (0 - 7.2 mM). Immediately after each addition of NAC, a soret spectrum was recorded. In the spin trap sensitivity experiment, TEMPO was added to MetHb-H_2O_2 complex prior to NAC.

5.2.6 Optical Spectral studies: Optical spectra were recorded in 100 mM Tris pH 7.2 containing 1 µM MetHb in a Cary 100 UV/VIS spectrophotometer at 25°C.

5.2.7 E. P. R. Studies: E. P. R. Spectra were recorded in 0.5 ml 100 mM Tris-HCl buffer, pH 7.2, MetHb (2 µM), 1 mM H_2O_2, 0.8 mM NAC, 1 mM DTPA and 90 µM dimethylpyrroline-N-oxide (DMPO) in an E. P. R spectrometer Jeol-JES FA200. Instrument settings were: microwave power 50

mW, microwave frequency 9.43 GHz, time constant 0.03s, modulation frequency 100 kHz and gain $2x10^4$.

5.2.8 Heme dissociation and ESI-mass analysis: MetHb (300 µM) inactivated in the presence of NAC (400 µM) and H_2O_2 (100 µM) for a period of 10 min at 37°C in 100 mM Tris-HCl buffer, pH 7.2. Acetone-HCl mixture is used to dissociated and extract heme from the inactivated enzyme (0.5 ml) as described (Trivedi et al., 2005b). The extracted heme-NAC complex is directly processed in liquid chromatography mass spectrometer (LC/MS/MS) waters Q-Tof Premier & Aquity UPLC.

5.2.9 Statistical analysis: Student t-test (MICROSOFT EXCEL 2007) used for statistical analysis and a p-value < 0.05 was considered significant.

5.3 Results

5.3.1 NAC abrogates MetHb pseudoperoxidase activity to protect RBC from hemolysis: Development of oxidative stress by MetHb within RBCs causes osmotic stress and hemolysis in a dose dependent manner as reported in chapter 2. Several sulfur containing compounds tested to protect RBCs from oxidative stress induced hemolysis. Sodium nitrite mediated RBC hemolytic model is used to assess the potentials of different sulfur containing compounds such as NAC, GSH, N-acetyl methionine (NAM) and methionine. NAC and GSH restore cellular integrity to provide complete protection against sodium nitrite mediated hemolysis where as other sulfur containing compounds failed to protect cells from lysis (Figure 5.1-A). MetHb incubated with NAC, GSH, N-acetyl L-methionine (NAM) and L-methionine (0.4 mM) and peroxidase activity was measured by guaiacol oxidation. The MetHb incubated with PBS gives 100% peroxidase activity, GSH and NAC inhibited the MetHb peroxidase activity completely, whereas other compounds have no significant inhibitory effects (Figure 5.1-B). NAC protects RBCs from oxidative stress induced RBC lysis in a dose dependent manner with complete protection at 5 mM (Figure 5.1-C). To confirm whether NAC mediated protection in RBC is due to inactivation of MetHb peroxidase activity, RBCs treated with sodium nitrite (1mg/ml) for 2 hrs (to cause oxidative mM). Post treatment, RBCs were lysed and MetHb was purified from different concentration of NAC loaded RBC samples as described (Hong et al., 2003) and peroxidase activity was determined using guaiacol oxidation (Trivedi et al., 2005a). The peroxidase activity of MetHb isolated from sodium nitrite exposed RBCs considered as 100% activity, and RBCs loaded with NAC (0-2.5 mM) gives a dose dependent decrease in MetHb peroxidase activity with a complete inhibition at 2.5 mM (Figure 5.1-D). Hence, data presented in Figure 5.1 supports the notion that NAC provides protection against oxidative stress induced RBC lysis (hemolysis) through inhibition of pro-oxidant MetHb.

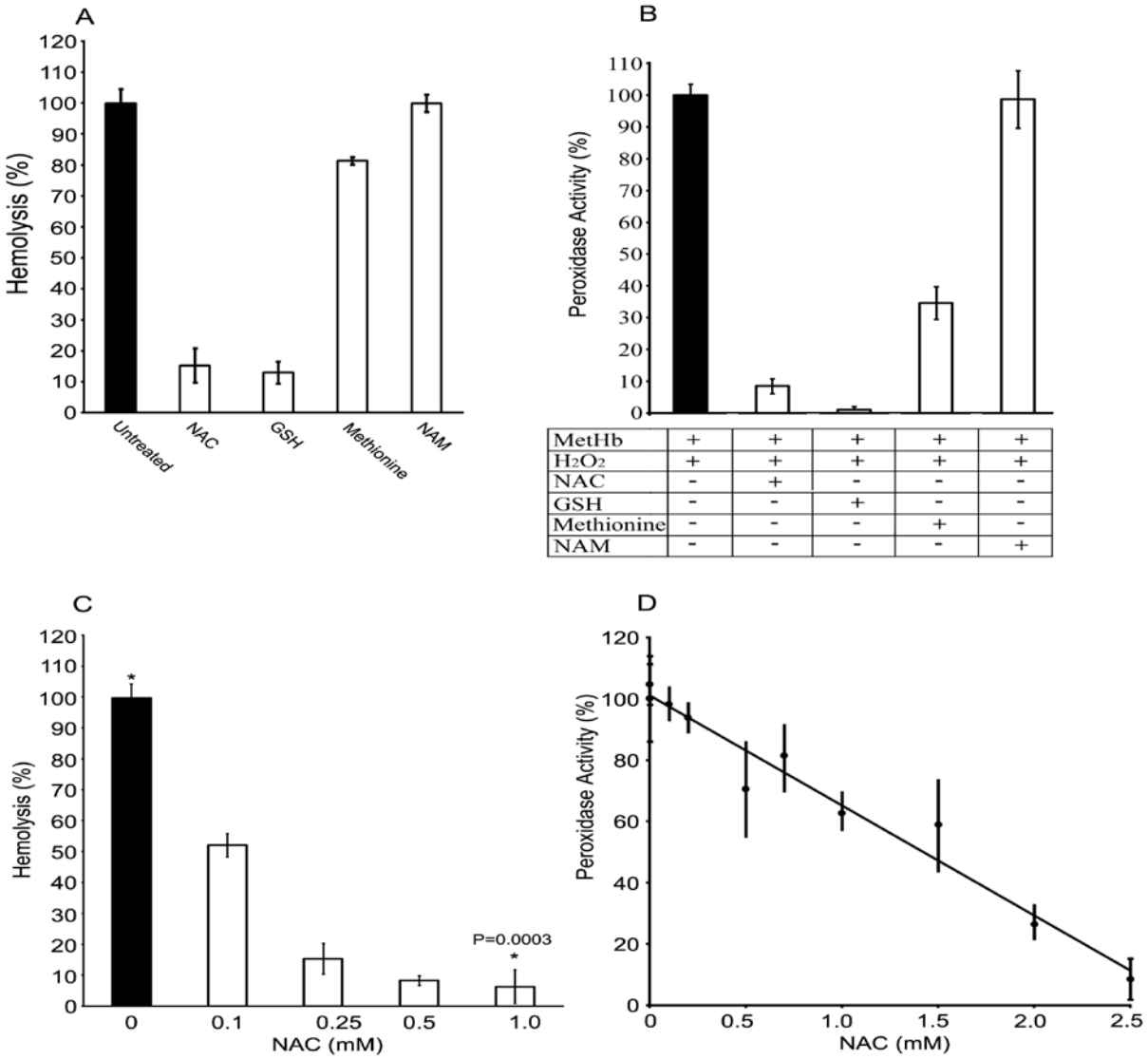

Figure 5.1 NAC protects RBC cells from oxidative stress induced hemolysis. (A) Thiols group containing compounds blocks oxidative stress induced hemolysis. 5% RBC hematocrit in PBS was exposed to sodium nitrite (1 mg/ml) for 2 hrs at 37°C in the presence of different sulfur containing compounds; N-acetyl l-cysteine (NAC), glutathione (GSH), N-acetyl l-methionine (NAM), L-methionine and hemolysis were measured. Hemolysis obtained in RBCs exposed to sodium nitrite alone considered as 100 % and used for comparison purposes. (B) MetHb (2 µM) was incubated with 0.4 mM of NAC, GSH, NAM, L-methionine in the presence of 50 µM H_2O_2 for 30 min at 37°C and peroxidase activity was measured by guaiacol oxidation as described in section 5.2.3. Peroxidase activity of MetHb incubated with buffer considered as 100% and used to analyze the degree of inhibition with sulfhydryl compounds. NAC dose dependently inhibits (C) hemolysis or (D) MetHb peroxidase activity from sodium nitrite exposed RBCs. RBC incubated with PBS was treated as a control. Data were from three independent experiments performed in triplicate. Standard deviations (SD) were calculated from triplicate measurements.

5.3.2 NAC follows mechanism based (suicidal) inactivation of MetHb: In a pure in-vitro system, pre-incubation of MetHb with varying concentration of NAC results in a concentration and time-dependent irreversible inactivation of MetHb following pseudo 1st order kinetics (Figure 5.2-A). When K_{obs} plotted against NAC concentration, a straight line (Figure 5.2-B) obtained, from which 2nd order rate constant calculated to 8.46 x 10^3 $M^{-1}min^{-1}$ at 37°C. The half-life of MetHb inactivation ($t_{1/2}$) at each NAC concentration was plotted against 1/NAC, gives a straight line (Figure 5.2-C).

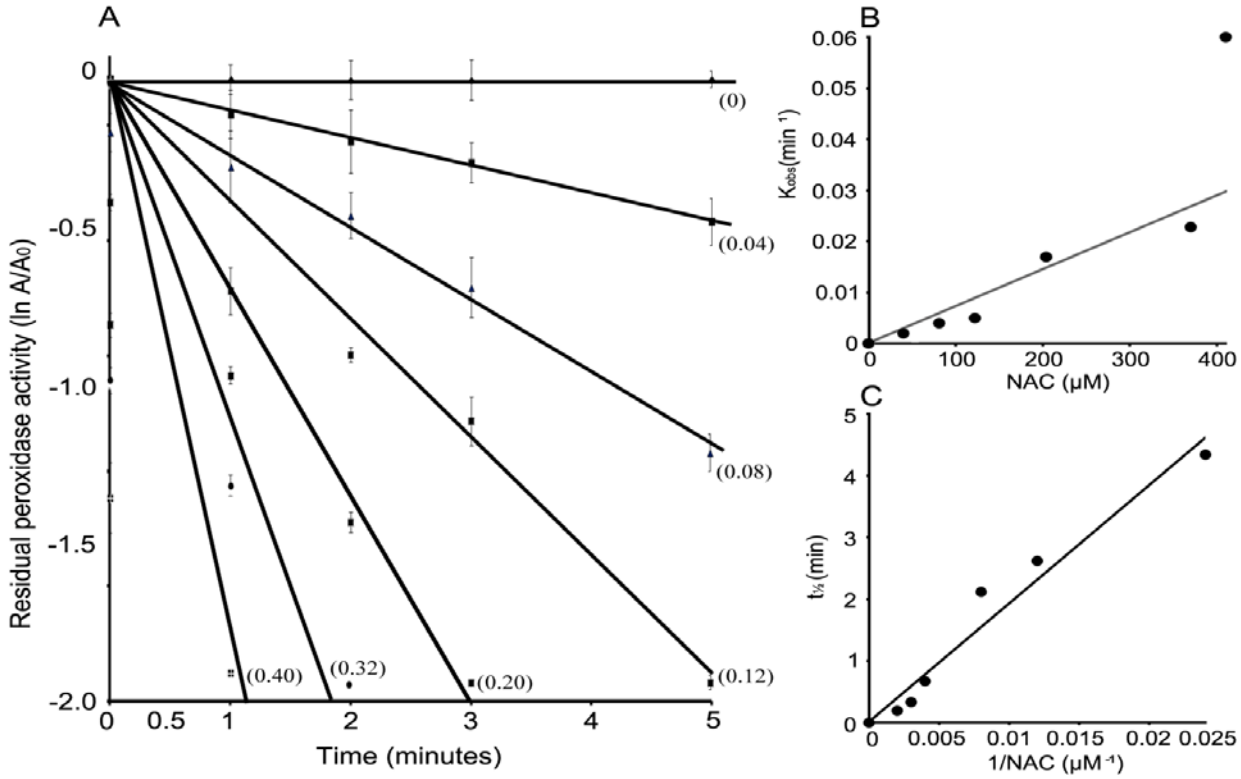

Figure 5.2 NAC mediated MetHb inactivation kinetics in the presence of hydrogen peroxide. (A) Calculation of the pseudo-first-order rate constant of MetHb inactivation by NAC for guaiacol oxidation. MetHb (2 μM) was incubated with different concentrations of NAC in the presence of 100 μM H_2O_2 at 37±1°C in a final volume of 0.3ml containing 100 mM Tris-HCl buffer pH 7.2. The concentration of NAC (in mM) used is given in parenthesis. (B) Determination of second-order rate constant of inactivation of guaiacol oxidation by MetHb. The slopes of the straight lines obtained in (A) were plotted against the concentration of NAC. The second-order rate constant of inactivation is $8.46x10^3M^{-1}min^{-1}$. (C) Time kinetics of mechanism-based inactivation of MetHb by NAC. The times required for half-time of inactivation at each concentration of NAC obtained from the straight lines of (A) were plotted against their corresponding reciprocal NAC concentrations. Data is mean ± SD (n= 3).

The affinity and kinetic constants k_i, k_{inact}, and $t_{1/2}$ found to 8.5 μM, 0.706 min^{-1} and 0.9 min respectively. No inactivation observed in the absence of H_2O_2 or NAC, indicating the role of MetHb catalyzed NAC oxidation (NAC_{ox}) in the inactivation process. The results indicate that H_2O_2 or NAC alone fails to inactivate MetHb, whereas the activity is 100% inhibited when MetHb incubated with NAC and H_2O_2 both (Table 5.1). In addition, no catalytic activity could be regained after diluting the reaction mixture or removing NAC and H_2O_2 by dialysis (Table 5.1). Thus, NAC inactivates MetHb following mechanism–based suicidal kinetics.

5.3.3 NAC Serve as the suicidal substrate of methemoglobin: Peroxidases mediated oxidation and inactivation mechanism involves the production of oxidation products of suicidal substrate through single electron transfer. To test the ability of MetHb to accept and oxidize NAC, an optical spectral study was performed. MetHb forms compound II in the presence of excess H_2O_2 with a spectral shift from 406 nm (Figure 5.3, spectrum a) to 416 nm (Figure 5.3, spectrum b). Addition of NAC shifts the soret peak at 410nm with a single electron transfer to NAC for oxidation (Figure

Table 5.1 NAC oxidation is essential for inhibition of MetHb peroxidase activity.

Reaction Mixtures	Residual Activity (%)
MetHb + NAC	99.9 ± 0.34
MetHb + H_2O_2	94.9 ± 0.21
MetHb + NAC + H_2O_2	2.9 ± 0.02
MetHb + NAC + H_2O_2 (after O/N dialysis)	6.67 ± 0.23
MetHb + Guiacol + NAC	98.3 ± 0.01
MetHb + KI + NAC	98.2 ± 0.02
MetHb + KCl + NAC	32.2 ± 0.08

Table 5.1 MetHb (2µM) was incubated with NAC (0.4mM) in the presence or absence of different electron donors such as guaiacol (100 mM), potassium iodide (100 mM), potassium chloride (100 mM) for 15mins at 37^0C and peroxidase activity was measured using guaiacol oxidation as described under "methods" section. The reaction mixture containing NAC inactivated MetHb was dialyzed over night (O/N) against PBS at 4^0C. Peroxidase activity from MetHb was considered as 100 % to calculate the residual peroxidase activity. The Data presented from experiment (n=4) performed in triplicate and values were expressed with standard deviation (SD).

5.3, spectrum c). These results can be attributed to the NAC mediated H_2O_2 scavenging activity, but the addition of excess H_2O_2 at this stage does not revert the soret peak to form compound II (data not shown). Hence, spectral studies indicate that MetHb oxidizes NAC to form NAC_{ox} through a single electron transfer mechanism involving high oxidation enzymatic spectral intermediates (compound II/compound I). To probe the NAC oxidation as a mechanism for MetHb inactivation,

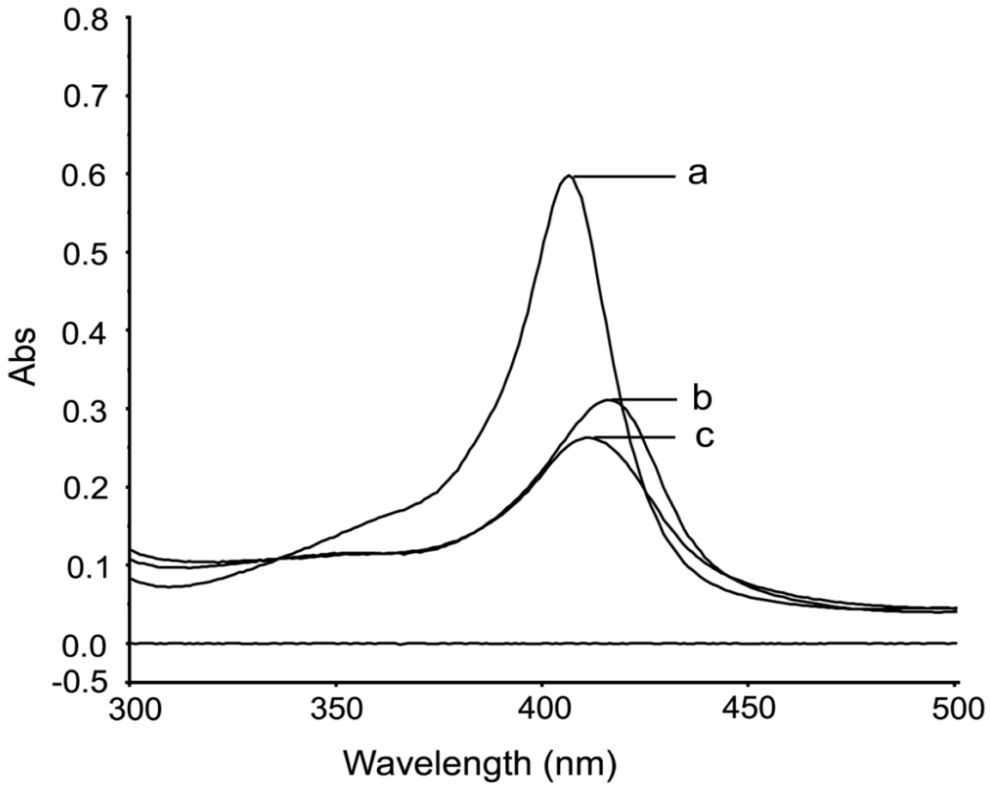

Figure 5.3 MetHb oxidizes NAC to NAC_{ox}. (A) Optical spectra of NAC oxidation by MetHb. In a total volume of 1ml containing 100 mM Tris-HCl buffer, pH 7.2, soret spectrum of (a) native MetHb (1.5µM), (b) a+ H_2O_2 (140µM); (c) b+NAC (7.2mM) were recorded.

the enzyme pre-incubated with other electron substrate donors such as iodide, chloride and guaiacol to test whether the presence of other substrate could reduce NAC oxidation and protects MetHb being inactivated. NAC inhibits more than 97% MetHb peroxidase activity, and presence of 100mM of electron donors give complete protection from inactivation except partial with KCl (Table 5.1). KCl is a poor substrate for MetHb and could not protect MetHb from NAC mediated inactivation clearly support the notion that protection depends on MetHb mediated preferential substrate oxidation.

5.3.4 Identification of NAC oxidation products: NAC acts as a suicidal substrate and gets oxidized by catalytically active MetHb to form NAC_{ox}. E.P.R. spectroscopy was performed to identify NAC free radicals (NAC_{ox}) as DMPO- NAC radical adduct (a^N = 15.2 G and a^H=16.78 G) in MetHb-H_2O_2 system (Figure 5.4-B, complete reaction). The radical is a sulphur centered thiyl radicals consistent with the previous reported NAC radicals (Hong et al., 2003; Jiang et al., 1996; Kwak et al., 1995). No radical is observed in the absence of H_2O_2 (Figure 5.4-B, -H_2O_2), NAC (Figure 5.4, NAC) or complete reaction containing inactivated MetHb (Figure 5.4, inactive MetHb).

