CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND OF THE STUDY

In the last decade, the prevalence of malaria has been escalating at an alarming rate, especially in Africa. A recent estimate of malaria incidence derived from routine surveillance data suggest 174 million episode occurred in African region in 2011 with estimated deaths of 596,000 (WHO, 2011). Malaria has been estimated to cause 2.3% of global disease and 9% of disease in Africa (WHO, 1997); it ranks third among major infectious disease threats in Africa after pneumococcal acute respiratory infections (3.5%) and tuberculosis (TB) (2.8%). Between 1994 and 1996, malaria epidemics in 14 countries of sub-Saharan Africa caused an unacceptably high number of deaths, of which many occurred in areas previously free of the disease (Anderson *et al.,* 1996). Adolescents and young adults are now dying of severe forms of the disease. Air travel has brought the threat of the disease to the doorsteps of industrialized countries, with an increasing incidence of imported cases and deaths from malaria by visitors to endemic-disease regions. The estimated annual direct and indirect cost of malaria was 800 million US dollars in 1987, this was expected to exceed 1.8 billion US dollars by 1995 (Anderson *et al.,* 1996). The vast majority of malaria deaths occur in Africa, south of the Sahara, where malaria also presents major obstacles to social and economic development. Malaria has been estimated to cost Africa more than 12 billion US dollars every year, even though it could be controlled for a fraction of that sum.

Malaria is Africa's leading cause of under-five mortality (20%) and constitutes 10% of the continent's overall disease burden. It accounts for 40% of public health expenditure, 30-50% of in-patient admissions, and up to 50% of outpatient visits in areas with high malaria transmission (WHO, 2002). There are several reasons why Africa bears an overwhelming proportion of the malaria burden. Most malaria infections in Africa south of the Sahara are caused by *Plasmodium falciparum,* the most severe and life threatening form of the disease. This region is also home to the most efficient, and therefore deadly, species of the mosquitoes, which transmit the disease. Moreover, many countries in Africa lack the infrastructures and resources necessary to mount sustainable campaigns against malaria and as a result few benefit from historical efforts to eradicate malaria. In Africa today, malaria is understood to be both a disease of poverty and a cause of poverty. Annual economic growth in countries with high malaria transmission has historically been lower than in countries without malaria. Economists believe that malaria is responsible for a growth penalty of up to 1.3% per year in some African countries (WHO, 2002). When compounded over the years, this penalty leads to substantial differences in GDP (Gross domestic product) between countries with and without malaria and severely restrains the economic growth of the entire region (DeLeire and Manning, 2004). Malaria also has a direct impact on Africa's human resources. Not only does malaria result in lost life and lost productivity due to illness and premature death, malaria also hampers children's schooling and social development through both absenteeism and permanent neurological and other damage associated with severe episodes of the disease.

1.2 STATEMENT OF PROBLEM

The interaction between malaria and nutrition is complex (McGregor, 1982) and has been the subject of controversy since the early 1950s. Several studies have shown associations between malaria and protein energy malnutrition, poor growth and certain micronutrient deficiencies among children (Mc Gregor *et al.*, 1956; Marsden, 1964; Rowland *et al.*, 1977; Nyakeriga *et al.*, 2004). Moreover, studies of malaria prevention involving insecticide-treated bed nets or chemoprophylaxis have been associated with improvement in the growth and micronutrient status of children (Archibald *et al.*, 1956; Bradley-Moore *et al.*, 1985; Snow *et al.*, 1997; Ter-Kuile *et al.*, 2003). Despite clear evidence of the impact of malaria on the nutritional status of affected individuals, the effect of nutritional status on host resistance to the acquisition and progression of malaria is still not clearly defined. Earlier studies suggested that poorly nourished individuals were to some extent protected against malaria (Edington, 1954; Hendrickse *et al.*, 1971; Murray *et al.*, 1975; Murray *et al.*, 1976; Murray *et al.*, 1978a; Murray *et al.*, 1978b). A review of the malaria–nutrition literature (Shankar, 2000; Augustin *et al.*, 2008), concluded that the earlier findings of a protective effect of malnutrition against malaria were mainly based on studies with several methodological shortcomings. Reappraisal of the data together with evidence from literature indicates that the effect of nutrition on host susceptibility to malaria is more complex and, in many cases, poor nutritional status predisposes the host to an increased risk of infection, symptomatic clinical malaria attacks, and a higher likelihood of mortality from malaria (Augustin *et al.*, 2008). Several factors influence malaria treatment decisions. These include the severity of the infection, the child's age, degree of background immunity, other host factors that may impair immune function

such as malnutrition or advanced HIV infection, local patterns of antimalarial drug resistance, availability of drugs, and the cost of antimalarial drugs (White, 1996). Hence, there is need to address the controversy surrounding the use of antioxidant micronutrients in malaria.

1.3 JUSTIFICATION FOR THE SELECTION OF MICRONUTRIENTS

Malaria infection is known to be associated with specific nutrient deficiency in children; the micronutrients implicated in this deficiency state were essentially vitamins and trace metals which have antioxidant properties. Among the vitamins implicated in malaria, vitamin A, C and E were selected based on the fact that they are commonly in use as supplements and their therapeutic role has not been clearly defined particularly with respect to their co-administration with antimalarials in humans. Moreso, trace metals such as zinc have been studied clinically in a few longititudinal cohort studies in children, however there is scanty information on therapeutic trial with this agent. In addition to this, no human study exist on the potential therapeutic benefit of selenium in malaria.

1.4 AIMS/OBJECTIVES

1.4.1 General Objective:

This study is designed to determine the potential antimalarial activity of selected antioxidant micronutrients in *Plasmodium berghei* parasitised mice as well as determine the antimalarial activity in uncomplicated *Plasmodium falciparum* infection.

1.4.2 Specific Objectives:

1) To determine antiplasmodial activity of selected antioxidant micronutrient with a 4-day suppression test as well as determine the chemopreventive and chemotherapeutic role of the selected micronutrients in malaria.
2) To evaluate synergistic effect of selected antioxidant combination and standard antimalarial agents as well as to provide reliable pre-clinical data for clinical trials of the combinations.
3) To conduct a clinical study that will elucidate the effect of selected antioxidant micronutrients when used in varying combinations as adjuvants in the treatment of uncomplicated *falciparum* malaria as well as determine the antimalarial effect of selected standard agents when used in combination with selected micronutrients in the therapy of uncomplicated *falciparum* malaria.
4) To elucidate possible mechanisms of antimalarial action for selected antioxidant micronutrients.
5) To evaluate serum vitamins; A, E and C levels and serum trace element (zinc and selenium) levels in under five's with uncomplicated *falciparum* malaria (pre-treatment day 0 and post-treatment day 28).

1.5 SIGNIFICANCE OF STUDY

This study is expected to provide an alternative treatment option in the management of uncomplicated *falciparum* malaria in children under the age of five years.

1.6 LIMITATION OF SCOPE OF STUDY

The use of *in vivo* models to evaluate the antimalarial effect of the selected micronutrients without corroboration with *in vitro* models.

1.7 LIST OF ABBREVIATIONS

- **ACT:** Artesmisin combination therapy
- **AMA:** Apical membrane antigen
- **CIDR:** Cysteine-rich interdomain region
- **CQ:** Chloroquine
- **CTRP:** Circumsporozoite and TRAP-related protein of Plasmodium
- **DBL:** Duffy binding like
- **DBP:** Duffy binding protein
- **EBA:** Erythrocyte Binding Antigen
- **EGF:** Epithelial Growth Factor
- **FCT:** Fever Clearance Time
- **KAHRP:** Knob-associated histidine rich protein
- **MCH:** Mean Corpuscular Haemoglobin
- **MCHC:** Mean Corpuscular Haemoglobin Concentration
- **MDA:** Malondialdehyde
- **MDR:** Multidrug resistant protein
- **MSP:** Merozoite surface protein
- **MST:** Mouse Survival Time
- **PCR:** Polymerase Chain Reaction
- **PCT:** Parasite Clearance Time

- **PCV:** Packed Cell Volume
- **PECAM 1:** Platelet endothelial cell adhesion molecule-1
- ***Pf*crt:** Plasmodium falciparum chloroquine resistant gene
- ***Pf*EMP-1:** Plasmodium falciparum erythrocyte membrane protein-1
- ***Pf*MDR1:** Plasmodium falciparum multidrug resistant gene-1
- **PUFA:** Polyunsaturated fatty acid
- **PVM:** Parasitophorous Vacoule Membrane
- **RBC:** Red Blood Cell
- **RESA:** Red Cell Surface Antigen
- **SSP2:** Plasmodium sporozoite surface protein-2.
- **TBARS:** Thiobarbituric Acid Reacting Substances
- **TRAP:** Thrombospondin-related adhesive protein.
- **VCAM:** Vascular cell Adhesion Molecule
- **WBC:** White Blood Cell Count

1.8 DEFINITION OF OPERATIONAL TERMS

Active Comparator: This is the control group in the clinical trial that is administered with standard interventional regimen.