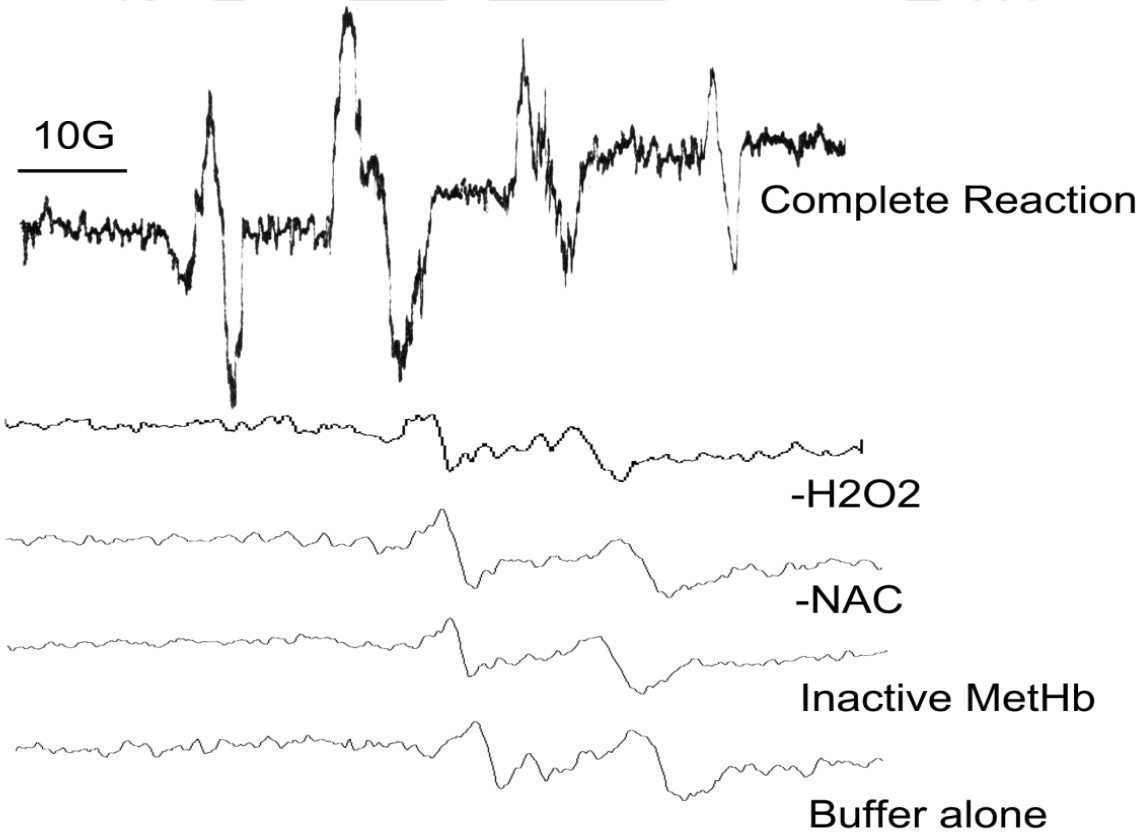

Figure 5.4 MetHb oxidizes NAC to form sulfur centered thiyl radical as NAC oxidation product (NAC_{ox}). EPR spectrum obtained from complete reaction containing 10µM MetHb, 0.8mM NAC, 100µM H_2O_2, 90mM DMPO and 1mM diethylenediaminetriaminopentaacetic acid (DTPA) in 100mM Tris-HCl buffer, pH7.2. -H_2O_2 or –NAC is complete reaction without H_2O_2 or NAC respectively. Complete reaction containing inactive MetHb or DMPO suspended in the buffer is also analyzed and presented. Instrumental setting is given in “experimental procedure”.

5.3.5 The NAC oxidation product is interacting and inactivating species: Next we asked, whether NAC oxidation product, NAC_{ox} is the interacting species to form an irreversible complex with MetHb. NAC binding to native and catalytically active MetHb complex (MetHb-H_2O_2) evaluated with difference spectroscopic studies. NAC binds to the catalytically active complex as evidenced in the decrease of absorbance at 413 nm (Figure 5.5-A) whereas no significant binding of NAC with the native enzyme (Figure 5.5-B) indicating NAC oxidation product (NAC_{ox}) generated by catalytically active MetHb is probably being the interacting species. The binding constant of NAC for catalytically active and native MetHb is 0.025mM, 0.269mM respectively. Ten folds difference in binding constant indicate an absolute requirement of NAC_{ox} formation for NAC binding to MetHb.

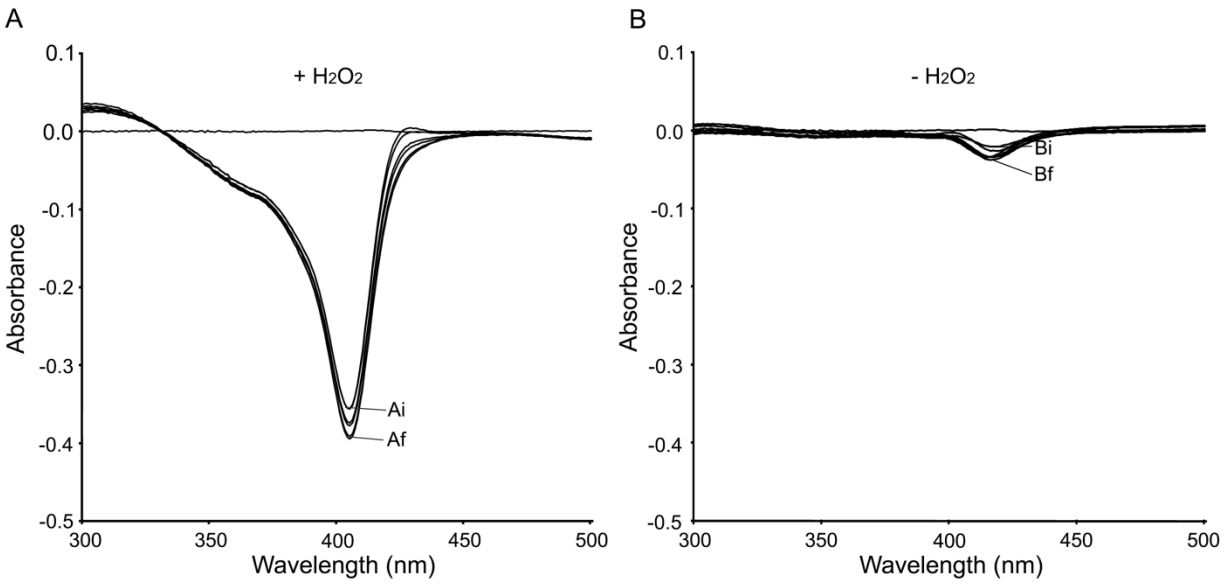

Figure 5.5 NAC_{ox} is the interacting species with MetHb. Optical difference spectra of NAC with (A) catalytically active (in presence of 10μM H_2O_2) or (B) native MetHb. (i, initial scan at 1.2mM; f, final scan at 7.2mM). The plot of 1/Δ Absorbance (nm) versus 1/[NAC] was used to calculate the K_D.

To explore the binding mode and nature of the NAC oxidation product (NAC_{ox}) binding to MetHb, the heme extracted from MetHb after reaction with NAC in the presence and absence of H_2O_2. In the absence of H_2O_2, a number of prominent peaks appeared in the ESI-MS analysis, but heme-NAC adduct peak was completely absent (Figure 5.6-A). In the presence of H_2O_2, a *m/z* of 1011.19 (Heme+2[NAC]+K) clearly indicates the formation of heme-NAC adduct (2xNAC/heme) and irreversible binding of NAC to the heme moiety of MetHb (Figure 5.6-B). Hence, it was clear that MetHb mediated NAC oxidation to form NAC_{ox} is the crucial event for binding and inactivation of MetHb.

To further probe such a mechanism, we pre-incubated the MetHb with two different spin traps, TEMPO and PBN to reduce the amount of NAC oxidation product (NAC_{ox}). MetHb was completely protected from NAC mediated irreversible inactivation in the presence of TEMPO and PBN (Figure 5.7-A). NAC binding to MetHb catalytically active complex (MetHb-H_2O_2) was done

in the presence of TEMPO to explore whether the protection is due to inability of NAC_{ox} to bind MetHb. In the presence of spin trap TEMPO, binding constant of NAC for active complex (MetHb-H_2O_2) was comparable to binding constant for native MetHb with insignificant NAC binding to catalytically active MetHb (Figure 5.7-B). Spin trap sensitivity to NAC binding further supports that NAC_{ox} is the interacting species (Figure 5.7-B) and probably be responsible for MetHb inactivation.

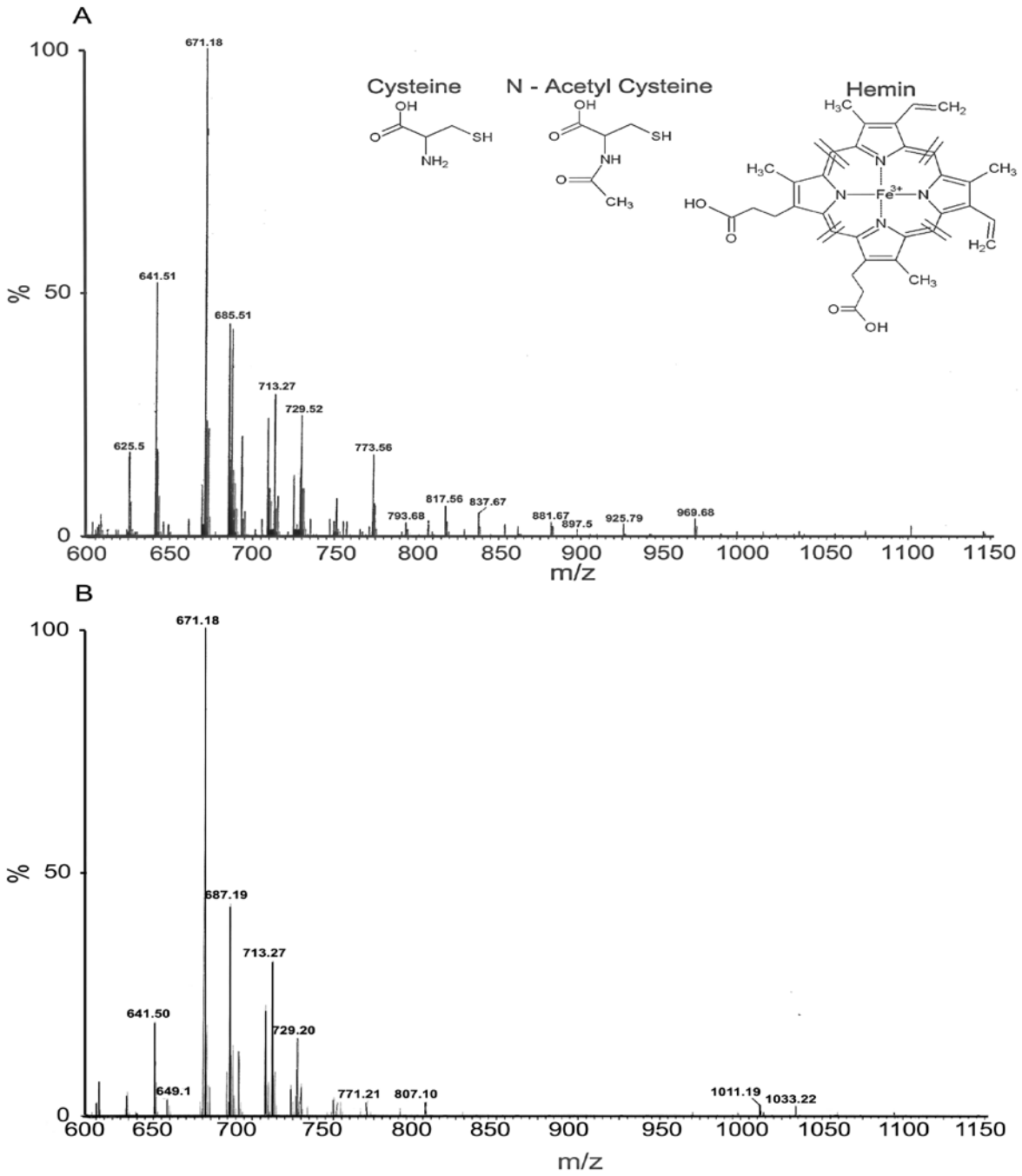

Figure 5.6 Characterization of Heme-NAC adduct by ESI-mass spectroscopy. The ESI - mass spectrum of the (A) Heme extracted from the MetHb (0.3mM) in the presence of NAC (0.4mM) or (B) as in A but in the presence of H_2O_2 (0.1 mM).

5.3.6 NACox is the molecular species to provide protection against oxidative stress induced hemolysis: Results in figure 5.7 indicate that, NAC oxidation product (NAC_{ox}) is interacting and

inactivating species for MetHb. Now we asked whether NAC_{ox} is the species responsible for antioxidant activity of NAC in RBC to protect it from oxidative stress induced MetHb production and resulting hemolysis. RBC under oxidative stress gives 100% hemolysis where as treatment with NAC (2.5mM) alone protects the RBC from hemolysis completely, but in the presence of spin trap, TEMPO and PBN, NAC has lost its ability to protect RBC from oxidative stress induced hemolysis (Figure 5.8). Both spin traps, TEMPO and PBN used in the experiment do not induce hemolysis alone or they have any stimulatory role in amplifying oxidative stress induced hemolysis (data not shown).

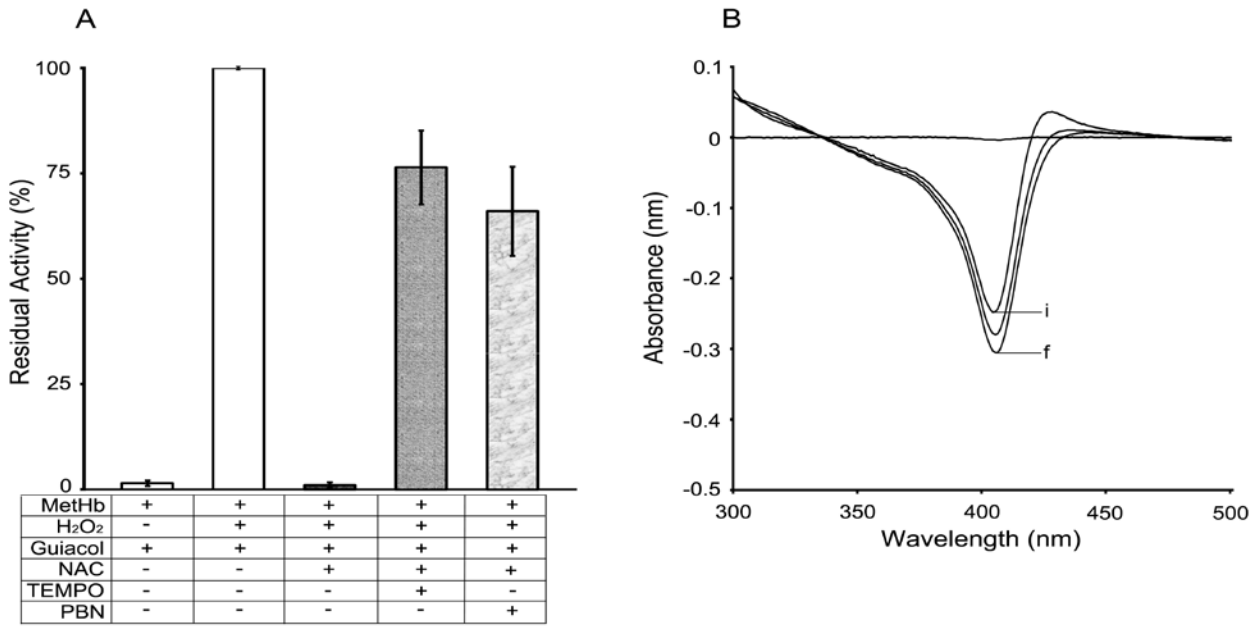

Figure 5.7 NAC oxidation product (NAC_{ox}) is the interacting and inactivating species for MetHb. (A) Protection against NAC mediated MetHb peroxidase inhibition by spin trap PBN and TEMPO. MetHb (2µM) was pre-incubated with TEMPO (0.5mM) or PBN (0.3mM) in the presence of NAC (0.4mM) and H_2O_2 in 20µl containing 100mM Tris-HCl buffer pH 7.2. The incubation mixture was transferred to the microtiter plate containing 0.28ml assay mixture containing guaiacol as substrate. Peroxidase activity of MetHb incubated with buffer was considered as 100% and used to calculate residual activity. (B) NAC binding to catalytically active MetHb is abolished in the presence of TEMPO. Optical difference spectra of NAC with MetHb-H_2O_2 complex in the presence of TEMPO (0.5mM). (i, initial scan at 1.2mM; f, final scan at 7.2mM). The plot of 1/Absorbance (nm) versus 1/[NAC] was used to calculate the K_D.

5.4 Discussion

Several lines of evidences are given to claim that the antioxidant activity of N-acetyl cysteine (NAC), associated with the inhibition of pro-oxidant MetHb to abolish ROS production and oxidative stress development (Figure 5.1). Kinetic evidence shows that NAC acts as a suicidal substrate to inactivate MetHb (Figure 5.2). MetHb has heme with Fe^{3+} and has potentials to oxidize

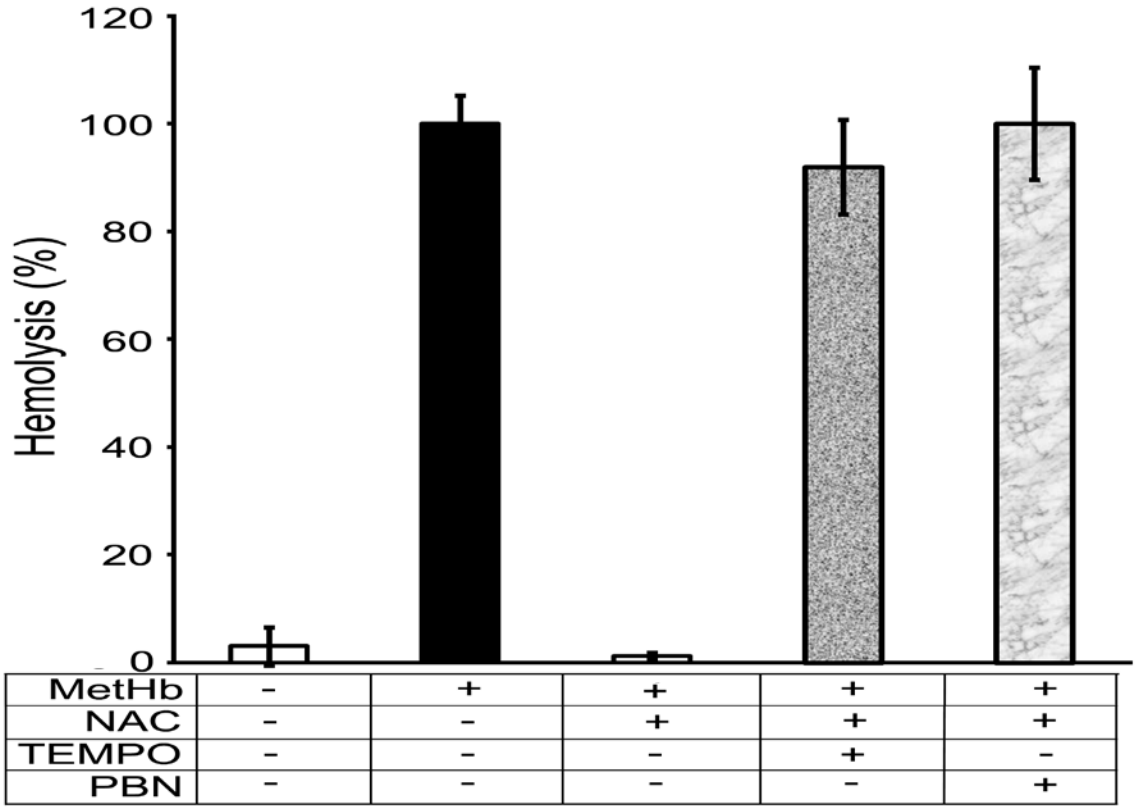

Figure 5.8 Spin Trap (TEMPO) abolishes NAC mediated protective effects against MetHb induced hemolysis. 5% RBC hematocrit in PBS was pre-incubated with PBN (2.5mM) or TEMPO (7.5mM) for 30mins and then treated with sodium nitrite (1mg/ml) for 10mins to give oxidative stress in the presence of NAC (2.5mM). RBCs exposed to PBS considered as control where as sodium nitrite treated RBCs considered as 100% hemolysis and used for comparison purposes. NAC in the presence of spin trap lost its ability to protect RBC from oxidative stress induced hemolysis. TEMPO or PBN at a concentration used has no visible hemolysis. Also, in combination with MetHb, the spin trap doesn't have any pro-stimulatory effects in hemolysis. All error bars indicate standard deviation (SD) and were calculated from triplicate measurements. If not visible, an error is smaller than symbols.

a variety of aromatic and halide substrates in the presence of H_2O_2 (Trivedi et al., 2005a). Aromatic and halide substrates protect MetHb inactivation against NAC, indicate a crucial role of NAC oxidation in the process (Table 5.1). Peroxidase hemo pocket has different micro-environment to provide substrate docking sites for halide and aromatic substrates (Huang et al., 2005). Competition of NAC thiyl radical with the docking sites for substrate radicals can be possible, but substrate mediated protection results correlate with substrate oxidation, clearly ruled out such a possibility. MetHb mediated oxidation product of NAC (NAC_{ox}) is highly reactive thiyl radical to irreversibly inactivate MetHb. MetHb mediated substrate oxidation to form substrate radicals interact with each other to form a stable product but suicidal substrate radicals interact with the enzyme to inactivate instead forming a stable product (Doerge, 1986). An active involvement of MetHb in generating NAC thiyl radicals was confirmed by EPR spectroscopy as NAC-DMPO adduct (Figure 5.4-B). EPR experiments done in the presence of DTPA (1mM) to rule out the possibility of the role of trace metals released from MetHb in the process. Difference spectroscopic studies indicate that NAC binds

at the heme moiety of active MetHb (MetHb- H_2O_2 complex) but not to the native enzyme indicating NAC_{ox} as the interacting species (Figure 5.5). LC-MS/MS analyses of NAC-heme adduct shows a molecular peak at m/z 1011.19 corresponds to the formation of 2:1 ratio of NAC/Heme in adduct (Figure 5.6). Optical spectroscopy studies in the presence of TEMPO show low affinity of NAC toward MetHb further supports NAC_{ox} as interacting and inactivating species (Figure 5.7). Moreover, pre-incubation of RBC with spin trap TEMPO abolishes the protective effect of NAC against oxidative stress induced hemolysis confirms that NAC_{ox} intermediate is crucial for antioxidant activity of NAC (Figure 5.8).