Adjuvant Therapy: The use of agents as supportive measure to standard chemotherapy.

Antioxidants: These are agents that protect biological system from oxidative damage.

Cure: Cure was defined as initial and sustained parasite and symptom clearance with no increase in asexual parasitemia 48 hours after the initiation of treatment and the absence of microscopically detected asexual parasitemia within 120 hours of the commencement of treatment until day 7, 14 and 28 (for 7 day, 14 day and 28 day cure rates respectively).

Cure Rates: Cure rates were calculated from the number of patients with clinical and parasitological cure by day 7, 14, or 28 divided by the total number of patients who could be evaluated (per protocol population).

Chemoprohylaxis: Chemoprophylaxis refers to the administration of a medication for the purpose of preventing disease or infection.

Chemosuppression: It is a term used to describe the inhibition of the growth of schizonts following drug administration.

Fever Clearance Time: Fever clearance time (FCT) is calculated from the start of treatment until the first of two consecutive temperature measurements remain below 37.5°C.

***In Vivo* Study:** An experimental study within a biological system.

Intention to Treat Analysis: Analysis of data for all participants based on the group to which they were randomized and not based on the actual treatment they received.

Inoculum: The titre of *Plasmodium berghei* infected blood obtained from donor mouse.

Malaria: A protozoan disease caused in humans by four species of the *Plasmodium* genus: *Plasmodium falciparum; Plasmodium vivax; Plasmodium ovale* and *Plasmodium malariae;* and transmitted by the bite of an infected female mosquito of the genus Anopheles. Malaria in animals is caused by other species of plasmodia.

Micronutrients: These are essential nutrients required in trace amounts for normal growth, development and metabolism

Mouse Survival Time: It is the average time of survival in days after administration of treatment to parasitized mice and compared to the control.

Parasite Clearance Time: The time required for parasite clearance between the beginning of treatment and the time when no asexual forms is found on the blood film.

Parasite Clearance Rate: It is the parasite reduction ratio, calculated as the rate between the parasite density before treatment and that at 48 hours from commencement of treatment.

Passage: The intraperitoneal administration of *Plasmodium berghei* infected blood from donor mouse to recipient mouse.

Protocol Population: Total number of participants who received actual treatment during the clinical trials.

Randomized Controlled Clinical Trials: A trial in which participants are randomly assigned to two or more different treatment or intervention groups.

Repository Activity: It is a measure of the prophylactic activity of a drug, that is, the ability of the drug to inhibit schizonts following a pre-passaging administration of the drug.

Recrudescence: The reoccurrence of parasitemia after an initial and complete clearance of parasite.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 EPIDEMIOLOGY

Malaria has been and still is the cause of much human morbidity and mortality. Although the disease has been eradicated in most temperate zones, it continues to be endemic throughout much of the tropics and subtropics. Forty percent of the world's population lives in endemic areas. Epidemics have devastated large populations and malaria poses a serious barrier to economic progress in many developing countries. There is an estimated 300-500 million cases of clinical disease per year with 1.5-2.7 million deaths (WHO, 1995). Some of the earliest known medical writings from China, Assyria, and India accurately describe the malaria-like intermittent fevers. Hippocrates, the 'father of medicine', is generally credited with the first description of the clinical symptoms in 500 BC, more than 2000 years before the parasite was described (Hoops, 1934).

Malaria is primarily a disease of the tropics and subtropics and is widespread in hot humid regions of Africa, Asia and South and Central America. The disease has been eradicated in many temperate areas including the USA, Europe and northern Eurasia and Asia. However, some areas which previously had malaria under control are experiencing resurgence (Cunha, 2008). The four human malarial species exhibit an overlapping geographical distribution. *P. vivax* and *P. falciparum* are the most commonly encountered species with *P. vivax* being the most widespread geographically. Mixed infections are common in endemic areas. Molecular methods suggest that ***P. malariae*** and ***P. ovale*** might be more widespread and prevalent than previously thought (Mueller *et al.,* 2007).