N-acetyl-l-cysteine, evaluated in a series of clinical studies to determine reduction of pathophysiological symptoms in malaria patients, during chemotherapy (Arreesrisom et al., 2007; Charunwatthana et al., 2009; Treeprasertsuk et al., 2003; Watt et al., 2002). The clinical trial in Thailand with 105 patients provides NAC as a safe molecule for adjuvant therapy based on lack of adverse effects (Treeprasertsuk et al., 2003) but a larger double blind clinical study concluded that NAC had no significant effect on mortality, lactate clearance times, coma recovery times or red cell rigidity (Charunwatthana et al., 2009). In-vitro studies suggest that NAC interferes with the antimalarial activity of artemisinin (Arreesrisom et al., 2007) and probably account for no significant improvement in clinical trials. (Arreesrisom et al., 2007; Charunwatthana et al., 2009; Treeprasertsuk et al., 2003; Watt et al., 2002). NAC reduces MetHb level in RBC, but it is not clear whether NAC has a direct role or through an indirect mechanism involving efficient removal of ROS and restoring of MetHb reductase activity (Takeoka et al., 1997). But several reports are also available where NAC used in conjugation with LPS in a lung injury model and the outcome indicates a dual role of NAC as cyto-protective Vs cyto-toxic effects linked to the dosing. At low dosage, NAC reduces hydrogen peroxide with an enhanced survival outcome but at higher dosage NAC has opposite effects (Sprong et al., 1998).

Peroxidase mediated suicidal substrate oxidation generates fast acting substrate radicals to interact with heme to prevent electron transfer (Ator et al., 1987; Trivedi et al., 2005b). Hence, cellular peroxidases participate in activating NAC to form thiyl radicals to scavenge toxic metabolites or drug induced free radicals. In the presence of drug or drug metabolites within cellular micro-environment, thiyl radicals interact with free radicals instead of making complex with the active site hemin. Protection of MetHb peroxidase activity from NAC thiyl radicals in the presence of small molecule electron donors supports such a mechanism (Table 5.1). MetHb mediated NAC oxidation and generation of thiyl radical allows us to speculate an active role of enzymes in modulating kinetics of NAC and free radical interaction. Although no kinetic studies performed so far between NAC and free radicals in the presence of MetHb or peroxidases, but such a study might give useful insight into the active role of the enzyme with antioxidant potentials of NAC. Hence, the

study highlights an additional role of NAC thiyl radicals in regulating oxidative stress through inhibition of pro-oxidant MetHb peroxidase activity. Although it is a difficult question to answer which molecular event is more crucial for antioxidant potential of NAC; MetHb inactivation to down-regulate the ROS level or MetHb mediated NAC activation and production of thiyl radical to modulate free radicals scavenging kinetics. Data presented in current work highlights an active role of MetHb with the antioxidant activity of NAC but additional experiments are required to get molecular details. The questions can be explored in the future are related to the additional molecular targets of NAC thiyl radical, interaction of NAC with free radicals or other antioxidant enzymes in the presence of MetHb. In addition, understanding of NAC dosing with cyto-protective to cytotoxic effects and role of MetHb mediated NAC thiyl radical generation might help to enhance therapeutic potential and reduces adverse effects of the molecule. In conclusion, NAC is a potent antioxidant molecule with potential to be used as adjuvant therapy along with regular antimalarial chemotherapy to surmount patho-physiological effects.

5.5 Summary

N-acetyl-L-cysteine (NAC) improves antioxidant potentials of RBCs to provide protections against oxidative stress induced hemolysis. The antioxidant mechanism of NAC to reduce oxidative stress in RBC studied through inactivation of pro-oxidant MetHb. NAC causes irreversible inactivation of the MetHb in an H_2O_2 dependent manner, and the inactivation follow the pseudo-first- order kinetics. The kinetic constants are ki = 8.5µM, k_{inact} = 0.706 min^{-1} and $t_{1/2}$ = 0.9 min. Spectroscopic studies indicate that MetHb accepts NAC as a substrate and oxidizes through a single electron transfer mechanism to the NAC_{ox}. The single e- oxidation product of NAC has been identified as the 5, 5'- dimethyl-1- pyrroline N- oxide (DMPO) adduct of the sulfur centered radical (a^N = 15.2 G and a^H=16. 78 G). Binding studies indicate that NAC_{ox} interacts at the heme moiety and NAC oxidation through MetHb is essential for NAC binding. Heme-NAC adduct dissociated from MetHb and identified (*m/z* 1011.19) as 2:1 ratio of NAC:heme in the adduct. TEMPO and PBN treatment reduces NAC binding to MetHb and protects against inactivation confirms the role of thiyl radical in the inactivation process. Furthermore, scavenging thiyl radicals by TEMPO abolishes the protective effect of NAC in hemolysis. This work is highlighting the antioxidant mechanism of NAC through NAC thiyl radical generation, MetHb inactivation to exhibit protection in RBC against oxidative stress induced hemolysis.

Antioxidant property of NAC is needed to evaluate further in order to imply as adjuvant therapy for various diseases. Since, we have not explored the adverse effects of NAC; we can explore the mechanism for NAC mediated toxicity in presence of various metabolic products. Together, these studies give an idea about NAC as a potential adjuvant therapy.

Bibliography

1. Acquah, S., Boampong, J.N., Eghan Jnr, B.A., And Eriksson, M. (2014). Evidence Of Insulin Resistance In Adult Uncomplicated Malaria: Result Of A Two-Year Prospective Study. Malaria Research And Treatment *2014*, 8.
2. Aditya, N.P., Chimote, G., Gunalan, K., Banerjee, R., Patankar, S., And Madhusudhan, B. (2012). Curcuminoids-Loaded Liposomes In Combination With Arteether Protects Against *Plasmodium* Berghei Infection In Mice. Experimental Parasitology *131*, 292-299.
3. Ahmed Ismail, H., Tijani, M.K., Langer, C., Reiling, L., White, M.T., Beeson, J.G., Wahlgren, M., Nwuba, R., And Persson, K.E. (2014). Subclass Responses And Their Half-Lives For Antibodies Against EBA175 And Pfrh2 In Naturally Acquired Immunity Against *Plasmodium falciparum* Malaria. Malaria Journal *13*, 425.
4. Akhtar, F., Rizvi, M.M., And Kar, S.K. (2012). Oral Delivery Of Curcumin Bound To Chitosan Nanoparticles Cured *Plasmodium* Yoelii Infected Mice. Biotechnology Advances *30*, 310-320.
5. Al-Fadhli, M.A., Saraya, M.A., And Qasem, J.A. (2014). Evaluation Of Leptin, Interleukin-1β And Tumor Necrosis Factor-A In Serum Of Malaria Patients As Prognostic Markers Of Treatment Outcome. Asian Pacific Journal Of Tropical Biomedicine *4*, 441-445.
6. Aley, S.B., Atkinson, C.T., Aikawa, M., Maloy, W.L., And Hollingdale, M.R. (1987). Ultrastructural Localization Of *Plasmodium falciparum* Circumsporozoite Protein In Newly Invaded Hepatoma Cells. The Journal Of Parasitology *73*, 1241-1245.
7. Allison, J.L., O'Brien, R.L., And Hahn, F.E. (1965). Nature Of The Deoxyribonucleic Acid-Chloroquine Complex. Antimicrobial Agents And Chemotherapy (Bethesda) *5*, 310-314.
8. Amino, R., Thiberge, S., Martin, B., Celli, S., Shorte, S., Frischknecht, F., And Menard, R. (2006). Quantitative Imaging Of *Plasmodium* Transmission From Mosquito To Mammal. Nature Medicine *12*, 220-224.
9. Anand, A.C., And Puri, P. (2005). Jaundice In Malaria. Journal Of Gastroenterology Hepatology *20*, 1322-1332.
10. Angrisano, F., Tan, Y.H., Sturm, A., Mcfadden, G.I., And Baum, J. (2012). Malaria Parasite Colonisation Of The Mosquito Midgut--Placing The *Plasmodium* Ookinete Centre Stage. International Journal Of Parasitology *42*, 519-527.
11. Ansar, W., BAndyopadhyay, S.M., Chowdhury, S., Habib, S.H., And MAndal, C. (2006). Role Of C-Reactive Protein In Complement-Mediated Hemolysis In Malaria. Glycoconjugate Journal *23*, 233-240.
12. Anstey, N.M., Hassanali, M.Y., Mlalasi, J., Manyenga, D., And Mwaikambo, E.D. (1996). Elevated Levels Of Methemoglobin In Tanzanian Children With Severe And Uncomplicated Malaria. Transactions Of The Royal Society Of Tropical Medicine And Hygiene *90*, 147-151.
13. Anthony, M.P., Burrows, J.N., Duparc, S., Moehrle, J.J., And Wells, T.N. (2012). The Global Pipeline Of New Medicines For The Control And Elimination Of Malaria. Malaria Journal *11*, 316.
14. Arreesrisom, P., Dondorp, A.M., Looareesuwan, S., And Udomsangpetch, R. (2007). Suppressive Effects Of The Anti-Oxidant N-Acetylcysteine On The Anti-Malarial Activity Of Artesunate. Parasitol International *56*, 221-226.
15. Aruoma, O.I., Halliwell, B., Hoey, B.M., And Butler, J. (1989). The Antioxidant Action Of N-Acetylcysteine: Its Reaction With Hydrogen Peroxide, Hydroxyl Radical, Superoxide, And Hypochlorous Acid. Free Radical Biology And Medicine *6*, 593-597.
16. Arya, R., Layton, D.M., And Bellingham, A.J. (1995). Hereditary Red Cell Enzymopathies. Blood Reviews *9*, 165-175.
17. Asada, K., Sasaki, S., Suda, T., Chida, K., And Nakamura, H. (2004). Antiinflammatory Roles Of Peroxisome Proliferator-Activated Receptor Γ In Human Alveolar Macrophages. American Journal Of Respiratory And Critical Care Medicine *169*, 195-200.
18. Ates, B., Abraham, L., And Ercal, N. (2008). Antioxidant And Free Radical Scavenging Properties Of N-Acetylcysteine Amide (NACA) And Comparison With N-Acetylcysteine (NAC).Free Radical Research *42*, 372-377.
19. Ator, M.A., David, S.K., And Ortiz De Montellano, P.R. (1987). Structure And Catalytic Mechanism Of Horseradish Peroxidase. Regiospecific Meso Alkylation Of The Prosthetic Heme Group By Alkylhydrazines. The Journal Of Biological Chemistry *262*, 14954-14960.
20. Autino, B., Corbett, Y., Castelli, F., And Taramelli, D. (2012b). Pathogenesis Of Malaria In Tissues And Blood. Mediterranean Journal Of Hematology And Infectious Diseases *4*, E2012061.
22. Avula, B., Tekwani, B.L., Chaurasiya, N.D., Nanayakkara, N.P., Wang, Y.H., Khan, S.I., Adelli, V.R., Sahu, R., Elsohly, M.A., Mcchesney, J.D., *Et Al.* (2013). PrOfiling Primaquine Metabolites In Primary Human

Hepatocytes Using UHPLC-QTOF-MS With 13C Stable Isotope Labeling. Journal Of Mass Spectrometry *48*, 276-285.
23. Awandare, G.A., Kempaiah, P., Ochiel, D.O., Piazza, P., Keller, C.C., And Perkins, D.J. (2011). Mechanisms Of Erythropoiesis Inhibition By Malarial Pigment And Malaria-Induced Proinflammatory Mediators In An In Vitro Model. American Journal Of Hematology *86*, 155-162.
24. Baird, J.K. (2012). Primaquine Toxicity Forestalls Efective Therapeutic Management Of The Endemic Malarias. International Journal For Parasitology *42*, 1049-1054.
25. Baird, J.K., And Hoffman, S.L. (2004). Primaquine Therapy For Malaria. Clinical Infectious Diseases: An Official Publication Of The Infectious Diseases Society Of America *39*, 1336-1345.
26. Balla, J., Jacob, H.S., Balla, G., Nath, K., Eaton, J.W., And Vercellotti, G.M. (1993). Endothelial-Cell Heme Uptake From Heme Proteins: Induction Of Sensitization And Desensitization To Oxidant Damage. Proceedings Of The National Academy Of Sciences Of The United States Of America *90*, 9285-9289.
27. Barrera, V., Hiscott, P.S., Craig, A.G., White, V.A., Milner, D.A., Beare, N.A., Maccormick, I.J., Kamiza, S., Taylor, T.E., Molyneux, M.E., (2015). Severity Of Retinopathy Parallels The Degree Of Parasite Sequestration In The Eyes And Brains Of Malawian Children With Fatal Cerebral Malaria. Journal Of Infectious Disease *211*, 1977-1986.
28. Beaudoin, R.L., And Aikawa, M. (1968). Primaquine Induced Changes In Morphology Of Exoerythrocytic Stages Of Malaria. Science *160*, 1233-1234.
29. Beck, J.S., And Saari, J.T. (1977). Permeability Coefficients By The Hemolytic Method: A Correction. Biophysical Journal *17*, 281-282.
30. Beers, R.F., Jr., And Sizer, I.W. (1952). A Spectrophotometric Method For Measuring The Breakdown Of Hydrogen Peroxide By Catalase. *The Journal* Of *Biological Chemistry 195*, 133-140.
31. Beier, M.S., Pumpuni, C.B., Beier, J.C., And Davis, J.R. (1994). Effects Of Para-Aminobenzoic Acid, Insulin, And Gentamicin On *Plasmodium falciparum* Development In Anopheline Mosquitoes (Diptera: Culicidae). Journal Of Medical Entomology *31*, 561-565.
32. Berra, L., Pinciroli, R., Stowell, C.P., Wang, L., Yu, B., FernAndez, B.O., Feelisch, M., Mietto, C., Hod, E.A., Chipman, D., *Et Al.* (2014). Autologous Transfusion Of Stored Red Blood Cells Increases Pulmonary Artery Pressure. American Journal Of Respiratory And Critical Care Medicine *190*, 800-807.
33. Beutler, E., Duparc, S., And Group, G.P.D.W. (2007). Glucose-6-Phosphate Dehydrogenase Deficiency And Antimalarial Drug Development. The American Journal Of Tropical Medicine And Hygiene *77*, 779-789.
34. Binh, T.Q., Davis, T.M., Johnston, W., Thu, L.T., Boston, R., Danh, P.T., And Anh, T.K. (1997). Glucose Metabolism In Severe Malaria: Minimal Model Analysis Of The Intravenous Glucose Tolerance Test Incorporating A Stable Glucose Label. Metabolism *46*, 1435-1440.
35. Birinyi-Strachan, L.C., Davies, M.J., Lewis, R.J., And Nicholson, G.M. (2005). Neuroprotectant Effects Of Iso-Osmolar D-Mannitol To Prevent Pacific Ciguatoxin-1 Induced Alterations In Neuronal Excitability: A Comparison With Other Osmotic Agents And Free Radical Scavengers. Neuropharmacology *49*, 669-686.
36. Biryukov, S., And Stoute, J.A. (2014). Complement Activation In Malaria: Friend Or Foe? Trends In Molecular Medicine *20*, 293-301.
37. Bokori-Brown, M., Petrov, P.G., Khafaji, M.A., Mughal, M.K., Naylor, C.E., Shore, A.C., Gooding, K.M., Casanova, F., Mitchell, T.J., Titball, R.W., *Et Al.* (2016). Red Blood Cell Susceptibility To Pneumolysin: Correlation With Membrane Biochemical And Physical Properties. *The Journal* Of *Biological Chemistry 291*, 10210-10227.
38. Bolli, R., Jeroudi, M.O., Patel, B.S., Aruoma, O.I., Halliwell, B., Lai, E.K., And Mccay, P.B. (1989). Marked Reduction Of Free Radical Generation And Contractile Dysfunction By Antioxidant Therapy Begun At The Time Of Reperfusion. Evidence That Myocardial "Stunning" Is A Manifestation Of Reperfusion Injury. Circulatory Research *65*, 607-622.
39. Boretti, F.S., Buehler, P.W., D'Agnillo, F., Kluge, K., Glaus, T., Butt, O.I., Jia, Y., Goede, J., Pereira, C.P., Maggiorini, M., *Et Al.* (2009). Sequestration Of Extracellular Hemoglobin Within A Haptoglobin Complex Decreases Its Hypertensive And Oxidative Effects In Dogs And Guinea Pigs. The Journal Of Clinical Investigation *119*, 2271-2280.
40. Bowman, Z.S., Jollow, D.J., And Mcmillan, D.C. (2005a). Primaquine Induced Hemolytic Anemia: Role Of Splenic Mmacrophages In The Fate Of 5-Hydroxyprimaquine Treated Rat Erythrocytes. The Journal Of Pharmacology And Experimental Therapeutics *315*, 980-986.
41. Bowman, Z.S., Morrow, J.D., Jollow, D.J., And Mcmillan, D.C. (2005b). Primaquine Induced Hemolytic Anemia: Role Of Membrane Lipid Peroxidation And Cytoskeletal Protein Alterations In The Hemotoxicity Of 5-Hydroxyprimaquine. The Journal Of Pharmacology And Experimental Therapeutics *314*, 838-845.
42. Bowman, Z.S., Oatis, J.E., Jr., Whelan, J.L., Jollow, D.J., And Mcmillan, D.C. (2004). Primaquine Induced Hemolytic Anemia: Susceptibility Of Nnormal Versus Glutathione Depleted Rat Erythrocytes To 5-Hydroxyprimaquine. The Journal Of Pharmacology And Experimental Therapeutics *309*, 79-85.
43. Bradberry, S.M. (2003). Occupational Methaemoglobinaemia. Mechanisms Of Production, Features, Diagnosis And Management Including The Use Of Methylene Blue. Toxicological Reviews *22*, 13-27.

44. Bratosin, D., Mazurier, J., Tissier, J.P., Estaquier, J., Huart, J.J., Ameisen, J.C., AminOff, D., And Montreuil, J. (1998). Cellular And Molecular Mechanisms Of Senescent Erythrocyte Phagocytosis By Macrophages. A Review. Biochimie *80*, 173-195.
45. Bruce-Chwatt, L.J., Wernsdorfer, W., And Mcgregor, I. (1988). History Of Malaria From Prehistory To Eradication. Malaria: Principles And Practice Of Malariology Volume 1, 1-59.
46. Brumen, M., And Heinrich, R. (1984). A Metabolic Osmotic Model Of Human Erythrocytes. Bio Systems *17*, 155-169.
47. Buehler, P.W., And D'Agnillo, F. (2010). Toxicological Consequences Of Extracellular Hemoglobin: Biochemical And Physiological Perspectives. Antioxidants & Redox Signaling *12*, 275-291.
48. Buffet, P.A., Safeukui, I., Deplaine, G., Brousse, V., Prendki, V., Thellier, M., Turner, G.D., And Mercereau-Puijalon, O. (2011). The Pathogenesis Of *Plasmodium falciparum* Malaria In Humans: Insights From Splenic Physiology. Blood *117*, 381-392.
49. Camus, S.M., De Moraes, J.A., Bonnin, P., Abbyad, P., Le Jeune, S., Lionnet, F., Loufrani, L., Grimaud, L., Lambry, J.C., Charue, D., *Et Al.* (2015). Circulating Cell Membrane Microparticles Transfer Heme To Endothelial Cells And Trigger Vasoocclusions In Sickle Cell Disease. Blood *125*, 3805-3814.
50. Carmona-Fonseca, J., Alvarez, G., And Maestre, A. (2009). Methemoglobinemia And Adverse Events In *Plasmodium Vivax* Malaria Patients Associated With High Doses Of Primaquine Treatment. The American Journal Of Tropical Medicine And Hygiene *80*, 188-193.
51. Carrolo, M., Giordano, S., Cabrita-Santos, L., Corso, S., Vigario, A.M., Silva, S., Leiriao, P., Carapau, D., Armas-Portela, R., Comoglio, P.M., *Et Al.* (2003). Hepatocyte Growth Factor And Its Receptor Are Required For Malaria Infection. Nature Medicine *9*, 1363-1369.
52. Carson, P.E., Hohl, R., Nora, M.V., Parkhurst, G.W., Ahmad, T., Scanlan, S., And Frischer, H. (1981). Toxicology Of The 8-Aminoquinolines And Genetic Factors Associated With Their Toxicity In Man. Bulletin Of The World Health Organization *59*, 427-437.
53. Chapman, L.M., Aggrey, A.A., Field, D.J., Srivastava, K., Ture, S., Yui, K., Topham, D.J., Baldwin, W.M., 3rd, And Morrell, C.N. (2012). Platelets Present Antigen In The Context Of MHC Class I. Journal Of Immunology *189*, 916-923.
54. Charunwatthana, P., Abul Faiz, M., Ruangveerayut, R., Maude, R.J., Rahman, M.R., Roberts, L.J., Moore, K., Bin Yunus, E., Hoque, M.G., Hasan, M.U., (2009). N-Acetylcysteine As Adjunctive Treatment In Severe Malaria: A RAndomized, Double-Blinded Placebo-Controlled Clinical Trial. Critical Care Medicine *37*, 516-522.
55. Chiabrando, D., Mercurio, S., And Tolosano, E. (2014). Heme And Erythropoieis: More Than A Structural Role. Haematologica *99*, 973-983.
56. Chien, S., Jan, K.M., And Usami, S. (1975). Roles Of Electrostatic Repulsion And Mechanical Shearing In Red Cell Aggregation. Bibliotheca Anatomica *13*, 93-94.
57. Chiu, D.T., And Liu, T.Z. (1997). Free Radical And Oxidative Damage In Human Blood Cells. Journal Of Biomedical Science *4*, 256-259.
58. Chotivanich, K., Udomsangpetch, R., Chierakul, W., Newton, P.N., Ruangveerayuth, R., Pukrittayakamee, S., Looareesuwan, S., And White, N.J. (2004). In Vitro Efficacy Of Antimalarial Drugs Against *Plasmodium Vivax* On The Western Border Of ThailAnd. The American Journal Of Tropical Medicine And Hygiene *70*, 395-397.
59. Chotivanich, K., Udomsangpetch, R., Mcgready, R., Proux, S., Newton, P., Pukrittayakamee, S., Looareesuwan, S., And White, N.J. (2002a). Central Role Of The Spleen In Malaria Parasite Clearance. The Journal Of Infectious Diseases *185*, 1538-1541.
60. Chotivanich, K., Udomsangpetch, R., Pattanapanyasat, K., Chierakul, W., Simpson, J., Looareesuwan, S., And White, N. (2002b). Hemoglobin E: A Balanced Polymorphism Protective Against High Parasitemias And Thus Severe *Plasmodium falciparum* Malaria. Blood *100*, 1172-1176.
61. Chotivanich, K.T., Dondorp, A.M., White, N.J., Peters, K., Vreeken, J., Kager, P.A., And Udomsangpetch, R. (2000). The Resistance To Physiological Shear Stresses Of The Erythrocytic Rosettes Formed By Cells Infected With *Plasmodium falciparum*. Annals Of Tropical Medicine And Parasitology *94*, 219-226.
62. Chua, C.L., Brown, G., Hamilton, J.A., Rogerson, S., And Boeuf, P. (2013). Monocytes And Macrophages In Malaria: Protection Or Pathology? Trends In Parasitology *29*, 26-34.
63. Combrinck, J.M., Mabotha, T.E., Ncokazi, K.K., Ambele, M.A., Taylor, D., Smith, P.J., Hoppe, H.C., And Egan, T.J. (2013). Insights Into The Role Of Heme In The Mechanism Of Action Of Antimalarials. ACS Chemical Biology *8*, 133-137.
64. Condon, M.R., Kim, J.E., Deitch, E.A., Machiedo, G.W., And Spolarics, Z. (2003). Appearance Of An Erythrocyte Population With Decreased Deformability And Hemoglobin Content Following Sepsis. American Journal Of Physiology Heart And Circulatory Physiology *284*, H2177-2184.
65. Cook, G.C., And Zumla, A. (2009). Manson's Tropical Diseases (Saunders).
66. Couper, K.N., Barnes, T., Hafalla, J.C., Combes, V., Ryffel, B., Secher, T., Grau, G.E., Riley, E.M., And De Souza, J.B. (2010). Parasite Derived Plasma Microparticles Contribute Significantly To Malaria Infection Induced Inflammation Through Potent Macrophage Stimulation. PLOS Pathogensology *6*, E1000744.
67. Cox, F.E. (2002). History Of Human Parasitology. Clinical Microbiology Reviews *15*, 595-612.

68. Croft, S. (2001). Antimalarial Chemotherapy: Mechanisms Of Action, Resistance And New Directions In Drug Discovery. Drug Discovery Today *6*, 1151.
69. Cui, L., And Su, X.Z. (2009). Discovery, Mechanisms Of Action And Combination Therapy Of Artemisinin. Expert Review Of Anti-Infective Therapy *7*, 999-1013.
70. Dambach, P., Louis, V.R., Kaiser, A., Ouedraogo, S., Sie, A., Sauerborn, R., And Becker, N. (2014). Efficacy Of *Bacillus Thuringiensis* Var. Israelensis Against Malaria Mosquitoes In Northwestern Burkina Faso. Parasites & Vectors *7*, 371.
71. Danquah, I., Bedu-Addo, G., And Mockenhaupt, F.P. (2010). Type 2 Diabetes Mellitus And Increased Risk For Malaria Infection. Emerging Infectious Diseases *16*, 1601-1604.
72. De Back, D.Z., Kostova, E.B., Van Kraaij, M., Van Den Berg, T.K., And Van Bruggen, R. (2014a). Of Macrophages And Red Blood Cells; A Complex Love Story. Frontiers In Physiology *5*, 9.
74. Deans, J.A., Thomas, A.W., Inge, P.M., And Cohen, S. (1983). Stage Specific Protein Synthesis By Asexual Blood Stage Parasites Of *Plasmodium falciparum*. Molecular & Biochemical Parasitology *8*, 45-51.
75. Deshmukh, R., And Trivedi, V. (2014). Role Of Pro-Oxidants From Infected RBCS In Disturbing Homeostasis And Pathogenesis During Malaria. Austin Journal Of Biotechnology & Bioengineering *1*, 1-5.
76. Dinarello, C.A. (2000). Proinflammatory Cytokines. Chest *118*, 503-508.
77. Diou, J., Tardif, M.R., Barat, C., And Tremblay, M.J. (2010). Dendritic Cells Derived From Hemozoin-Loaded Monocytes Display A Partial Maturation Phenotype That Promotes HIV-1 Trans-Infection Of CD4+ T Cells And Virus Replication. Journal Of Immunology *184*, 2899-2907.
78. Doerge, D.R. (1986). Mechanism Based Inhibition Of Lactoperoxidase By Thiocarbamide Goitrogens. Biochemistry *25*, 4724-4728.
79. Dondorp, A.M., Kager, P.A., Vreeken, J., And White, N.J. (2000). Abnormal Blood Flow And Red Blood Cell Deformability In Ssevere Malaria. Parasitology Today *16*, 228-232.
80. Dondorp, A.M., Pongponratn, E., And White, N.J. (2004). Reduced Microcirculatory Flow In Severe Falciparum Malaria: Pathophysiology And Electron Microscopic Pathology. Acta Tropica *89*, 309-317.
81. Dongre, V.G., Karmuse, P.P., Rao, P.P., And Kumar, A. (2008). Development And Validation Of UPLC Method For Determination Of Primaquine Phosphate And Its Impurities. Journal Of Pharmaceutical And Biomedical Analysis *46*, 236-242.
82. Eaton, P., Zuzarte-Luis, V., Mota, M.M., Santos, N.C., And Prudencio, M. (2012). Infection By *Plasmodium* Changes Shape And Stiffness Of Hepatic Cells. Nanomedicine: Nanotechnology, Biology, And Medicine *8*, 17-19.
83. Edgcomb, J.H., Arnold, J., Yount, E.H., Jr., Alving, A.S., Eichelberger, L., Jeffery, G.M., Eyles, D., And Young, M.D. (1950). Primaquine, SN 13272, A New Curative Agent In Vivax Malaria; A Preliminary Report. Journal Of National Malaria Society *9*, 285-292.
84. Ehrich, J.H., And Eke, F.U. (2007). Malaria Induced Renal Damage: Facts And Myths. Pediatrics Nephrology *22*, 626-637.
85. El-Assaad, F., Wheway, J., Hunt, N.H., Grau, G.E., And Combes, V. (2014). Production, Fate And Pathogenicity Of Plasma Microparticles In Murine Cerebral Malaria. PLOS Pathogensology *10*, E1003839.
86. Eltahir, E.M., El Ghazali, G., TM, A.E., IE, A.E., Elbashir, M.I., And Giha, H.A. (2010). Raised Plasma Insulin Level And Homeostasis Model Assessment (HOMA) Score In Cerebral Malaria: Evidence For Insulin Resistance And Marker Of Virulence. Acta Biochimica Polonica *57*, 513-520.
87. Erta, M., Quintana, A., And Hidalgo, J. (2012). Interleukin-6, A Major Cytokine In The Central Nervous System. International Journal Of Biological Science *8*, 1254-1266.
88. Faille, D., Combes, V., Mitchell, A.J., Fontaine, A., Juhan-Vague, I., Alessi, M.C., Chimini, G., Fusai, T., And Grau, G.E. (2009). Platelet Microparticles: A New Player In Malaria Parasite Cytoadherence To Human Brain Endothelium. FASEB Journal *23*, 3449-3458.
89. Fairhurst, R.M., And Wellems, T.E. (2010). *Plasmodium* Species (Malaria). MAndell, Douglas, And Bennett's Principles And Practice Of Infectious Diseases *2*, 3437-3462.
90. Fibach, E., And Rachmilewitz, E. (2008). The Role Of Oxidative Stress In Hemolytic Anemia. Curruent Molecular Medicine *8*, 609-619.
91. Fitch, C.D. (2004). Ferriprotoporphyrin IX, Phospholipids And The Antimalarial Actions Of Quinoline Drugs. Life Sciences *74*, 1957-1972.
92. Fitri, L.E., Sardjono, T.W., Simamora, D., Sumarno, R.P., And Setyawati, S.K. (2011). High Dose Of N-Acetylcysteine Increase HO And MDA Levels And Decrease GSH Level Of Huvecs Exposed With Malaria Serum. Tropical Biomedicine *28*, 7-15.
93. Fletcher, K.A., Barton, P.F., And Kelly, J.A. (1988). Studies On The Mechanisms Of Oxidation In The Erythrocyte By Metabolites Of Primaquine. Biochemical Pharmacology *37*, 2683-2690.
94. Frischer, H., Mellovitz, R.L., Ahmad, T., And Nora, M.V. (1991). The Conversion Of Primaquine Into Primaquine-Aldehyde, Primaquine-Alcohol And Carboxyprimaquine, A Major Plasma Metabolite. Journal Of Laboratory Clinical Medicine *117*, 468-476.

95. Fulton, J.D., And Flewett, T.H. (1956). The Relation Of *Plasmodium Berghei* And *Plasmodium Knowlesi* To Their Respective Red Cell Hosts. Transactions Of the Royal Society Of Tropical Medicine & Hygiene *50*, 150-156.
96. Fusman, R., Zeltser, D., Rotstein, R., Chapman, J., Avitzour, D., Shapira, I.I., Eldor, A., Elkayam, O., Caspi, D., Arber, N., (2000). Inflamet: An Image Analyzer To Display Erythrocyte Adhesiveness/Aggregation. European Journal Of Internal Medicine *11*, 271-276.
97. Ganesan, S., Chaurasiya, N.D., Sahu, R., Walker, L.A., And Tekwani, B.L. (2012). Understanding The Mechanisms For Metabolism Linked Hemolytic Toxicity Of Primaquine Against Glucose 6 Phosphate Dehydrogenase Deficient Human Erythrocytes: Evaluation Of Eryptotic Pathway. Toxicology *294*, 54-60.
98. Ganesan, S., Tekwani, B.L., Sahu, R., Tripathi, L.M., And Walker, L.A. (2009). Cytochrome P(450)-Dependent Toxic Effects Of Primaquine On Human Erythrocytes. Toxicology And Applied Pharmacology *241*, 14-22.
99. Garcia-Garcia, A., Rodriguez-Rocha, H., Madayiputhiya, N., Pappa, A., Panayiotidis, M.I., And Franco, R. (2012). Biomarkers Of Protein Oxidation In Human Disease. Curruent Molecular Medicine *12*, 681-697.
100. Gaudreault, V., Wirbel, J., Jardim, A., Rohrbach, P., And Scorza, T. (2015). Red Blood Cells Preconditioned With Hemin Are Less Permissive To *Plasmodium* Invasion In Vivo And In Vitro. PLOS ONE *10*, E0140805.
101. Gaunt, S.D., Baker, D.C., And Green, R.A. (1981). Clinicopathologic Evaluation N-Acetylcysteine Therapy In Acetaminophen Toxicosis In The Cat. American Journal Of Veternary Research *42*, 1982-1984.
102. Geurts, N., Opdenakker, G., And Van Den Steen, P.E. (2012). Matrix Metalloproteinases As Therapeutic Targets In Protozoan Parasitic Infections. Pharmacology & Therapeuctics *133*, 257-279.
103. Giha, H., Elghazali, G., A-Elgadir, T., A-Elbasit, I., And Elbashir, M.I. (2009). Severe Malaria In An Unstable Setting: Clinical And Laboratory Correlates Of Cerebral Malaria And Severe Malarial Anemia And A Paradigm For A Simplified Severity Scoring. European Journal Of Clinical Microbiology & Infectious Diseases *28*, 661-665.
104. Giribaldi, G., Ulliers, D., Mannu, F., Arese, P., And Turrini, F. (2001). Growth Of *Plasmodium falciparum* Induces Stage-Dependent Hemichrome Formation, Oxidative Aggregation Of BAnd 3, Membrane Deposition Of Complement And Antibodies, And Phagocytosis Of Parasitized Erythrocytes. British Journal Of Haematology *113*, 492-499.
105. Goldman, D.W., Breyer, R.J., 3rd, Yeh, D., Brockner-Ryan, B.A., And Alayash, A.I. (1998). Acellular Hemoglobin Mediated Oxidative Stress Toward Endothelium: A Role For Ferryl Iron. The American Journal Of Physiology *275*, H1046-1053.
106. Gram, M., Sveinsdottir, S., Ruscher, K., Hansson, S.R., Cinthio, M., Akerstrom, B., And Ley, D. (2013). Hemoglobin Induces Inflammation After Preterm Intraventricular Hemorrhage By Methemoglobin Formation. Journal Of Neuroinflammation *10*, 100.
107. Grau, G.E., Frei, K., Piguet, P.F., Fontana, A., Heremans, H., Billiau, A., Vassalli, P., And Lambert, P.H. (1990). Interleukin 6 Production In Experimental Cerebral Malaria: Modulation By Anticytokine Antibodies And Possible Role In Hypergammaglobulinemia. Journal Of Experimental Medicine *172*, 1505-1508.
108. Grau, G.E., Mackenzie, C.D., Carr, R.A., Redard, M., Pizzolato, G., Allasia, C., Cataldo, C., Taylor, T.E., And Molyneux, M.E. (2003). Platelet Accumulation In Brain Microvessels In Fatal Pediatric Cerebral Malaria. Journal Of Infectious Disease *187*, 461-466.
109. Graves, P., And GelbAnd, H. (2006). Vaccines For Preventing Malaria (Spf66). The Cochrane Database Of Systematic Reviews iews, CD005966.
110. Group, P.S., Pekyi, D., Ampromfi, A.A., Tinto, H., Traore Coulibaly, M., Tahita, M.C., Valea, I., Mwapasa, V., Kalilani-Phiri, L., KalAnda, G., *Et Al.* (2016). Four Artemisinin-Based Treatments In African Pregnant Women With Malaria. New EnglAnd Journal Of Medicine *374*, 913-927.
111. Grubina, R., Huang, Z., Shiva, S., Joshi, M.S., Azarov, I., Basu, S., Ringwood, L.A., Jiang, A., Hogg, N., Kim-Shapiro, D.B., *Et Al.* (2007). Concerted Nitric Oxide Formation And Release From The Simultaneous Reactions Of Nitrite With Deoxy- And Oxyhemoglobin. *The Journal* Of *Biological Chemistry 282*, 12916-12927.
112. Guha, M., Kumar, S., Choubey, V., Maity, P., And BAndyopadhyay, U. (2006). Apoptosis In Liver During Malaria: Role Of Oxidative Stress And Implication Of Mitochondrial Pathway. FASEB Journal *20*, 1224-1226.
113. Gutierrez-Correa, J., And Stoppani, A.O. (1999). Inactivation Of Myocardial Dihydrolipoamide Dehydrogenase By Myeloperoxidase Systems: Effect Of Halides, Nitrite And Thiol Compounds. Free Radical Research *30*, 105-117.
114. Habig, W.H., Pabst, M.J., And Jakoby, W.B. (1974). Glutathione S-Transferases. The First Enzymatic Step In Mercapturic Acid Formation. *The Journal* Of *Biological Chemistry 249*, 7130-7139.
115. Hall, A.H., Kulig, K.W., And Rumack, B.H. (1986). Drug And Chemical Induced Methemoglobinaemia. Clinical Features And Management. Medical Toxicology *1*, 253-260.
116. Halliwell, B., And Whiteman, M. (2004). Measuring Reactive Species And Oxidative Damage In Vivo And In Cell Culture: How Should You Do It And What Do The Results Mean? British Journal Of Pharmacology *142*, 231-255.