The epidemiology of malaria can be viewed in terms of being stable (or endemic) or unstable (or epidemic). Stable malaria refers to a situation in which there is a measurable incidence of natural transmission over several years. This would also include areas that experience seasonal transmission. Different areas can experience different levels of incidence rates and this is often denoted by: hypoendemic, mesoendemic, hyperendemic, and holoendemic. Persons living in highly endemic areas usually exhibit a high level of immunity and tolerate the infection well.

Unstable or epidemic malaria refers to an increase in malaria in areas of low endemicity or to outbreaks in areas previously without malaria or among non-immune persons. These outbreaks can usually be attributed to changes in human behavior or effects on the environment. For example, human migration and resettlement can either introduce malaria into an area or expose a previously non-immune population to endemic transmission. Changes in the ecology caused by natural disasters or public works projects such as building roads can also impact malaria transmission and lead to epidemics.

The above emphasizes the complexity of malaria and the many facets the disease exhibits. Different communities will experience different malaria and consequently different control and treatment strategies may be necessary. The intricate interactions between host, parasite, and vector are the major factors in this epidemiological complexity. For example, as with all vector-transmitted diseases, the parasite must be able to establish a chronic infection within the host to maximize the opportunities for transmission. This is especially true in the case of seasonal transmission and in areas of low endemicity. In general, malaria infections are characterized by an initial acute phase followed by a longer relatively asymptomatic chronic phase. This is due in part to the

ability of the parasite to avoid complete clearance by the immune system. For example, *P. falciparum* exhibits an antigenic variation that allows it to stay one step ahead of the immune system. In addition, *P. vivax* and *P. ovale* exhibit the hypnozoite stage and are capable of relapses. This allows the parasite to maintain the infection within the human host even after the blood stage of the infection has been cleared. The relative long interval between relapses in some *P. vivax* isolates probably explains its ability to maintain transmission cycles in some temperate climates. Several molecular epidemiology studies have indicated that *P. falciparum* can also produce long-term chronic infections (Roper, 1996).

With regards to the host, humans are the only significant reservoir for the parasite and sustained transmission depends upon maintaining a pool of infected individuals and contact between humans and anopheline mosquitoes. Several factors influence the susceptibility of humans to infection. Obviously the immune status of the individual and their prior experience with malaria will influence the course of the infection. Pregnant women, especially during the first pregnancy, are more susceptible to *falciparum* malaria as evidenced by a higher prevalence of infection and higher parasitemias. In addition, certain genetic diseases and polymorphisms have been associated with a decrease in infection or disease.

The potential of the mosquito to serve as a vector depends on the ability to support sporogony, mosquito abundance, and contact with humans, which are all influenced by climatic and ecological factors. The ability to support sporogony is largely dependent upon species in that not all species of ***Anopheles*** are susceptible to ***Plasmodium*** infection (Ahmed, 1987). Temperature and mosquito longevity are other key factors affecting the

parasite's interaction with the vector. Development of *P. falciparum* requires a minimum temperature of 20^{o}C, whereas the minimum temperature for the other species is 16^{o}C. Temperature also affects the time of development in that the duration of sporogony is substantially shorter at higher temperatures (Amerasinghe *et al.*, 1999). A shorter duration of sporogony increases the chances that the mosquito will transmit the infection within its lifespan.

2.2 PLASMODIUM SPECIES INFECTING HUMANS

Malaria is caused by members of the genus *Plasmodium*. *Plasmodium* species are apicomplexa and exhibit a heteroxenous life cycle involving a vertebrate host and an arthropod vector. Vertebrate hosts include: reptiles, birds, rodents, monkeys and humans. *Plasmodium* species are generally host specific and vector specific in that each species will only infect a limited range of hosts and vectors. The species differ in regards to their morphology, details of their life cycles, and their clinical manifestations. However, molecular methods have revealed the possible existence of other species or morphological variants. For example, sequencing of the gene for the circumsporozoite surface protein (CSP) revealed that some individuals diagnosed with *P. vivax* infections were actually infected with a distinct species more closely related to *P. simiovale*, a simian malaria parasite that is morphologically identical to *P. vivax*. In addition, molecular analysis indicates that *P. ovale* consists of two clades that are as divergent as distinct species (Cox-Singh and Singh, 2008).

Similarly, molecular analyses indicate that some morphological variants of *P. malariae* are distinct parasites related to *P. malariae* and *P. brasilianum* (David, 2007). *P. brasilianum* is a simian parasite of South and Central America that is often speculated to have originated from humans as a result of colonization of the New World.

In addition, humans naturally infected with the simian parasite *P. knowlesi* have been identified in Malaysia. Nearly all of the cases identified by microscopy as *P. malariae* were determined to be *P. knowlesi* by PCR (Polymerase chain reaction). Four fatalities associated with *P. knowlesi* infection were also reported from Malaysia. Humans infected

with *P. knowlesi* may not be such a rare occurence and may be widespread in Malaysia and perhaps other parts of South-East Asia (Cox-Singh, 2008).

The four major human *Plasmodium* species are found in tropical and subtropical regions throughout the world and exhibit overlapping geographical distributions. Differences between the species include:

- Blood-stage morphology
- Minor life cycle variations (Cox-Singh, 2008)
    - *P. vivax* and *P. ovale* exhibit the hypnozoite stage and can cause true relapses.
    - Trophozoite and schizont-infected erythrocytes of *P. falciparum* sequester in the microvasculature and are not found in the peripheral circulation.
- Host erythrocyte preference
    - *P. vivax* and *P. ovale* prefer reticulocytes (immature erythrocytes)
    - *P. malariae* prefers senescent erythrocytes
    - *P. falciparum* exhibits no preference
- Disease and clinical manifestation

The older designations for the various types of malaria reflect the differences in the diseases caused by the different *Plasmodium* species. *P. falciparum* causes the most severe disease, hence the malignant designation. The increase morbidity and mortalilty correlates with the higher parasitemia associated with *P. falciparum* infections and the complications arising from sequestration. Factors contributing to the higher parasitemias include: large number of merozoites per schizont, lack of host erythrocyte preference, and

the immune evasion (ie, spleen avoidance) provided by sequestration. Tertian and quartan refer to the differences in the periodicity of paroxysms. Tertian patterns exhibit 48 hour periodicities and quartan refers to a 72 hour periodicity. Note that in Roman counting the first attack is on day one followed by a symptom-free day and then the next attack on day three. The species also exhibit other differences in disease severity and duration.

2.2.1 Morphological Differences

The blood-stage parasites of human *Plasmodium* species exhibit differences in their morphology and modify the host erythrocyte differently. These differences can be used to distinguish the four species. *P. falciparum* blood smears are characterized by the presence of young trophozoites (that is, rings) in the absence of mature trophozoites and schizonts. The ring stages of *P. falciparum* tend to be slightly smaller than the other species and are generally more numerous. Multiple infected erythrocytes and appliqué forms are seen more often in *P. falciparum* than in the other species. The crescent-shaped gametocytes of *P. falciparum* are very distinctive, but tend to only appear late in the infection. The most distinctive features of *P. vivax* are the enlarged infected erythrocytes and the appearance of granules, called 'Schüffner's dots', over the erythrocyte cytoplasm. These granules are manifestation of caveola-vesicle complexes that form on the erythrocyte membrane. The growing trophozoite of *P. vivax* often has an ameboid apearance and the schizonts can have more than 20 merozoites.

The ring forms of all four species are very similar and difficult to distinguish. *P. falciparum* rings tend to be a little smaller and more numerous than the other species. The presence of a large number of rings in the absence of more mature stages, as well as

multiply infected erythrocytes, is highly suggestive of *P. falciparum*. Erythrocytes infected with *P. vivax* and *P. ovale* are enlarged and exhibit Schüffner's dots as the rings mature into trophozoites. The trophozoites of *P. vivax* are often ameboid, whereas *P. ovale* tends to be more compact. The *P. malariae* trophozoite is very compact and the host erythrocyte is not enlarged.