117. Han, J.M., Patterson, S.J., Speck, M., Ehses, J.A., And Levings, M.K. (2014). Insulin Inhibits IL-10 Mediated Regulatory T Cell Function: Implications For Obesity. Journal Of Immunology *192*, 623-629.
118. Hanson, J., Lam, S.W., Mahanta, K.C., Pattnaik, R., Alam, S., Mohanty, S., Hasan, M.U., Hossain, A., Charunwatthana, P., Chotivanich, K., *Et Al.* (2012). Relative Contributions Of Macrovascular And Microvascular Dysfunction To Disease Severity In Falciparum Malaria. Journal Of Infectious Disease *206*, 571-579.
119. Harman, L.S., Carver, D.K., Schreiber, J., And Mason, R.P. (1986). One And Two Electron Oxidation Of Reduced Glutathione By Peroxidases. *The Journal* Of *Biological Chemistry 261*, 1642-1648.
120. Hemmer, C.J., Lehr, H.A., Westphal, K., Unverricht, M., Kratzius, M., And Reisinger, E.C. (2005). *Plasmodium falciparum* Malaria: Reduction Of Endothelial Cell Apoptosis In Vitro. Infection & Immunity *73*, 1764-1770.
121. Herdman, M.T., Sriboonvorakul, N., Leopold, S.J., Douthwaite, S., Mohanty, S., Hassan, M.M., Maude, R.J., Kingston, H.W., Plewes, K., Charunwatthana, P., *Et Al.* (2015). The Role Of Previously Unmeasured Organic Acids In The Pathogenesis Of Severe Malaria. Critical Care *19*, 317.
122. Hill, A.V. (2006). Pre-Erythrocytic Malaria Vaccines: Towards Greater Efficacy. Nature Reviews Immunology *6*, 21-32.
123. Hogman, C.F., Eriksson, L., Ericson, A., And Reppucci, A.J. (1991). Storage Of Saline Adenine-Glucose-Mannitol-Suspended Red Cells In A New Plastic Container: Polyvinylchloride Plasticized With Butyryl-N-Trihexyl-Citrate. Transfusion *31*, 26-29.
124. Hong, S.Y., Yang, J.O., Lee, E.Y., And Lee, Z.W. (2003). Effects Of N-Acetyl-L-Cysteine And Glutathione On Antioxidant Status Of Human Serum And 3T3 Fibroblasts. Journal Of Korean Medical Science *18*, 649-654.
125. Hortola, P. (1992). SEM Analysis Of Red Blood Cells In Aged Human Bloodstains. Forensic Science International *55*, 139-159.
126. Hoshen, M.B., Heinrich, R., Stein, W.D., And Ginsburg, H. (2000). Mathematical Modelling Of The Within-Host Dynamics Of *Plasmodium falciparum*. Parasitology *121 (Pt 3)*, 227-235.
127. Howland, S.W., Poh, C.M., And Renia, L. (2015). Activated Brain Endothelial Cells Cross-Present Malaria Antigen. Plos Pathology *11*, E1004963.
128. Huang, H., Lamikanra, A.A., Alkaitis, M.S., Thezenas, M.L., Ramaprasad, A., Moussa, E., Roberts, D.J., And Casals-Pascual, C. (2014). Interleukin-10 Regulates Hepcidin In *Plasmodium falciparum* Malaria. PLOS ONE *9*, E88408.
129. Huang, L., Wojciechowski, G., And Ortiz De Montellano, P.R. (2005). Prosthetic Heme Modification During Halide Ion Oxidation. Demonstration Of Chloride Oxidation By Horseradish Peroxidase. Journal Of American Chemical Society *127*, 5345-5353.
130. Humeida, H., Pradel, G., Stich, A., And Krawinkel, M. (2011). The Effect Of Glucose And Insulin On In Vitro Proliferation Of *Plasmodium falciparum*. Journal Of Diabetology *3*, 6.
131. Idro, R., Marsh, K., John, C.C., And Newton, C.R. (2010). Cerebral Malaria: Mechanisms Of Brain Injury And Strategies For Improved Neurocognitive Outcome. Pediatric Research *68*, 267-274.
132. Islam, M.N., ChAnda, S., Sen, P., And Mitra, C. (2011). Stress-Mediated Alteration In Membrane Fluidity Regulates Calcium Atpase Activity In Plasma Membrane Of Duodenal Enterocytes Of Oophorectomized Rats. Al Ameen Journal Of Medical Sciences 4(1), 28-37.
133. Jacobs, G.H., Aikawa, M., Milhous, W.K., And Rabbege, J.R. (1987). An Ultrastructural Study Of The Effects Of Mefloquine On Malaria Parasites. The American Journal Of Tropical Medicine And Hygiene *36*, 9-14.
134. Jain, K., Sood, S., And Gowthamarajan, K. (2013). Modulation Of Cerebral Malaria By Curcumin As An Adjunctive Therapy. The Brazilian Journal Of Infectious Diseases *17*, 579-591.
135. James, M.A., Fajfar-Whetstone, C.J., Kakoma, I., Buese, M.M., Clabaugh, G.W., Hansen, R., And Ristic, M. (1991). Immunogenicity And Protective Efficacy Of Affinity-Purified *Plasmodium falciparum* Exoantigens In Aotus Nancymai Monkeys. Tropical Medicine and Parasitology *42*, 49-54.
136. Jayavanth, S., And Park, B.C. (2007). Microrheologic Dysfunctions In Blood During Malaria. Indian Journal Of Experimental Biology *45*, 111-120.
137. Jiang, J.J., Liu, K.J., Jordan, S.J., Swartz, H.M., And Mason, R.P. (1996). Detection Of Free Radical Metabolite Formation Using In Vivo EPR Spectroscopy: Evidence Of Rat Hemoglobin Thiyl Radical Formation Following Administration Of Phenylhydrazine. Archieves of Biochemistry and Biophysics *330*, 266-270.
138. Jin, M., Chen, Y., He, S., Ryan, S.J., And Hinton, D.R. (2004). Hepatocyte Growth Factor And Its Role In The Pathogenesis Of Retinal Detachment. Investigative Ophthalmology and Visual Science *45*, 323-329.
139. John, C.C., Kutamba, E., Mugarura, K., And Opoka, R.O. (2010). Adjunctive Therapy For Cerebral Malaria And Other Severe Forms Of *Plasmodium falciparum* Malaria. Expert Review Of Anti-Infective Therapy *8*, 997-1008.
140. Kamchonwongpaisan, S., SamOff, E., And Meshnick, S.R. (1997). Identification Of Hemoglobin Degradation Products In *Plasmodium falciparum*. Molecular And Biochemical Parasitology *86*, 179-186.

141. Karunaweera, N.D., Grau, G.E., Gamage, P., Carter, R., And Mendis, K.N. (1992). Dynamics Of Fever And Serum Levels Of Tumor Necrosis Factor Are Closely Associated During Clinical Paroxysms In *Plasmodium* Vivax Malaria. Proceeding of the Natlonal Academy Science USA *89*, 3200-3203.
142. Kelly, G.S. (1998). Clinical Applications Of N-Acetylcysteine. Alternative Medicine Review *3*, 114-127.
143. Khadjavi, A., Valente, E., Giribaldi, G., And Prato, M. (2014). Involvement Of P38 MAPK In Haemozoin-Dependent MMP-9 Enhancement In Human Monocytes. Cell Biochemistry and Function *32*, 5-15.
144. Kiefer, C.R., And Snyder, L.M. (2000). Oxidation And Erythrocyte Senescence. Current Opinion In Hematology *7*, 113-116.
145. King, T., And Lamb, T. (2015). Interferon?: The Jekyll And Hyde Of Malaria. PLOS Pathogens *11*, E1005118.
146. Klinger, G., Morad, Y., Westall, C.A., Laskin, C., Spitzer, K.A., Koren, G., Ito, S., And Buncic, R.J. (2001). Ocular Toxicity And Antenatal Exposure To Chloroquine Or Hydroxychloroquine For Rheumatic Diseases. The Lancet *358*, 813-814.
147. Kochar, D.K., Agarwal, P., Kochar, S.K., Jain, R., Rawat, N., Pokharna, R.K., Kachhawa, S., And Srivastava, T. (2003a). Hepatocyte Dysfunction And Hepatic Encephalopathy In *Plasmodium falciparum* Malaria. QJM An International Journal Of Medicine *96*, 505-512.
148. Kochar, D.K., Singh, P., Agarwal, P., Kochar, S.K., Pokharna, R., And Sareen, P.K. (2003b). Malarial Hepatitis. Journal Of Associate Physicians In India *51*, 1069-1072.
149. Koeppen, B.M. (2009). The Kidney And Acid-Base Regulation. Advances in Physiology Education *33*, 275-281.
150. Konigk, E., Mirtsch, S., Putfarken, B., And Abdel-Rasoul, S. (1981). *Plasmodium* Chabaudi-Infection Of Mice: Effects Of Chloroquine And Mefloquine. Inhibition Of Ornithine Decarboxylase Activity. Tropenmedizin Parasitologie *32*, 73-76.
151. Koshiar, R.L., Somajo, S., Norstrom, E., And Dahlback, B. (2014). Erythrocyte-Derived Microparticles Supporting Activated Protein C-Mediated Regulation Of Blood Coagulation. PLOS ONE *9*, E104200.
152. Kristoff, J. (2007). Malaria Stage-Specific Vaccine CAndidates. Current Pharmaceutical Design *13*, 1989-1999.
153. Kumaravel, M., And Singh, M. (1995). Aggregation And Deformability Of Erythrocytes In Leprosy. Indian Journal Of Experimental Biology *33*, 408-415.
154. Kwak, H.S., Yim, H.S., Chock, P.B., And Yim, M.B. (1995). Endogenous Intracellular Glutathionyl Radicals Are Generated In Neuroblastoma Cells Under Hydrogen Peroxide Oxidative Stress. Proceeding of the Natlonal Academy Science USA *92*, 4582-4586.
155. Lamy, M.L., Daily, E.K., Brichant, J.F., Larbuisson, R.P., Demeyere, R.H., VAndermeersch, E.A., Lehot, J.J., Parsloe, M.R., Berridge, J.C., Sinclair, C.J., *Et Al.* (2000). Randomized Trial Of Diaspirin Cross-Linked Hemoglobin Solution As An Alternative To Blood Transfusion After Cardiac Surgery. The Dclhb Cardiac Surgery Trial Collaborative Group. Anesthesiology *92*, 646-656.
156. Lasonder, E., Ishihama, Y., Andersen, J.S., Vermunt, A.M.W., Pain, A., Sauerwein, R.W., Eling, W.M.C., Hall, N., Waters, A.P., Stunnenberg, H.G., *Et Al.* (2002). Analysis Of The *Plasmodium falciparum* Proteome By High-Accuracy Mass Spectrometry. Nature *419*, 537-542.
157. Lee, C.C., Kinter, L.D., And Heiffer, M.H. (1981). Subacute Toxicity Of Primaquine In Dogs, Monkeys, And Rats. Bulletin Of The World Health Organization *59*, 439-448.
158. Lee, C.T., Zhong, L., Mace, T.A., And Repasky, E.A. (2012). Elevation In Body Temperature To Fever Range Enhances And Prolongs Subsequent Responsiveness Of Macrophages To Endotoxin Challenge. PLOS ONE *7*, E30077.
159. Lees, R.S., Gilles, J.R.L., Hendrichs, J., Vreysen, M.J.B., And Bourtzis, K. (2015). Back To The Future: The Sterile Insect Technique Against Mosquito Disease Vectors. Current Opinion In Insect Science *10*, 156-162.
160. Legorreta-Herrera, M., Retana-Ugalde, R., Ventura-Gallegos, J.L., And Narvaez, V. (2010). Pyrimethamine Induces Oxidative Stress In *Plasmodium* Yoelii 17XL-Infected Mice: A Novel Immunomodulatory Mechanism Of Action For An Old Antimalarial Drug? Experimental Parasitology *126*, 381-388.
161. Leoratti, F.M.D.S., Trevelin, S.C., Cunha, F.Q., Rocha, B.C., Costa, P.A.C., Gravina, H.D., Tada, M.S., Pereira, D.B., Golenbock, D.T., Do Valle Antonelli, L.R., *Et Al.* (2012). Neutrophil Paralysis In *Plasmodium vivax* Malaria. PLOS Neglected Tropical Diseases *6*, E1710.
162. Li, X.Q., Bjorkman, A., Andersson, T.B., Gustafsson, L.L., And Masimirembwa, C.M. (2003). Identification Of Human Cytochrome P(450)S That Metabolise Anti-Parasitic Drugs And Predictions Of In Vivo Drug Hepatic Clearance From In Vitro Data. European Journal Of Clinical Pharmacology *59*, 429-442.
163. Lipinski, B. (2011). Hydroxyl Radical And Its Scavengers In Health And Disease. Oxidative Medicine And Cellular Longevity *2011*, 809696.
164. Lisovskaia, I.L., Ataullakhanov, F.I., Tuzhilova, E.G., And Vitvitskii, V.M. (1994). Analysis Of Geometric Parameters And Mechanical Properties Of Erythrocytes By Filtration Through Nuclear Membrane Filters. II. Experimental Verification Of A Mathematical Model. Biofizika *39*, 864-871.
165. Liu, H., Tekwani, B.L., Nanayakkara, N.P., Walker, L.A., And Doerksen, R.J. (2013). Methemoglobin Generation By 8-Aminoquinolines: Effect Of Substitution At 5-Position Of Primaquine. Chemical Research In Toxicology *26*, 1801-1809.

166. Liu, X., And Spolarics, Z. (2003). Methemoglobin Is A Potent Activator Of Endothelial Cells By Stimulating IL-6 And IL-8 Production And E-Selectin Membrane Expression. American Journal Of Physiology Cell Physiology*285*, C1036-1046.
167. Lokken, K.L., Mooney, J.P., Butler, B.P., Xavier, M.N., Chau, J.Y., Schaltenberg, N., Begum, R.H., Müller, W., Luckhart, S., And Tsolis, R.M. (2014). Malaria Parasite Infection Compromises Control Of Concurrent Systemic Non-Typhoidal *Salmonella* Infection Via IL-10-Mediated Alteration Of Myeloid Cell Function. PLOS Pathogens *10*, E1004049.
168. Looareesuwan, S., Sjostrom, L., Krudsood, S., Wilairatana, P., Porter, R.S., Hills, F., And Warrell, D.A. (1999). Polyclonal Anti-Tumor Necrosis Factor-Alpha Fab Used As An Ancillary Treatment For Severe Malaria. The American Journal Of Tropical Medicine And Hygiene *61*, 26-33.
169. Looareesuwan, S., Wilairatana, P., Vannaphan, S., Wanaratana, V., Wenisch, C., Aikawa, M., Brittenham, G., Graninger, W., And Wernsdorfer, W.H. (1998). Pentoxifylline As An Ancillary Treatment For Severe *falciparum* Malaria In Thailand. The American Journal Of Tropical Medicine And Hygiene *58*, 348-353.
170. Lopez-Antunano, F.J. (1999). Is Primaquine Useful And Safe As True Exo-Erythrocytic Merontocidal, Hypnozoitocidal And Gametocidal Antimalarial Drug? Salud Publica Mex *41*, 410-419.
171. Lum, H., And Roebuck, K.A. (2001). Oxidant Stress And Endothelial Cell Dysfunction. American Journal Of Physiology Cell Physiology *280*, C719-741.
172. Lyke, K.E., Burges, R., Cissoko, Y., Sangare, L., Dao, M., Diarra, I., Kone, A., Harley, R., Plowe, C.V., Doumbo, O.K., *Et Al.* (2004). Serum Levels Of The Proinflammatory Cytokines Interleukin-1 Beta (IL-1beta), IL-6, IL-8, IL-10, Tumor Necrosis Factor Alpha, And IL-12(P70) In Malian Children With Severe *Plasmodium falciparum* Malaria And Matched Uncomplicated Malaria Or Healthy Controls. Infection and Immunity *72*, 5630-5637.
173. Maciel, A.T., And Park, M. (2009). Differences In Acid-Base Behavior Between Intensive Care Unit Survivors And Nonsurvivors Using Both A Physicochemical And A StAndard Base Excess Approach: A Prospective, Observational Study. Journal of Critical Care *24*, 477-483.
174. Macone, A., Fontana, M., Barba, M., Botta, B., Nardini, M., Ghirga, F., Calcaterra, A., Pecci, L., And Matarese, R.M. (2011). Antioxidant Properties Of Aminoethylcysteine Ketimine Decarboxylated Dimer: A Review. International Journal Of Molecular Sciences *12*, 3072-3084.
175. Macpherson, G.G., Warrell, M.J., White, N.J., Looareesuwan, S., And Warrell, D.A. (1985). Human Cerebral Malaria. A Quantitative Ultrastructural Analysis Of Parasitized Erythrocyte Sequestration. The American Journal Of Pathology *119*, 385-401.
176. Magistrado, P., Salanti, A., Tuikue Ndam, N.G., Mwakalinga, S.B., Resende, M., Dahlback, M., Hviid, L., Lusingu, J., TheAnder, T.G., And Nielsen, M.A. (2008). VAR2CSA Expression On The Surface Of Placenta-Derived *Plasmodium falciparum*-Infected Erythrocytes. The Journal of Infectious Diseases *198*, 1071-1074.
177. Marinkovic, M., Diez-Silva, M., Pantic, I., Fredberg, J.J., Suresh, S., And Butler, J.P. (2009). Febrile Temperature Leads To Significant Stiffening Of *Plasmodium falciparum* Parasitized Erythrocytes. American Journal Of Physiology Cell Physiology*296*, C59-64.
178. Martinelli, A., Rodrigues, L.A., And Cravo, P. (2008). *Plasmodium* Chabaudi: Efficacy Of Artemisinin + Curcumin Combination Treatment On A Clone Selected For Artemisinin Resistance In Mice. Experimental Parasitology *119*, 304-307.
179. Maruyama, N., Kakuta, Y., Yamauchi, K., Ohkawara, Y., Aizawa, T., Ohrui, T., Nara, M., Oshiro, T., Ohno, I., Tamura, G., *Et Al.* (1994). Quinine Inhibits Production Of Tumor Necrosis Factor-Alpha From Human Alveolar Macrophages. American Journal Of Respiratory Cell And Molecular Biology *10*, 514-520.
180. Masocha, W., And Kristensson, K. (2012). Passage Of Parasites Across The Blood-Brain Barrier. Virulence *3*, 202-212.
181. Matarrese, P., Straface, E., Pietraforte, D., Gambardella, L., Vona, R., Maccaglia, A., Minetti, M., And Malorni, W. (2005). Peroxynitrite Induces Senescence And Apoptosis Of Red Blood Cells Through The Activation Of Aspartyl And Cysteinyl Proteases. FASEB Journal *19*, 416-418.
182. Mayor, A., Hafiz, A., Bassat, Q., Rovira-Vallbona, E., Sanz, S., Machevo, S., Aguilar, R., Cistero, P., Sigauque, B., Menendez, C., Association Of Severe Malaria Outcomes With Platelet-Mediated Clumping And Adhesion To A Novel Host Receptor. PLOS ONE *6*, E19422.
183. Mayor, A., Hafiz, A., Bassat, Q., Rovira-Vallbona, E., Sanz, S., Machevo, S., Aguilar, R., Cistero, P., Sigauque, B., Menendez, C., *Et Al.* (2011). Association Of Severe Malaria Outcomes With Platelet-Mediated Clumping And Adhesion To A Novel Host Receptor. PLOS ONE *6*, E19422.
184. Mazor, D., Golan, E., Philip, V., Katz, M., Jafe, A., Ben-Zvi, Z., And Meyerstein, N. (1996). Red Blood Cell Permeability To Thiol Compounds Following Oxidative Stress. European Journal Of Haematology *57*, 241-246.
185. Mcqueen, P.G., And Mckenzie, F.E. (2004). Age-Structured Red Blood Cell Susceptibility And The Dynamics Of Malaria Infections. Proceeding of the National Academy Science USA *101*, 9161-9166.
186. Mebius, R.E., And Kraal, G. (2005). Structure And Function Of The Spleen. Nature Reviews Immunology *5*, 606-616.

187. Megnekou, R., Tenou, S., Bigoga, J.D., Djontu, J.C., Medou, F.M., And Lissom, A. (2015). Placental Malaria And Modulation Of Immune And Hormonal Responses In Cameroonian Women. Acta Tropica *147*, 23-30.
188. Mehta, M., Sonawat, H.M., And Sharma, S. (2005). Malaria Parasite-Infected Erythrocytes Inhibit Glucose Utilization In Uninfected Red Cells. FEBS Letters *579*, 6151-6158.
189. Meshnick, S.R., Thomas, A., Ranz, A., Xu, C.M., And Pan, H.Z. (1991). Artemisinin (Qinghaosu): The Role Of Intracellular Hemin In Its Mechanism Of Antimalarial Action. Molecular And Biochemical Parasitology *49*, 181-189.
190. Mihaly, G.W., Ward, S.A., Edwards, G., Orme, M.L., And Breckenridge, A.M. (1984). Pharmacokinetics Of Primaquine In Man: Identification Of The Carboxylic Acid Derivative As A Major Plasma Metabolite. British Journal Of Clinical Pharmacology *17*, 441-446.
191. Mimche, P.N., Taramelli, D., And Vivas, L. (2011). The Plant-Based Immunomodulator Curcumin As A Potential CAndidate For The Development Of An Adjunctive Therapy For Cerebral Malaria. Malaria Journal *10*, 1-9.
192. MirAnda, A.S., Brant, F., Rocha, N.P., Cisalpino, D., Rodrigues, D.H., Souza, D.G., Machado, F.S., Rachid, M.A., Teixeira, A.L., Jr., And Campos, A.C. (2013). Further Evidence For An Anti-Inflammatory Role Of Artesunate In Experimental Cerebral Malaria. Malaria Journal *12*, 388.
193. Mohandas, N., And Evans, E. (1994). Mechanical Properties Of The Red Cell Membrane In Relation To Molecular Structure And Genetic Defects. Annual Review Of Biophysics And Biomolecular Structure *23*, 787-818.
194. Mohanty, J.G., Nagababu, E., And Rifkind, J.M. (2014). Red Blood Cell Oxidative Stress Impairs Oxygen Delivery And Induces Red Blood Cell Aging. Frontiers In Physiology *5*, 84.
195. Mohanty, S., P, D., Pati, S.S., And Mishra, S.K. (2006a). Adjuvant Therapy In Cerebral Malaria. Indian Journal Of Medical Research *124*.
196. Mohanty, S., Patel, D.K., Pati, S.S., And Mishra, S.K. (2006b). Adjuvant Therapy In Cerebral Malaria. Indian Journal Of Medical Research *124*, 245-260.
197. Monk, L.S., Fagerstedt, K.V., And Crawford, R.M. (1987). Superoxide Dismutase As An Anaerobic Polypeptide : A Key Factor In Recovery From Oxygen Deprivation In Iris Pseudacorus? Plant Physiology *85*, 1016-1020.
198. Morimatsu, H., Toda, Y., Egi, M., Shimizu, K., Matsusaki, T., Suzuki, S., Iwasaki, T., And Morita, K. (2009). Acid-Base Variables In Patients With Acute Kidney Injury Requiring Peritoneal Dialysis In The Pediatric Cardiac Care Unit. Journal Of Anesthesia *23*, 334-340.
199. Mosser, D.M., And Karp, C.L. (1999). Receptor Mediated Subversion Of Macrophage Cytokine Production By Intracellular Pathogens. Current Opinion in Immunology *11*, 406-411.
200. Moxon, C.A., Grau, G.E., And Craig, A.G. (2011). Malaria: Modification Of The Red Blood Cell And Consequences In The Human Host. British Journal Of Haematology *154*, 670-679.
201. Mueller, I., Zimmerman, P.A., And Reeder, J.C. (2007). *Plasmodium malariae* And *Plasmodium ovale*--The "Bashful" Malaria Parasites. Trends In Parasitology *23*, 278-283.
202. Nantakomol, D., Dondorp, A.M., Krudsood, S., Udomsangpetch, R., Pattanapanyasat, K., Combes, V., Grau, G.E., White, N.J., Viriyavejakul, P., Day, N.P., *Et Al.* (2011). Circulating Red Cell-Derived Microparticles In Human Malaria. The Journal of Infectious Diseases *203*, 700-706.
203. Nayak, A.P., Tiyaboonchai, W., Patankar, S., Madhusudhan, B., And Souto, E.B. (2010). Curcuminoids-Loaded Lipid Nanoparticles: Novel Approach Towards Malaria Treatment. Colloids And Surfaces B: Biointerfaces *81*, 263-273.
204. Nerlich, A.G., Schraut, B., Dittrich, S., Jelinek, T., And Zink, A.R. (2008). *Plasmodium falciparum* In Ancient Egypt. Emerging Infectious Diseases *14*, 1317-1319.
205. Newton, C.R., Crawley, J., Sowumni, A., Waruiru, C., Mwangi, I., English, M., Murphy, S., Winstanley, P.A., Marsh, K., And Kirkham, F.J. (1997). Intracranial Hypertension In Africans With Cerebral Malaria. Archives Of Disease In Childhood *76*, 219-226.
206. Newton, C.R., Hien, T.T., And White, N. (2000). Cerebral Malaria. Journal of Neurology, Neurosurgery & Psychiatry *69*, 433-441.
207. Nguansangiam, S., Day, N.P., Hien, T.T., Mai, N.T., Chaisri, U., Riganti, M., Dondorp, A.M., Lee, S.J., Phu, N.H., Turner, G.D., (2007). A Quantitative Ultrastructural Study Of Renal Pathology In Fatal *Plasmodium falciparum* Malaria. Tropical Medicine & International Health *12*, 1037-1050.
208. Niikura, M., Inoue, S., And Kobayashi, F. (2011). Role Of Interleukin-10 In Malaria: Focusing On Coinfection With Lethal And Nonlethal Murine Malaria Parasites. Journal Of Biomedicine And Biotechnology *2011*, 383962.
209. Nontprasert, A., Nosten-Bertrand, M., Pukrittayakamee, S., Vanijanonta, S., Angus, B.J., And White, N.J. (1998). Assessment Of The Neurotoxicity Of Parenteral Artemisinin Derivatives In Mice. The American Journal Of Tropical Medicine And Hygiene *59*, 519-522.
210. Nuchsongsin, F., Chotivanich, K., Charunwatthana, P., Fausta, O.-S., Taramelli, D., Day, N.P., White, N.J., And Dondorp, A.M. (2007a). Effects Of Malaria Heme Products On Red Blood Cell Deformability. The American Journal Of Tropical Medicine And Hygiene *77*, 617-622.

211. Nuchsongsin, F., Chotivanich, K., Charunwatthana, P., Omodeo-Sale, F., Taramelli, D., Day, N.P., White, N.J., And Dondorp, A.M. (2007b). Effects Of Malaria Heme Products On Red Blood Cell Deformability. The American Journal Of Tropical Medicine And Hygiene *77*, 617-622.
212. Nweneka, C.V., Doherty, C.P., Cox, S., And Prentice, A. (2010). Iron Delocalisation In The Pathogenesis Of Malarial Anaemia. Transactions Of The Royal Society Of Tropical Medicine And Hygiene *104*, 175-184.
213. Nye, E.R. (2002). Alphonse Laveran (1845-1922): Discoverer Of The Malarial Parasite And Nobel Laureate, 1907. Journal Of Medical Biography *10*, 81-87.
214. O'Neill, P.M., Barton, V.E., And Ward, S.A. (2010). The Molecular Mechanism Of Action Of Artemisinin--The Debate Continues. Molecules *15*, 1705-1721.
215. Oo, M.M., Aikawa, M., Than, T., Aye, T.M., Myint, P.T., Igarashi, I., And Schoene, W.C. (1987). Human Cerebral Malaria: A Pathological Study. Journal Of Neuropathology And Experimental Neurology *46*, 223-231.
216. Opal, S.M., And Depalo, V.A. (2000). Anti-Inflammatory Cytokines. Chest *117*, 1162-1172.
217. Pai, S., Qin, J., Cavanagh, L., Mitchell, A., El-Assaad, F., Jain, R., Combes, V., Hunt, N.H., Grau, G.E., And Weninger, W. (2014). Real-Time Imaging Reveals The Dynamics Of Leukocyte Behaviour During Experimental Cerebral Malaria Pathogenesis. PLOS Pathogens *10*, E1004236.
218. Pakpour, N., Corby-Harris, V., Green, G.P., Smithers, H.M., Cheung, K.W., Riehle, M.A., And Luckhart, S. (2012a). Ingested Human Insulin Inhibits The Mosquito NF-Kappab-Dependent Immune Response To *Plasmodium falciparum*. Infection and Immunity *80*, 2141-2149.
219. Pakpour, N., Corby-Harris, V., Green, G.P., Smithers, H.M., Cheung, K.W., Riehle, M.A., And Luckhart, S. (2012b). Ingested Human Insulin Inhibits The Mosquito NF-Kb-Dependent Immune Response To *Plasmodium falciparum*. Infection And Immunity *80*, 2141-2149.
220. Pamplona, A., Hanscheid, T., Epiphanio, S., Mota, M.M., And Vigario, A.M. (2009). Cerebral Malaria And The Hemolysis/Methemoglobin/Heme Hypothesis: Shedding New Light On An Old Disease. The International Journal Of Biochemistry And Cell Biology *41*, 711-716.
221. Panisko, D.M., And Keystone, J.S. (1990). Treatment Of Malaria--1990. Drugs *39*, 160-189.
222. Parcell, S. (2002). Sulfur In Human Nutrition And Applications In Medicine. Alternative Medicine Review : A Journal Of Clinical Therapeutic *7*, 22-44.
223. Parker, F.S., And Irvin, J.L. (1952). The Interaction Of Chloroquine With Nucleic Acids And Nucleoproteins. The Journal Of Biological Chemistry *199*, 897-909.
224. Pascual, V., Allantaz, F., Arce, E., Punaro, M., And Banchereau, J. (2005). Role Of Interleukin-1 (IL-1) In The Pathogenesis Of Systemic Onset Juvenile Idiopathic Arthritis And Clinical Response To IL-1 Blockade. The Journal Of Experimental Medicine *201*, 1479-1486.
225. Pasvol, G. (2005). The Treatment Of Complicated And Severe Malaria. British Medical Bulletin *75-76*, 29-47.
226. Pasvol, G., Wainscoat, J.S., And Weatherall, D.J. (1982). Erythrocytes Deficiency In Glycophorin Resist Invasion By The Malarial Parasite *Plasmodium falciparum*. Nature *297*, 64-66.
227. Pavithra, S.R., Banumathy, G., Joy, O., Singh, V., And Tatu, U. (2004). Recurrent Fever Promotes *Plasmodium falciparum* Development In Human Erythrocytes. The Journal Of Biological Chemistry *279*, 46692-46699.
228. Percario, S., Moreira, D.R., Gomes, B.A., Ferreira, M.E., Goncalves, A.C., Laurindo, P.S., Vilhena, T.C., Dolabela, M.F., And Green, M.D. (2012). Oxidative Stress In Malaria. International Journal Of Molecular Sciences *13*, 16346-16372.
229. Perkins, D.J., Were, T., Davenport, G.C., Kempaiah, P., Hittner, J.B., And Ong'echa, J.M. (2011). Severe Malarial Anemia: Innate Immunity And Pathogenesis. Int J Biol Sci *7*, 1427-1442.
230. Piantadosi, C.A. (2008). Carbon Monoxide, Reactive Oxygen Signaling, And Oxidative Stress. Free Radical Biology & Medicine *45*, 562-569.
231. Pichyangkul, S., Saengkrai, P., And Webster, H.K. (1994). *Plasmodium falciparum* Pigment Induces Monocytes To Release High Levels Of Tumor Necrosis Factor-Alpha And Interleukin-1 Beta. The American Journal Of Tropical Medicine And Hygiene *51*, 430-435.
232. Polimeni, M., And Prato, M. (2014). Host Matrix Metalloproteinases In Cerebral Malaria: New Kids On The Block Against Blood-Brain Barrier Integrity? Fluids And Barriers Of The CNS *11*, 1.
233. Polimeni, M., Valente, E., Ulliers, D., Opdenakker, G., Van Den Steen, P.E., Giribaldi, G., And Prato, M. (2013). Natural Haemozoin Induces Expression And Release Of Human Monocyte Tissue Inhibitor Of Metalloproteinase-1. PLOS ONE *8*, E71468.
234. Pong, C.K., Thevenon, A.D., Zhou, J.A., And Taylor, D.W. (2009). Influence Of Human Chorionic Gonadotropin (Hcg) On In Vitro Growth Of *Plasmodium falciparum*. Malaria Journal *8*, 101.
235. Prasad, K., And Garner, P. (2000). Steroids For Treating Cerebral Malaria. The Cochrane Database Of Systematic Reviews , CD000972.
236. Prato, M., And Giribaldi, G. (2011). Matrix Metalloproteinase-9 And Haemozoin: Wedding Rings For Human Host And *Plasmodium falciparum* Parasite In Complicated Malaria. Journal Of Tropical Medicine *2011*, 628435.

237. Price, R., Van Vugt, M., Phaipun, L., Luxemburger, C., Simpson, J., Mcgready, R., Ter Kuile, F., Kham, A., Chongsuphajaisiddhi, T., White, N.J., *Et Al.* (1999). Adverse Effects In Patients With Acute Falciparum Malaria Treated With Artemisinin Derivatives. The American Journal Of Tropical Medicine And Hygiene *60*, 547-555.
238. Prudencio, M., Rodriguez, A., And Mota, M.M. (2006). The Silent Path To ThousAnds Of Merozoites: The *Plasmodium* Liver Stage. Nature Reviews Microbiology*4*, 849-856.
239. Przybelski, R.J., Daily, E.K., Micheels, J., Sloan, E., Mols, P., Corne, L., Koenigsberg, M.D., Bickell, W.H., Thompson, D.R., Harviel, J.D., *Et Al.* (1999). A Safety Assessment Of Diaspirin Cross-Linked Hemoglobin (Dclhb) In The Treatment Of Hemorrhagic, Hypovolemic Shock. Prehospital And Disaster Medicine *14*, 251-264.
240. Ramirez, J.L., Garver, L.S., And Dimopoulos, G. (2009). Challenges And Approaches For Mosquito Targeted Malaria Control. Current Molecular Medicine *9*, 116-130.
241. Ramirez, J.L., Short, S.M., Bahia, A.C., Saraiva, R.G., Dong, Y., Kang, S., Tripathi, A., Mlambo, G., And Dimopoulos, G. (2014). Chromobacterium Csp_P Reduces Malaria And Dengue Infection In Vector Mosquitoes And Has Entomopathogenic And In Vitro Anti-Pathogen Activities. PLOS Pathogens *10*, E1004398.
242. Rao, A., Kumar, M.K., Joseph, T., And Bulusu, G. Cerebral Malaria: Insights From Host-Parasite Protein-Protein Interactions. Malaria Journal *9*, 155.
243. RazakAndrainibe, R., Pelleau, S., Grau, G.E., And Jambou, R. (2012). Antigen Presentation By Endothelial Cells: What Role In The Pathophysiology Of Malaria? Trends In Parasitology *28*, 151-160.
244. Reddy, R.C., Vatsala, P.G., Keshamouni, V.G., Padmanaban, G., And Rangarajan, P.N. (2005). Curcumin For Malaria Therapy. Biochemical And Biophysical Research Communications *326*, 472-474.
245. Reed, Z.H., Friede, M., And Kieny, M.P. (2006). Malaria Vaccine Development: Progress And Challenges. Current Molecular Medicine *6*, 231-245.
246. Reeder, B.J. (2010). The Redox Activity Of Hemoglobins: From Physiologic Functions To Pathologic Mechanisms. Antioxidants & Redox Signaling *13*, 1087-1123.
247. Reeder, B.J., Svistunenko, D.A., Cooper, C.E., And Wilson, M.T. (2004). The Radical And Redox Chemistry Of Myoglobin And Hemoglobin: From In Vitro Studies To Human Pathology. Antioxidants & Redox Signaling *6*, 954-966.
248. Rifkind, J.M., Mohanty, J.G., And Nagababu, E. (2014). The Pathophysiology Of Extracellular Hemoglobin Associated With Enhanced Oxidative Reactions. Frontiers In Physiology *5*, 500.
249. Roberts, N.J. (1979). Temperature And Host Defense. Microbiological Reviews *43*, 241-259.
250. Rodkey, F.L., Hill, T.A., Pitts, L.L., And Robertson, R.F. (1979). Spectrophotometric Measurement Of Carboxyhemoglobin And Methemoglobin In Blood. Clinical Chemistry *25*, 1388-1393.
251. Rodopulo, A.K. (1951). Oxidation Of Tartaric Acid In Wine In The Presence Of Heavy Metal Salts (Activation Of Oxygen By Iron). Izv Akad Nauk SSSR Biol *3*, 115-128.
252. Rohrig, G., Maier, W.A., And Seitz, H.M. (1999). Growth-Stimulating Influence Of Human Chorionic Gonadotropin (Hcg) On *Plasmodium falciparum* In Vitro. Zentralbl Bakteriol *289*, 89-99.
253. Rosenblum, W.I., And El-Sabban, F. (1982). Dimethyl Sulfoxide (DMSO) And Glycerol, Hydroxyl Radical Scavengers, Impair Platelet Aggregation Within And Eliminate The Accompanying Vasodilation Of, Injured Mouse Pial Arterioles. Stroke; A Journal Of Cerebral Circulation *13*, 35-39.
254. Rosenthal, P.J. (2001). Antimalarial Chemotherapy: Mechanisms Of Action, Resistance, And New Directions In Drug Discovery, British Journal Of Clinical Pharmacology 52(4), 464-470.
255. Rosenthal, P.J., And Meshnick, S.R. (1996). Hemoglobin Catabolism And Iron Utilization By Malaria Parasites. Molecular And Biochemical Parasitology *83*, 131-139.
256. Ross, R. (1897). On Some Peculiar Pigmented Cells Found In Two Mosquitos Fed On Malarial Blood. British Medical Journal *2*, 1786-1788.
257. Rother, R.P., Bell, L., Hillmen, P., And Gladwin, M.T. (2005). The Clinical Sequelae Of Intravascular Hemolysis And Extracellular Plasma Hemoglobin: A Novel Mechanism Of Human Disease. The Journal Of The American Medical Association *293*, 1653-1662.
258. Roumenina, L.T., Rayes, J., Lacroix-Desmazes, S., And Dimitrov, J.D. (2016). Heme: Modulator Of Plasma Systems In Hemolytic Diseases. Trends In Molecular Medicine *22*, 200-213.
259. RTS, S.C.T.P. (2015). Efficacy And Safety Of RTS,S/AS01 Malaria Vaccine With Or Without A Booster Dose In Infants And Children In Africa: Final Results Of A Phase 3, Individually Randomised, Controlled Trial. Lancet *386*, 31-45.
260. Russell, P.F., And Mohan, B.N. (1942). The Immunization Of Fowls Against Mosquito-Borne *Plasmodium Gallinaceum* By Injections Of Serum And Of Inactivated Homologous Sporozoites. The Journal Of Experimental Medicine *76*, 477-495.
261. Sachs, J., And Malaney, P. (2002). The Economic And Social Burden Of Malaria. Nature *415*, 680-685.
262. Sagrista, M.L., Garcia, A.E., Africa De Madariaga, M., And Mora, M. (2002). Antioxidant And Pro-Oxidant Effect Of The Thiolic Compounds N-Acetyl-L-Cysteine And Glutathione Against Free Radical-Induced Lipid Peroxidation.Free Radical Research *36*, 329-340.

263. Santos-Martinez, M.J., Medina, C., Jurasz, P., And Radomski, M.W. (2008). Role Of Metalloproteinases In Platelet Function. Thrombosis Research *121*, 535-542.
264. Sarkar, P.K., Ahluwalia, G., Vijayan, V.K., And Talwar, A. (2010). Critical Care Aspects Of Malaria. J Intensive Care Medicineicine *25*, 93-103.
265. Schaer, D.J., Buehler, P.W., Alayash, A.I., Belcher, J.D., And Vercellotti, G.M. (2013). Hemolysis And Free Hemoglobin Revisited: Exploring Hemoglobin And Hemin Scavengers As A Novel Class Of Therapeutic Proteins. Blood *121*, 1276-1284.
266. Schmidt, N.W., Butler, N.S., And Harty, J.T. (2011). *Plasmodium*-Host Interactions Directly Influence The Threshold Of Memory CD8 T Cells Required For Protective Immunity. J Immunol *186*, 5873-5884.
267. SchOfield, L., And Grau, G.E. (2005). Immunological Processes In Malaria Pathogenesis. Nature Reviews Immunology *5*, 722-735.
268. SchOfield, L., Villaquiran, J., Ferreira, A., Schellekens, H., Nussenzweig, R., And Nussenzweig, V. (1987). Gamma Interferon, CD8+ T Cells And Antibodies Required For Immunity To Malaria Sporozoites. Nature *330*, 664-666.
269. Schwarzer, E., Turrini, F., Ulliers, D., Giribaldi, G., Ginsburg, H., And Arese, P. (1992). Impairment Of Macrophage Functions After Ingestion Of *Plasmodium falciparum*-Infected Erythrocytes Or Isolated Malarial Pigment. The Journal Of Experimental Medicine *176*, 1033-1041.
270. Scott, K.L., Lecak, J., And Acker, J.P. (2005). Biopreservation Of Red Blood Cells: Past, Present, And Future. Transfusion Medicine Reviews *19*, 127-142.
271. Seilmaier, M., Hartmann, W., Beissner, M., Fenzl, T., Haller, C., Guggemos, W., Hesse, J., Harle, A., Bretzel, G., Sack, S., *Et Al.* (2014). Severe *Plasmodium* Knowlesi Infection With Multi-Organ Failure Imported To Germany From ThailAnd/Myanmar. Malaria Journal *13*, 422.
272. Shah, M.S., And Brownlee, M. (2016). Molecular And Cellular Mechanisms Of Cardiovascular Disorders In Diabetes. Circulation Research *118*, 1808-1829.
273. Sheffner, A.L., Medler, E.M., Bailey, K.R., Gallo, D.G., Mueller, A.J., And Sarett, H.P. (1966). Metabolic Studies With Acetylcysteine. Biochemical Pharmacology*15*, 1523-1535.
274. Sherman, I. (1998). A Brief History Of Malaria And Discovery Of The Parasite's Life Cycle. Malaria: Parasite Biology, Pathogenesis, And Protection, 3-10.
275. Sherman, I.W. (1979). Biochemistry Of *Plasmodium* (Malarial Parasites). Microbiological Reviews *43*, 453-495.
276. Silva, G., Jeney, V., Chora, A., Larsen, R., Balla, J., And Soares, M.P. (2009). Oxidized Hemoglobin Is An Endogenous Proinflammatory Agonist That Targets Vascular Endothelial Cells. The Journal Of Biological Chemistry *284*, 29582-29595.
277. Sinclair, D., Donegan, S., Isba, R., And Lalloo, D.G. (2012). Artesunate Versus Quinine For Treating Severe Malaria. The Cochrane Database Of Systematic Reviews *6*, CD005967.
278. Singer, S.T., And Ataga, K.I. (2008). Hypercoagulability In Sickle Cell Disease And Beta-Thalassemia. Current Molecular Medicine *8*, 639-645.
279. Sinniah, R., Rui-Mei, L., And Kara, A. (1999). Up-Regulation Of Cytokines In Glomerulonephritis Associated With Murine Malaria Infection. International Journal Of Experimental Pathology *80*, 87-95.
280. Siregar, J.E., Kurisu, G., Kobayashi, T., Matsuzaki, M., Sakamoto, K., Mi-Ichi, F., Watanabe, Y.-I., Hirai, M., Matsuoka, H., Syafruddin, D., *Et Al.* (2015). Direct Evidence For The Atovaquone Action On The *Plasmodium* Cytochrome Bc1 Complex. Parasitology International *64*, 295-300.
281. Slater, A.F. (1993). Chloroquine: Mechanism Of Drug Action And Resistance In *Plasmodium falciparum*. Pharmacology & Therapeutics*57*, 203-235.
282. Sloan, E.P., Koenigsberg, M., Gens, D., Cipolle, M., Runge, J., Mallory, M.N., And Rodman, G., Jr. (1999). Diaspirin Cross-Linked Hemoglobin (Dclhb) In The Treatment Of Severe Traumatic Hemorrhagic Shock: A Randomized Controlled Efficacy Trial. The Journal of the American Medical Association *282*, 1857-1864.
283. Smith, A., And Mcculloh, R.J. (2015). Hemopexin And Haptoglobin: Allies Against Heme Toxicity From Hemoglobin Not Contenders. Frontiers In Physiology *6*, 187.
284. Smith, H.J., And Meremikwu, M. (2003). Iron Chelating Agents For Treating Malaria. The Cochrane Database Of Systematic Reviews , CD001474.
285. Sola, E., Vaya, A., Martinez, M., Moscardo, A., Corella, D., Santaolaria, M.L., Espana, F., And HernAndez-Mijares, A. (2009). Erythrocyte Membrane Phosphatidylserine Exposure In Obesity. Obesity (Silver Spring) *17*, 318-322.
286. Soulard, V., Bosson-Vanga, H., Lorthiois, A., Roucher, C., Franetich, J.F., Zanghi, G., Bordessoulles, M., Tefit, M., Thellier, M., Morosan, S., *Et Al.* (2015). *Plasmodium falciparum* Full Life Cycle And *Plasmodium* Ovale Liver Stages In Humanized Mice. Nature Communications *6*, 7690.
287. Spottiswoode, N., Duffy, P.E., And Drakesmith, H. (2015). Iron, Anemia And Hepcidin In Malaria. The Importance Of Iron In Pathophysiologic Conditions, Frontiers in Pharmacology 6(26), 268.
288. Sprong, R.C., Winkelhuyzen-Janssen, A.M., Aarsman, C.J., Van Oirschot, J.F., Van Der Bruggen, T., And Van Asbeck, B.S. (1998). Low-Dose N-Acetylcysteine Protects Rats Against Endotoxin-Mediated Oxidative Stress, But High-Dose Increases Mortality. American Journal Of Respiratory And Critical Care Medicine *157*, 1283-1293.