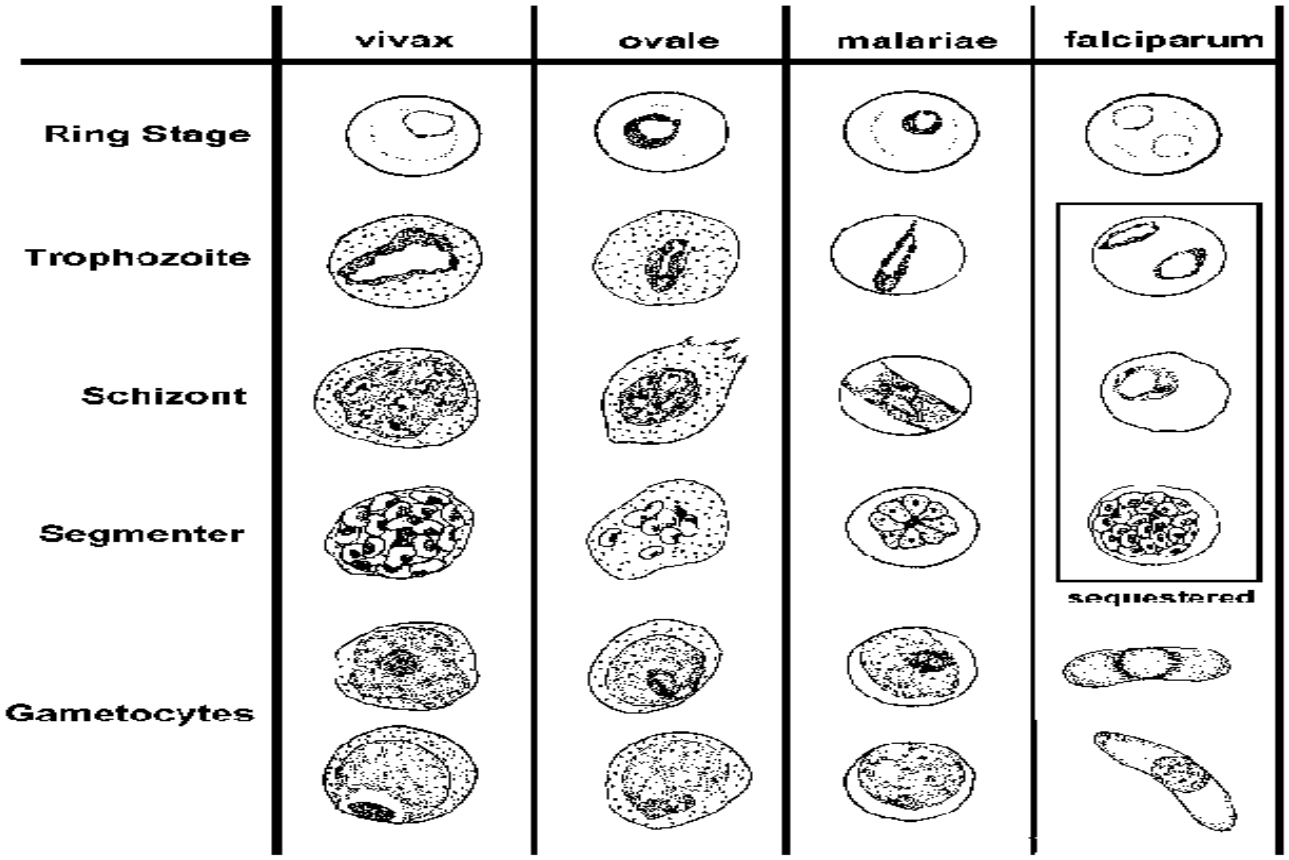


Plate 1 : Different Stages of Plasmodium Species (Adapted from Holder *et al.*, 1994)

Mature asexual forms of *P. falciparum* are rarely found in the peripheral circulation. The typical number of merozoites produced per schizont is: *P. vivax* 14-20 (up to 24), *P. ovale* 6-12 (up to 18), *P. malariae* 8-10 (up to 12), and *P. falciparum* 16-24 (up to 36). *P. falciparum* exhibits crescent-shaped gametocytes whereas the other species are all round to oval. *P. vivax* and *P. ovale* gametocytes are in enlarged erythrocytes with Schüffner's dots and are difficult to distinguish from each other. *P. malariae* gametocytes do not modify the host erythrocyte. Gametocytes can be distinguished from trophozoites by their large size (nearly filling the erythrocyte) and a single nucleus. Mature microgametocytes tend to stain lighter than macrogametocytes and have a more diffuse nucleus.

P. ovale also exhibits Schüffner's dots and an enlarged erythrocyte, making it difficult to distinguish from *P. vivax*. In general, *P. ovale* is a more compact parasite than *P. vivax*. This compactness is most evident in the growing trophozite stage and fewer merozoites are found per schizont. *P. ovale* also has more of a tendency to form elongated host erythrocytes. *P. malariae* is characterized by a compact parasite (all stages) and does not alter the host erythrocyte or cause enlargment. Elongated trophozoites stretching across the erythrocyte, called band forms, are sometimes observed. Schizonts will typically have 8-10 merozoites that are often arranged in a rosette pattern with a clump of pigment in the center.