289. Srivastava, K., Cockburn, I.A., Swaim, A., Thompson, L.E., Tripathi, A., Fletcher, C.A., Shirk, E.M., Sun, H., Kowalska, M.A., Fox-Talbot, K., *Et Al.* (2008). Platelet Factor 4 Mediates Inflammation In Experimental Cerebral Malaria. Cell Host And Microbe *4*, 179-187.
290. Staines, H.M., Rae, C., And Kirk, K. (2000). Increased Permeability Of The Malaria-Infected Erythrocyte To Organic Cations. Biochimica Biophysica Acta *1463*, 88-98.
291. Stanton, R.C. (2012). Glucose-6-Phosphate Dehydrogenase, NADPH, And Cell Survival. IUBMB Life *64*, 362-369.
292. Stocker, R., Hunt, N.H., Buffinton, G.D., Weidemann, M.J., Lewis-Hughes, P.H., And Clark, I.A. (1985). Oxidative Stress And Protective Mechanisms In Erythrocytes In Relation To *Plasmodium vinckei* Load. Proceeding of the Natlonal Academy Science USA *82*, 548-551.
293. Strother, A., Fraser, I.M., Allahyari, R., And Tilton, B.E. (1981). Metabolism Of 8-Aminoquinoline Antimalarial Agents. Bulletin Of The World Health Organization *59*, 413-425.
294. Subramanian, R., Tam, J., Aidasani, D., Reid, D.L., And Skiles, G.L. (2011). Novel Cytochrome P450 Bioactivation Of A Terminal Phenyl Acetylene Group: Formation Of A One-Carbon Loss Benzaldehyde And Other Oxidative Products In The Presence Of N-Acetyl Cysteine Or Glutathione. Chemical Research In Toxicology *24*, 677-686.
295. Sugita, Y., Nomura, S., And Yoneyama, Y. (1971). Purification Of Reduced Pyridine Nucleotide Dehydrogenase From Human Erythrocytes And Methemoglobin Reduction By The Enzyme. The Journal Of Biological Chemistry *246*, 6072-6078.
296. Surachetpong, W., Pakpour, N., Cheung, K.W., And Luckhart, S. (2011). Reactive Oxygen Species-Dependent Cell Signaling Regulates The Mosquito Immune Response To *Plasmodium falciparum*. Antioxidants & Redox Signaling *14*, 943-955.
297. Surolia, N., And Padmanaban, G. (1991). Chloroquine Inhibits Heme-Dependent Protein Synthesis In *Plasmodium falciparum*. Proceeding of the Natlonal Academy Science USA *88*, 4786-4790.
298. Takashima, M., Shichiri, M., Hagihara, Y., Yoshida, Y., And Niki, E. (2012). Reactivity Toward Oxygen Radicals And Antioxidant Action Of Thiol Compounds. Biofactors *38*, 240-248.
299. Takeoka, S., Sakai, H., Kose, T., Mano, Y., Seino, Y., Nishide, H., And Tsuchida, E. (1997). Methemoglobin Formation In Hemoglobin Vesicles And Reduction By Encapsulated Thiols. Bioconjugate Chemistry *8*, 539-544.
300. Takeuchi, O., And Akira, S. (2010). Pattern Recognition Receptors And Inflammation. Cell *140*, 805-820.
301. Taramelli, D., Basilico, N., Pagani, E., GrAnde, R., Monti, D., Ghione, M., And Olliaro, P. (1995). The Heme Moiety Of Malaria Pigment (Beta-Hematin) Mediates The Inhibition Of Nitric Oxide And Tumor Necrosis Factor-Alpha Production By Lipopolysaccharide-Stimulated Macrophages. Experimental Parasitology *81*, 501-511.
302. Tayal, V., And Kalra, B.S. (2008). Cytokines And Anti-Cytokines As Therapeutics--An Update. European Journal Of Pharmacology *579*, 1-12.
303. Taylor, W.R., And White, N.J. (2004). Antimalarial Drug Toxicity: A Review. Drug Safety *27*, 25-61.
304. Team, W. (2000). Severe Falciparum Malaria. World Health Organization, Communicable Diseases Cluster. Transactions Of The Royal Society Of Tropical Medicine And Hygiene *94 Suppl 1*, S1-90.
305. Thawani, N., Tam, M., Bellemare, M.-J., Bohle, D.S., Olivier, M., De Souza, J.B., And Stevenson, M.M. (2013). *Plasmodium* Products Contribute To Severe Malarial Anemia By Inhibiting Erythropoietin-Induced Proliferation Of Erythroid Precursors. Journal Of Infectious Diseases, Jit417.
306. Thomé, R., Lopes, S.C.P., Costa, F.T.M., And Verinaud, L. (2013). Chloroquine: Modes Of Action Of An Undervalued Drug. Immunology Letters *153*, 50-57.
307. Thompson, M.G., Breiman, R.F., Hamel, M.J., Desai, M., Emukule, G., Khagayi, S., Shay, D.K., Morales, K., Kariuki, S., Bigogo, G.M., *Et Al.* (2012). Influenza And Malaria Coinfection Among Young Children In Western Kenya, 2009-2011. The Journal of Infectious Diseases *206*, 1674-1684.
308. Tietjen, G.W., Chien, S., Leroy, E.C., Gavras, I., Gavras, H., And Gump, F.E. (1975). Blood Viscosity, Plasma Proteins, And Raynaud Syndrome. Archives Of Surgery *110*, 1343-1346.
309. Tombe, M., Bhatt, K.M., And Obel, A.O. (1993). Clinical Surprises And Challenges Of Severe Malaria At Kenyatta National Hospital, Kenya. East African Medical Journal *70*, 117-119.
310. Totino, P.R., Magalhaes, A.D., Silva, L.A., Banic, D.M., Daniel-Ribeiro, C.T., And Ferreira-Da-Cruz Mde, F. (2010). Apoptosis Of Non-Parasitized Red Blood Cells In Malaria: A Putative Mechanism Involved In The Pathogenesis Of Anaemia. Malaria Journal *9*, 350.
311. Trager, W., And Jensen, J.B. (1976). Human Malaria Parasites In Continuous Culture. Science *193*, 673-675.
312. Trager, W., Rudzinska, M.A., And Bradbury, P.C. (1966). The Fine Structure Of *Plasmodium falciparum* And Its Host Erythrocytes In Natural Malarial Infections In Man. Bulletin World Health Organ *35*, 883-885.
313. Treeprasertsuk, S., Krudsood, S., Tosukhowong, T., Maek, A.N.W., Vannaphan, S., Saengnetswang, T., Looareesuwan, S., Kuhn, W.F., Brittenham, G., And Carroll, J. (2003). N-Acetylcysteine In Severe Falciparum Malaria In Thailand. Southeast Asian Journal Of Tropical Medicine Public Health *34*, 37-42.
314. Tripette, J., Alexy, T., Hardy-Dessources, M.D., Mougenel, D., Beltan, E., Chalabi, T., Chout, R., Etienne-Julan, M., Hue, O., Meiselman, H.J., *Et Al.* (2009). Red Blood Cell Aggregation, Aggregate Strength And

Oxygen Transport Potential Of Blood Are Abnormal In Both Homozygous Sickle Cell Anemia And Sickle-Hemoglobin C Disease. Haematologica *94*, 1060-1065.
315. Trivedi, V., Chand, P., Maulik, P.R., And BAndyopadhyay, U. (2005a). Mechanism Of Horseradish Peroxidase-Catalyzed Heme Oxidation And Polymerization (Beta-Hematin Formation). Biochimica Biophysica Acta *1723*, 221-228.
316. Trivedi, V., Chand, P., Srivastava, K., Puri, S.K., Maulik, P.R., And BAndyopadhyay, U. (2005b). Clotrimazole Inhibits Hemoperoxidase Of *Plasmodium falciparum* And Induces Oxidative Stress. Proposed Antimalarial Mechanism Of Clotrimazole. The *The Journal* Of *Biological Chemistry 280*, 41129-41136.
317. Trivedi, V., Srivastava, K., Puri, S.K., Maulik, P.R., And BAndyopadhyay, U. (2005c). Purification And Biochemical Characterization Of A Heme Containing Peroxidase From The Human Parasite *P. falciparum*. Protein Expression And Purification *41*, 154-161.
318. Tu, Y. (2011). The Discovery Of Artemisinin (Qinghaosu) And Gifts From Chinese Medicine. Nature Medicine *17*, 1217-1220.
319. Umbreit, J. (2007). Methemoglobin--It's Not Just Blue: A Concise Review. American Journal Of Hematology *82*, 134-144.
320. Valero, M.V., Amador, R., Aponte, J.J., Narvaez, A., Galindo, C., Silva, Y., Rosas, J., Guzman, F., And Patarroyo, M.E. (1996). Evaluation Of Spf66 Malaria Vaccine During A 22-Month Follow-Up Field Trial In The Pacific Coast Of Colombia. Vaccine *14*, 1466-1470.
321. Van't Veer, M., And Haferlach, T. (2014). Should Clinical Hematologists Put Their Microscopes On Ebay? Haematologica *99*, 1533-1534.
322. Van Dyke, K., Lantz, C., And Szustkiewicz, C. (1970). Quinacrine: Mechanisms Of Antimalarial Action. Science *169*, 492-493.
323. Viriyavejakul, P., Khachonsaksumet, V., And Punsawad, C. (2014). Liver Changes In Severe *Plasmodium falciparum* Malaria: Histopathology, Apoptosis And Nuclear Factor Kappa B Expression. Malaria Journal *13*, 1-10.
324. Wagner, M.A., Andemariam, B., And Desai, S.A. (2003). A Two-Compartment Model Of Osmotic Lysis In *Plasmodium falciparum*-Infected Erythrocytes. Biophysical Journal *84*, 116-123.
325. Wassmer, S.C., Lepolard, C., Traore, B., Pouvelle, B., Gysin, J., And Grau, G.E. (2004). Platelets Reorient *Plasmodium falciparum*-Infected Erythrocyte Cytoadhesion To Activated Endothelial Cells. The Journal of Infectious Diseases *189*, 180-189.
326. Wassmer, S.C., Taylor, T.E., Rathod, P.K., Mishra, S.K., Mohanty, S., Arevalo-Herrera, M., Duraisingh, M.T., And Smith, J.D. (2015). Investigating The Pathogenesis Of Severe Malaria: A Multidisciplinary And Cross-Geographical Approach. The American Journal Of Tropical Medicine And Hygiene *93*, 42-56.
327. Watt, G., Jongsakul, K., And Ruangvirayuth, R. (2002a). A Pilot Study Of N-Acetylcysteine As Adjunctive Therapy For Severe Malaria. QJM *95*, 285-290.
29. Weatherall, D.J., And Abdalla, S. (1982). The Anaemia Of *Plasmodium falciparum* Malaria. British Medical Bulletin *38*, 147-151.
330. Weatherall, D.J., Higgs, D.R., Clegg, J.B., And Wood, W.G. (1982). The Significance Of Haemoglobin H In Patients With Mental Retardation Or Myeloproliferative Disease. British Journal Of Haematology *52*, 351-355.
331. Were, T., Davenport, G.C., Hittner, J.B., Ouma, C., Vulule, J.M., Ong'echa, J.M., And Perkins, D.J. (2011). Bacteremia In Kenyan Children Presenting With Malaria. Journal Of Clinical Microbiology *49*, 671-676.
332. White, N.J. (2011). Determinants Of Relapse Periodicity In *Plasmodium* Vivax Malaria. Malaria Journal *10*, 297.
333. White, N.J., Pukrittayakamee, S., Hien, T.T., Faiz, M.A., Mokuolu, O.A., And Dondorp, A.M. (2014). Malaria. Lancet *383*, 723-735.
334. Whittam, R., And Ager, M.E. (1964). Vectorial Aspects Of Adenosine-Triphosphatase Activity In Erythrocyte Membranes. The Biochemical Journal *93*, 337-348.
335. WHO (2016). WHO World Annual Malaria Report, 2015. WHO Bulletin.
336. Widmer, C.C., Pereira, C.P., Gehrig, P., Vallelian, F., Schoedon, G., Buehler, P.W., And Schaer, D.J. (2010). Hemoglobin Can Attenuate Hydrogen Peroxide-Induced Oxidative Stress By Acting As An Antioxidative Peroxidase. Antioxidants & Redox Signaling *12*, 185-198.
338. Wiewiora, M., Sosada, K., Wylezol, M., Slowinska, L., And Zurawinski, W. (2007). Red Blood Cell Aggregation And Deformability Among Patients Qualified For Bariatric Surgery. Obes Surg *17*, 365-371.
339. Wright, R.O., LewAnder, W.J., And Woolf, A.D. (1999). Methemoglobinemia: Etiology, Pharmacology, And Clinical Management. Annals Of Emergency Medicine *34*, 646-656.
340. Wu, X., Gowda, N.M., Kumar, S., And Gowda, D.C. (2010). Protein-DNA Complex Is The Exclusive Malaria Parasite Component That Activates Dendritic Cells And Triggers Innate Immune Responses. Journal Of Immunology *184*, 4338-4348.
341. Yayon, A., Cabantchik, Z.I., And Ginsburg, H. (1985). Susceptibility Of Human Malaria Parasites To Chloroquine Is Ph Dependent. Proceeding of the Natlonal Academy Science USA *82*, 2784-2788.

342. Yayon, A., Timberg, R., Friedman, S., And Ginsburg, H. (1984). Effects Of Chloroquine On The Feeding Mechanism Of The Intraerythrocytic Human Malarial Parasite *Plasmodium falciparum*. Journal Of Protozool *31*, 367-372.
343. Zaki, S.A., And Shanbag, P. (2011). Atypical Manifestations Of Malaria. Journal Of Research & Reports in Tropical Medicine *2*, 9-22.
344. Zhao, Y.O., Kurscheid, S., Zhang, Y., Liu, L., Zhang, L., Loeliger, K., And Fikrig, E. (2012). Enhanced Survival Of *Plasmodium*-Infected Mosquitoes During Starvation. PLOS ONE *7*, E40556.
345. Zilletti, L., Ciuffi, M., Franchi-Micheli, S., Fusi, F., Gentilini, G., Moneti, G., Valoti, M., And Sgaragli, G.P. (1994). Cyclooxygenase Activity Of Hemoglobin. Methods in Enzymology *231*, 562-573.
346. Ziment, I. (1988). Acetylcysteine: A Drug That Is Much More Than A Mucokinetic. Biomedicine and Pharmacotherapy *42*, 513-519.

List of Publications

1. **S N Balaji** and Vishal Trivedi*, **Extracellular methemoglobin mediated early ROS spike triggers osmotic fragility and RBC destruction: An insight into the enhanced hemolysis during malaria**, Indian J Clin Biochem. 2012 Apr;27(2):178-85. doi: 10.1007/s12291-011-0176-5. PMID: 23543627.

2. **S N Balaji** and Vishal Trivedi*, **Extracellular methemoglobin primes red blood cell aggregation in malaria: An in vitro mechanistic study**, FEBS Lett. 2013 Feb 14;587(4):350-7. doi: 10.1016/j.febslet.2012.12.015. PMID: 23313944**.**

3. **S N Balaji** and Vishal Trivedi*, **Methemoglobin incites primaquine toxicity through single electron oxidation and modification**, J Basic Clin Physiol Pharmacol. 2013;24(2):105-14. doi: 10.1515/jbcpp-2012-0058. PMID: 23412873**.**

4. **S N Balaji** and Vishal Trivedi*, **Suicidal inactivation of methemoglobin by generation thiyl radical: insight into NAC mediated protection in RBC**, Curr Mol Med. 2013 Jul;13(6):1000-9. PMID: 23745587.

5. **S N Balaji**, Mohamed Jawed Ahsan*, Surender Singh Jadav, Vishal Trivedi, **Molecular modeling, synthesis, and antimalarial potential of curcumin analogues containing heterocyclin ring**, Arabian Journal of Chemistry, 2015., doi:10.1016/j.arabjc.2015.04.011.

6. Balakrishna Muthuraj, Saurav Layek, **S N Balaji**, Vishal Trivedi*, Parameswar Krishnan Iyer*, **Multiple Function Fluorescein probe performs metal chelation, disaggregation and modulation of aggregated Aβ and Aβ-Cu complex**, ACS Chem Neurosci. 2015 Nov 18;6(11):1880-91. doi: 10.1021/acschemneuro.5b00205. PMID: 26332658.

7. Arnish Chakraborty, **S N Balaji**, Vishal Trivedi*, **Cloning, overexpression, purification and immunolocalization of PFD0975W from the malaria parasite Plasmodium falciparum**, Infect Disord Drug Targets. 2016 May 6., PMID: 27150807.

8. Nag, S., Chouhan, DK., **Balaji, SN.**, Chakraborty, A., Lhouvum, K., Bal, C., Sharon, A and Trivedi, V* (2013) Comprehensive screening of heterocyclic compound libraries to identify novel inhibitors for PfRIO-2 kinase through docking and substrate competition studies. Med Chem Res, 22; 4737-4744.

Conference Presentations

1. Presented poster entitled **"Extracellular Methemoglobin Mediated Oxidative Stress Causes RBC Destruction: An Insight into Enhanced Hemolysis"** at 80th Annual Meeting of SBC(I) on "Metabolic Pathway Modulations-Application in Health and Agriculture" organized by CSIR-CIMAP, Lucknow, 12-15 Nov 2011.

2. Presented poster entitled **"Understanding Mistakes Drive Solution for Adjuvant Therapy Against Malaria: A Mechanistic Study"** at 83th Annual Meeting of SBC(I) on **"Evolution: Molecules to Life"** organized by ILS, NISER and KIIT University, Bhubaneswar, 18-21 Dec 2014.