2.2.2 History of *Plasmodium*

The genus *Plasmodium* was described in 1885 by Ettore Marchiafava and Angelo Celli. Currently over 200 species of this genus are recognized and new species continue to be described (Chavatte *et al.*, 2007; Perkins and Austin, 2008). The organism was first seen

by Laveran on November 6, 1880 at a military hospital in Constantine, Algeria, when he discovered a microgametocyte exflagellating. In 1885, similar organisms were discovered within the blood of birds in Russia. There was a brief speculation that birds might be involved in the transmission of malaria. In 1894 Patrick Manson hypothesized that mosquitoes could transmit malaria. This hypothesis was independently confirmed by the Italian physician Giovanni Battista Grassi working in Italy and the British physician Ronald Ross working in India, both in 1898 (Hoops, 1934). Ross demonstrated the existence of *Plasmodium* in the wall of the midgut and salivary glands of a *Culex* mosquito using bird species as the vertebrate host. For this discovery he won the Nobel Prize in 1902. Grassi showed that human malaria could only be transmitted by *Anopheles* mosquitoes. It is worth noting, however, that for some species the vector may not be a mosquito (Chavatte *et al.*, 2007).

2.2.3 Plasmodium Life Cycle

The malaria parasite exhibits a complex life cycle involving an insect vector (mosquito) and a vertebrate host (human). The infection is initiated when sporozoites are injected with the saliva of a feeding mosquito. Sporozoites are carried by the circulatory system to the liver and invade hepatocytes. The intracellular parasite undergoes an asexual replication known as exoerythrocytic schizogony within the hepatocyte. Exoerythrocytic schizogony culminates in the production of merozoites, which are released into the bloodstream. A proportion of the liver-stage parasites from *P. vivax* and *P. ovale* go through a dormant period instead of immediately undergoing asexual replication. These hypnozoites will reactivate several weeks to months (or years) after the primary infection

and are responsible for relapses. Merozoites invade erythrocytes and undergo a trophic period in which the parasite enlarges. The early trophozoite is often referred to as 'ring form' because of its morphology. Trophozoite enlargement is accompanied by an active metabolism including the ingestion of host cytoplasm and the proteolysis of hemoglobin into amino acids. The end of the trophic period is manifested by multiple rounds of nuclear division without cytokinesis resulting in a schizont. Merozoites bud from the mature schizont, also called a segmenter, and the merozoites are released following rupture of the infected erythrocyte. Invasion of erythrocytes reinitiates another round of the blood-stage replicative cycle. The blood stage is responsible for the pathology associated with malaria. The intermittent fever paroxyms are due to the synchronous lysis of the infected erythrocytes. *P.malariae* exhibits a 72 hour periodicity, whereas the other three species exhibit 48 hour cycles. However, *P. falciparum* often exhibits a continuous fever rather than the periodic paroxyms. *P. falciparum* also is responsible for more morbidity and mortality than the other species. This increase virulence is due in part to the higher levels of parasitemia associated with *P. falciparum* infections. In addition, more complications are associated with *P. falciparum* because of the sequestration of the trophozoite and schizont-infected erythrocytes in the deep tissues. As an alternative to the asexual replicative cycle, the parasite can differentiate into sexual forms known as macro- or microgametocytes. The gametocytes are large parasites, which fill up the erythrocyte, but only contain one nucleus. Ingestion of gametocytes by the mosquito vector induces gametogenesis (that is, the production of gametes) and escape from the host erythrocyte. Factors, which participate in the induction of gametogenesis, include a drop in temperature, an increase in carbon dioxide, and an increase in mosquito metabolites.

Microgametes, formed by a process known as exflagellation, are flagellated forms, which will fertilize the macrogamete leading to a zygote. The zygote develops into a motile ookinete, which penetrates the gut epithelial cells and develops into an oocyst. The oocyst undergoes multiple rounds of asexual replication resulting in the production of sporozoites. Rupture of the mature oocyst releases the sporozoites into the hemocoel (body cavity) of the mosquito. The sporozoites migrate to and invade the salivary glands, thus completing the life cycle.

Sexual reproduction occurs with the switch from vertebrate to invertebrate host and leads to the formation of the invasive ookinete. All invasive stages are characterized by the apical organelles typical of apicomplexan species.