3. Participated in National conference on **"Recent Advances in Cancer Biology and Therapetics 2014"** at Indian Institute of Technology, Guwahati, held on 5 Dec 2014.

4. Participated in **"Model presentation"** in National Level symposium on **"Bioinsilicans – Motif '08"** organized by Bharathiar University, Coimbatore-641046 held on 24 Sep' 2008.

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ORIGINAL ARTICLE

Extracellular Methemoglobin Mediated Early ROS Spike Triggers Osmotic Fragility and RBC Destruction: An Insight into the Enhanced Hemolysis During Malaria

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Abstract Malaria infection is known to cause severe hemolysis due to production of abnormal RBCs and enhanced RBC destruction through apoptosis. Infected RBC lysis exposes uninfected RBC to the large amount of pro-oxidant molecules such as methemoglobin. Methemoglobin (MetHb) exposure dose dependently makes RBCs susceptible to osmotic stress and causes hemolysis. MetHb mediated oxidative stress in RBC correlated well with osmotic fragility and hemolysis. Interestingly, a reactive oxygen species (ROS) spike at 15 min was responsible for the observed effects on RBC cells. Two natural antioxidants N-acetyl cysteine and mannitol protected the RBC from MetHb-mediated defects, which clearly indicated involvement of oxidative stress in the process. MetHb due to its pseudo-peroxidase activity produces ROS in the external microenvironment. Therefore, classical peroxidase inhibitors were tested to probe peroxidase activity mediated ROS production with defects in RBCs. Clotrimazole (CLT), which irreversibly inactivates the MetHb (CLT-MetHb) and abolishes peroxidase activity, did not produce significant ROS outside RBC and was inefficient to cause osmotic fragility and hemolysis. Hence, initiating a chain reaction, MetHb released from ruptured RBC produces significant ROS in the external microenvironment to make RBC membrane leaky and enhanced hemolysis. Together data presented in the current work explored the role of MetHb in accelerated humorless during malaria which could be responsible for severe outcomes of pathological disorders.

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Keywords RBC · Methemoglobin · Reactive-oxygen species · Hemolysis · Antioxidant · Malaria

Abbreviations

GSH	Reduced glutathione
NAC	N-acetyl cysteine
ROS	Reactive oxygen species
MetHb	Methemoglobin
CLT-MetHb	Clotrimazole modified methemoglobin

Introduction

Red blood cell (RBC) is a biconcave disc shape cell with an average diameter of 7.5 μm. RBC is produced in bone marrow with an average life span of 120 days. During this period, RBC needs to do several cycles of gaseous exchange (CO_2/O_2), experience shear stress (pass through small blood vessels) and other physiological or non-physiological stresses. As a result, RBC inevitably aged out and express specific cell surface molecule to get cleared by circulating phagocytes [1]. The whole process of RBC destruction within reticulo-endothelial system doesn't cause inflammatory reactions [2]. However during malaria, an accelerated RBC lysis occurs, leading to the development of hemolytic anemia [3]. According to a rough estimate, during malaria every infected RBC (IRBC) causes lysis of ten or more uninfected RBC to exhibit hemolytic anemia [4]. A recent study correlates the enhanced uninfected RBC destruction through apoptosis with the observed development of anemia during malaria [5]. The mechanistic details of apoptosis in uninfected RBC or inducer molecules are not known but results indicate pro-oxidant molecules released from infected RBC might be

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Extracellular methemoglobin primes red blood cell aggregation in malaria: An in vitro mechanistic study

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ABSTRACT

Toxic byproducts from infected RBC cause rheological alteration and RBC aggregation. Malaria culture supernatant has the ability to exhibit RBC aggregation. Ammonium sulfate fractionation and immunodepletion of methemoglobin from culture supernatant confirms methemoglobin as a major aggregant. In vitro treatment of RBC with methemoglobin induces irreversible high order RBC aggregates, resistant to shear stress and physical forces. Methemoglobin-mediated ROS generation in the external micro-environment to develop oxidative stress close to RBC membrane seems to be responsible for initiating and forming high order RBC aggregates through phosphatidyl-serine externalization. Removal of oxidative stress through antioxidant treatment abolishes high order RBC aggregate formation. In conclusion, we discovered a novel pathway of methemoglobin-mediated RBC aggregation and its potential role in patho-physiological effects during malaria.

1. Introduction

Malaria caused by *Plasmodium falciparum* has a high mortality rate due to anemia, fever, multiple organ damage, renal failure, liver dysfunction and neurological disorders [1–4]. A number of clinical, biochemical and immunological studies tried to explain patho-physiological mechanisms during malaria but our understanding is still limited and underlying molecular events are not clear [5–9]. The main process responsible for tissue damage and dysfunction is a mechanical blockage of blood vessels. Vesicular obstruction is caused by infected RBC adherence to the endothelial lining of blood vessels and aggregation of uninfected RBC [1].

RBC aggregation during malaria is a complex process governed by adhesion ligand on infected RBC cells, receptor expression on uninfected RBC, coagulation factors, platelet mediated agglutination and plasma proteins [10–16]. The sticky forces between infected and un-infected RBCs slow down micro-circulatory flow but molecular mechanism and master player are not known.

A number of factors such as changes in shear stress, protein–protein interactions and erythrocyte membrane charge alteration regulate the RBC aggregate formation [10,17,18]. During malaria oxidative insult makes RBC membrane sticky to bind infected RBC cells expressing pfEMP-1 to form rosettes and aggregates [19]. In addition, infected RBCs express phosphatidyl serine (PS) to mimic aged or senescent RBCs and been cleared from circulation by macrophages [20,21]. Toxic by-products released from infected RBCs during parasite life-cycle develop pro-coagulant micro-environment. Heme released from infected RBC reduces the RBC deformability (RBC-D), an important property of normal RBC to pass through the small capillaries and avoid splenic clearance [22]. Both infected and normal RBC lysis releases large amount of hemoglobin in the blood. Hemoglobin released from ruptured RBC is oxidized by molecular oxygen to form methemoglobin (metHb). Extracellular metHb further accelerates the RBC lysis to contribute into elevated levels of metHb and hemoglobin degradation products [23]. Methemoglobin exposure to the endothelial cells produces a number of inflammatory markers and cellular adhesion molecules to promote endothelial cell-RBC adhesion [24–26]. In the current study, we explored the potential role of methemoglobin present in infected serum to cause RBC aggregation and its mechanism. Our results indicate that pseudo-peroxidase activity associated with methemoglobin causes production of free radicals to induce oxidative stress within RBC. Oxidative stress within RBC causes phosphatidyl serine (PS) externalization to generate sticky patches to initiate aggregation [27]. Hence methemoglobin induces RBC aggregation through membrane modification by oxidative stress mediated PS externalization.

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Methemoglobin incites primaquine toxicity through single-electron oxidation and modification

Abstract

Background: Primaquine (Pq) metabolic products are responsible for drug-associated hemotoxicity and limit primaquine usage.

Methods: Methemoglobin (MetHb)-Pq molecular modeling was used to identify the Pq binding pocket. UPLC, mass spectrometry, and other indirect analytical methods were used to predict the metabolite. MetHb generation, development of oxidative stress, inhibition of antioxidant enzymes, and scanning electron microscope (SEM) were used to characterize the hemotoxic potentials of oxidized Pq (Pq_{ox}).

Results: MetHb binded Pq at the heme site with K_D=6.4 μM as evidenced by a difference spectroscopy study. MetHb oxidized Pq through a single e-transfer mechanism to form Pq_{ox}. The analysis of Pq from MetHb-H_2O_2 peroxidase reaction mixture gave peaks at m/z 300.53 and m/z 243.42, corresponding to the hydroxyl and desamino derivative of Pq, respectively. Similar peaks were absent in Pq or Pq incubated with H_2O_2 in the same buffer system. A robust increase in MetHb formation, reactive oxygen species generation, and inhibition of antioxidant enzymes were found in red blood cells (RBCs) exposed to Pq_{ox} compared with a parent drug molecule. The RBC membrane exhibited visible damages to plasma membrane (holes) as evidenced by SEM analysis of Pq_{ox}-exposed RBCs.

Conclusions: The MetHb-H_2O_2 system transforms quiescent parent drug molecule to a highly reactive oxidative form to exhibit severe hemolysis. MetHb-H_2O_2-mediated Pq hemolytic potentiation that is sensitive to spin trap indicates the role of Pq* radical or other single e-species in the process. The result suggests that MetHb incites the molecular property of the Pq and peroxidase inhibitors can be explored to control drug-associated toxicity.

Keywords: hemolysis; methemoglobin; peroxidase; primaquine; RBC.

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Introduction

Malaria is a tropical disease caused by plasmodium species and leads to 200,000 deaths every year. In humans, the disease starts with the bite of the anopheles mosquito, which replicates in the host's liver and red blood cells (RBCs) [1]. In addition, the parasite resides within the hepatocytes under dormant stages (hypnozoites) and causes a disease on a preset time scale, a process known as relapse. Reappearance of the disease on a frequent basis (monthly or quarterly) without reinfection causes an immense burden on host machinery to contribute into the observed deaths and be responsible for an additional level of complication to treat malaria [2]. Chloroquine and artemisinin are the drugs used to target the blood stages of the parasite, whereas primaquine (Pq) is the only drug available used for clearing hypnozoites from hepatocytes [3, 4]. Pq dosing is more important for patients with *Plasmodium vivax* to avoid potential relapses within weeks or even months [5]. However, prolonged treatment is known to cause methemoglobinemia, hemolysis, abdominal cramps, and pathophysiological symptoms, which are more severe in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency [6, 7]. G6PD is involved in the regeneration of NADPH to support lipid biosynthesis, redox reactions, and maintenance of intracellular glutathione (GSH) level [8]. The G6PD-deficient RBCs accumulate reactive oxygen species (ROS), and this makes RBCs prone to further oxidative damage [9]. Pq is oxidatively metabolized to carboxy Pq (CPrq), 6-methoxy-8-aminoquinoline (MAQ), and hydroxylated derivatives [10, 11].

Suicidal Inactivation of Methemoglobin by Generation of Thiyl Radical: Insight into NAC Mediated Protection in RBC

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Abstract: N-acetyl-L-cysteine (NAC) improves antioxidant potentials of RBCs to provide protection against oxidative stress induced hemolysis. The antioxidant mechanism of NAC to reduce oxidative stress in RBC, studied through inactivation of pro-oxidant MetHb. NAC causes irreversible inactivation of the MetHb in an H_2O_2 dependent manner, and the inactivation follows the pseudo- first- order kinetics. The kinetic constants are k_i = 8.5μM, k_{inact} = 0.706 min^{-1} and $t_{1/2}$ = 0.9 min. Spectroscopic studies indicate that MetHb accepts NAC as a substrate and oxidizes through a single electron transfer mechanism to the NAC_{ox}. The single e^- oxidation product of NAC has been identified as the 5, 5'- dimethyl-1- pyrroline N- oxide (DMPO) adduct of the sulfur centered radical (a^N = 15.2 G and a^H=16. 78 G). Binding studies indicate that NAC_{ox} interacts at the heme moiety and NAC oxidation through MetHb is essential for NAC binding. Heme-NAC adduct dissociated from MetHb and identified (*m/z* 1011.19) as 2:1 ratio of NAC/heme in the adduct. TEMPO and PBN treatment reduces NAC binding to MetHb and protects against inactivation confirms the role of thiyl radical in the inactivation process. Furthermore, scavenging thiyl radicals by TEMPO abolish the protective effect of NAC in hemolysis. Current work highlights antioxidant mechanism of NAC through NAC thiyl radical generation, and MetHb inactivation to exhibit protection in RBC against oxidative stress induced hemolysis.

Keywords: Antioxidant, free radicals, hemolysis, malaria, NAC, RBC.

INTRODUCTION

Malaria caused by *Plasmodium falciparum* is associated with anemia, fever, multiple organ damage, renal failure, liver dysfunction and neurological disorders [1-4]. Reactive oxygen species (ROS) production and development of oxidative stress are crucial for disease pathology during malaria [5, 6]. Pro-oxidant molecules released from parasitized RBCs (PRBCs) or reactivity of immune cells towards PRBCs are mainly associated with development of oxidative stress [7]. Oxidative stress induces structural changes in RBC to activate immune cells and structurally altered RBCs tend to enhance vesicular blockage, cyto-adherence to promote cerebral malaria pathology [8, 9]. N-acetyl L-cysteine (NAC), a thiol (-SH) group containing antioxidant is used in adjuvant therapy against paracetamol toxicity in liver cells and mucolytic agent to treat respiratory diseases [10-12]. It improves antioxidant potential of RBCs to give protection against oxidative stress induced hemolysis [13]. N-acetyl-l-cysteine is tested in a series of *in vitro* drug toxicity studies or clinical studies to monitor malaria patient recovery during chemotherapy [14-17]. Hemin released from an infected RBC decreased the RBC deformability, a cellular property required for RBC splenic clearance. NAC improves RBC deformability through reduction of hemin mediated RBC membrane oxidation and replenishing GSH reserves [18]. A number of critical parameters monitored to assess potentials of NAC in an adjuvant therapy to support artesunate treatment in patients, but outcomes indicate that it give no significant improvement in lactate clearance times, mortality, coma recovery times or red cell rigidity [14].

Cyto-protective properties of NAC are due to direct interaction with a single electron containing free radical species, trapping toxic substances, inhibition of apoptotic and other inflammatory pathways or indirectly supports the synthesis of glutathione (GSH), a cellular antioxidant molecule [5, 19]. The antioxidant potential of a cell determined by the level of GSH within the cytosol and maintained by the GSSG reduction through enzymatic activity of glutathione reductase (GR), non-enzymatic reduction by other small molecular weight antioxidants or de-novo synthesis of GSH [20]. NAC mediated free radical scavenging is due to interaction of a free thiol group (-SH) to electrophilic ROS species [21-25]. The kinetic measurement indicates a rapid interaction of NAC with hypochlorous acid (HOCl) and hydroxyl radical (OH^*), slowly with H_2O_2 and no interaction with superoxide radicals (O^*) [22, 26]. NCB-20 cells treated with H_2O_2 found to reduce oxidative stress mediated NF-κB activation through the generation of NAC free radicals [27]. The formation of NAC thiyl radicals within the cells or in a cell free system seems to be essential for biological activity of

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ORIGINAL ARTICLE

Molecular modelling, synthesis, and antimalarial potentials of curcumin analogues containing heterocyclic ring

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KEYWORDS

Antimalarial;
Curcumin analogues;
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Plasmodium falciparum;
Pyrazole;
Pyrimidine

Abstract The molecular modelling approach was applied to a series of nineteen curcumin analogues to find the possible PfRIO2 kinase inhibitory action. A putative active site in flexible loop (S1) of PfRIO2 kinase was explored computationally to recognize the molecular basis of ligands binding. The ligands (curcumin analogues; **3a–3s**) were well accommodated in the selected active site (S1) due to their higher molecular size and length. Further all these synthesized compounds (**3a–3s**) were evaluated for their *in vitro* antimalarial activity according to the reported method. The antimalarial data showed that all these compounds to have parasiticidal activity with minimum killing concentrations (MKCs) range between 3.87 and 25.35 μM and schizonticidal activity with IC_{50} range between 1.48 and 23.09 μM. The compound **3p** showed the most significant result with maximum schizonticidal (IC_{50}; 1.48 ± 0.10 μM) and parasiticidal activities (MKC; 3.87 ± 0.36 μM) could be identified as promising lead for further investigations.

1. Introduction

Malaria is a life-threatening disease caused by five species of parasites and is transmitted through vector infected mosquito of genus *Anopheles*. An estimated 3.4 billion people are at risk of malaria and there are 107 countries and territories, where malaria is endemic. As per WHO report nearly 207 million cases of malaria and 627,000 death tolls occurred globally in 2012. Most of the cases and deaths occurred in unhygienic and developing African countries (WHO malaria report, 2013). Artemisinin combination therapy (ACTs) has been of much importance over the last decade however emergence of resistance has been reported in Cambodia's Pailin province (WHO malaria report, 2013; Phyo et al., 2012; Kumar et al., 2005) provides motivation to discover new potential antimalarial agents. More than 65 protein kinases (PKs) have been reported in the parasite kinome with ambiguous cellular target

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Multiple Function Fluorescein Probe Performs Metal Chelation, Disaggregation, and Modulation of Aggregated Aβ and Aβ-Cu Complex

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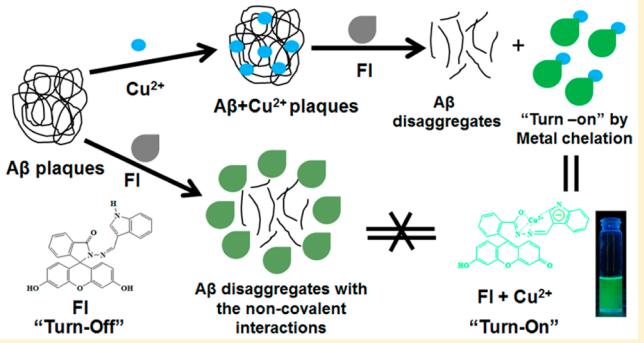
Supporting Information

ABSTRACT: An exceptional probe comprising indole-3-carboxaldehyde fluorescein hydrazone (FI) performs multiple tasks, namely, disaggregating amyloid β (Aβ) aggregates in different biomarker environments such as cerebrospinal fluid (CSF), Aβ1−40 fibrils, β-amyloid lysozyme aggregates (LA), and U87 MG human astrocyte cells. Additionally, the probe FI binds with Cu^{2+} ions selectively, disrupts the Aβ aggregates that vary from few nanometers to micrometers, and prevents their reaggregation, thereby performing disaggregation and modulation of amyloid-β in the presence as well as absence of Cu^{2+} ion. The excellent selectivity of probe FI for Cu^{2+} was effectively utilized to modulate the assembly of metal-induced Aβ aggregates by metal chelation with the "turn-on" fluorescence via spirolactam ring opening of FI as well as the metal-free Aβ fibrils by noncovalent interactions. These results confirm that FI has exceptional ability to perform multifaceted tasks such as metal chelation in intracellular conditions using Aβ lysozyme aggregates in cellular environments by the disruption of β-sheet rich Aβ fibrils into disaggregated forms. Subsequently, it was confirmed that FI had the ability to cross the blood-brain barrier and it also modulated the metal induced Aβ fibrils in cellular environments by "turn-on" fluorescence, which are the most vital properties of a probe or a therapeutic agent. Furthermore, the morphology changes were examined by atomic force microscopy (AFM), polarizable optical microscopy (POM), fluorescence microscopy, and dynamic light scattering (DLS) studies. These results provide very valuable clues on the Aβ (CSF Aβ fibrils, Aβ1−40 fibrils, β-amyloid lysozyme aggregates) disaggregation behavior via in vitro studies, which constitute the first insights into intracellular disaggregation of Aβ by "turn-on" method thereby influencing amyloidogenesis.



KEYWORDS: *Amyloid β, Alzheimer's disease, cerebrospinal fluid, neurodegenerative diseases, fluorescein, therapeutics, aggregation, modulator*

Alzheimer's disease (AD), the most prevalent cause of dementia among elderly population, affects >40 million people worldwide.[1,2] Early symptoms such as difficulty to remember newly acquired information and other severe symptoms such as mood and behavior changes, perplexity, severe memory loss, and judgment alteration coupled with difficulties in speaking, writing, and walking predominantly begin to appear with aging. As per the AD hypothesis, the aggregation of amyloid-β (Aβ) peptide is linked to the etiology of the disease, since soluble monomeric forms are found in the healthy brain while amyloid plaques are detected in an AD patient's brain.[3,4] These micrometer sized aggregates provide an important pharmacological target in the ability of drugs to (a) disrupt the already formed Aβ aggregates, (b) prevent Aβ aggregation, or (c) be capable of arresting and inverting the progression of AD.[5,6] Aβ40 and Aβ42 peptides are the primary species in the structure of the senile plaques found in the brain tissues of AD patients.[6−8] Metal ions, such as iron, copper, and zinc, are known to interact with Aβ peptides and promote their aggregation as well as generation of neurotoxicity.[9−18] They also have the ability to modulate the aggregation of Aβ peptides as observed by in vitro experiments.[9,19−22] Despite being an important pharmacological target for AD pathogenesis, separation of toxic metals from Aβ peptide fibrils along with their disruption and modulation remains unresolved.

The accumulation of metals such as iron, copper, and zinc within the senile plaques can reach levels of up to 400, 950, and 1100 mm, respectively, which is 3−5 times higher in concentration as compared to healthy brain.[23−27] To reduce the harmful effects of excess metal deposition, newer therapies that focus on metal ion chelation for AD are being developed.[28−30] Metal chelators are potential therapeutic prospects due to the metal ion hypothesis and the possible

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