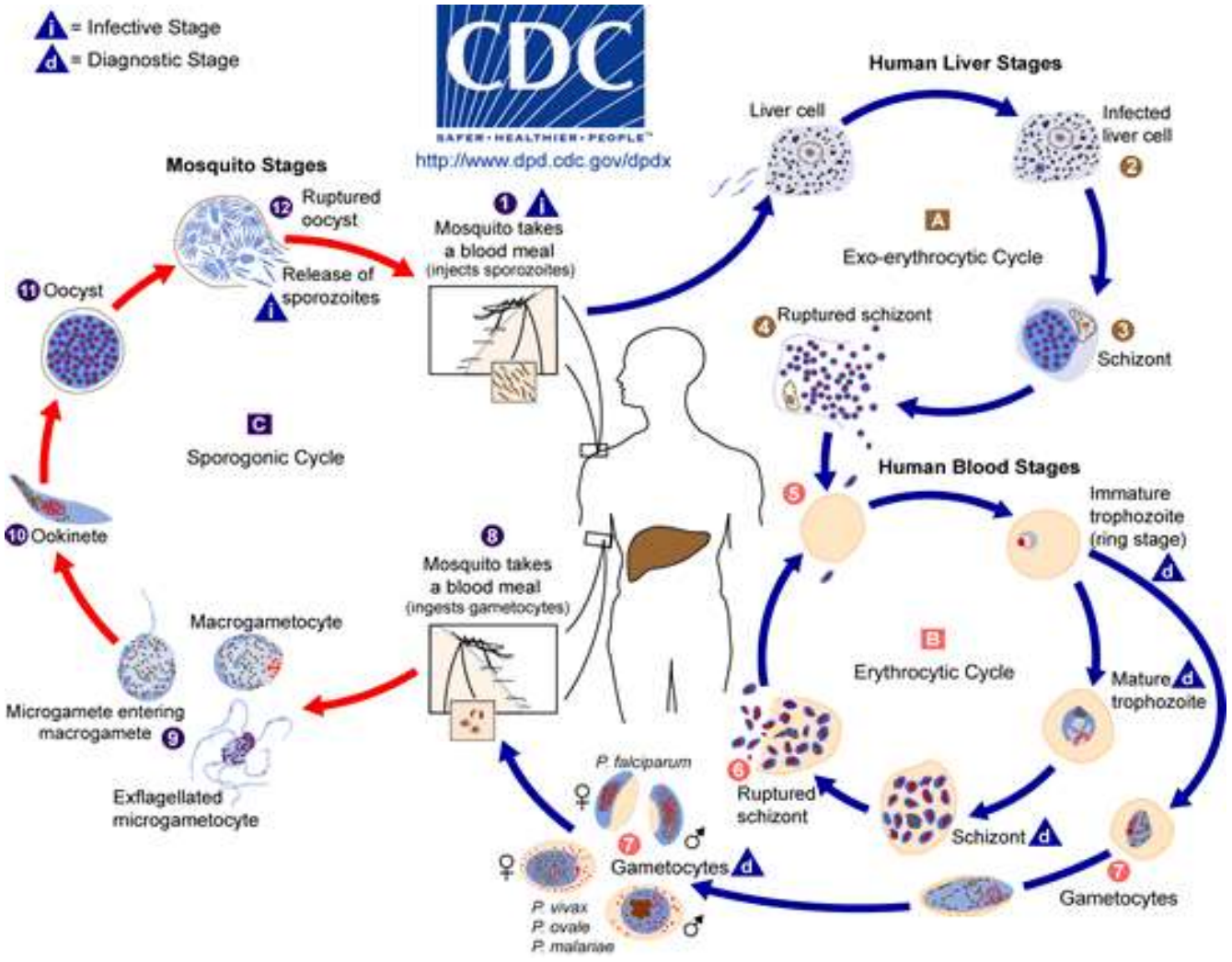


Plate 2: Life Cycle of *Plasmodium.* Adapted from Centre for Disease Control, (2010).

Uncomplicated malaria consists of symptomatic malaria parasitemia <5 percent without evidence of vital organ dysfunction, with the ability to take oral therapy. Young children, non-immune adults, and immunocompromised patients with malaria may deteriorate rapidly to severe malaria (WHO, 2010).

Genetic recrudescences or relapses can occur over months or years and develop severe complications (especially *P. falciparum*). Synchronous release of merozoites, antigens (for example, pyrogenic material) and high levels of tumor necrosis factor-α correlates with paroxysm. Between paroxysms temperature is normal and patient feels well; *P. falciparum* may not exhibit classic paroxysms (continuous fever). Paroxysms become less severe and irregular.

Human and other mammalian *Plasmodium* species are transmitted by anopheline mosquitoes. The parasite is injected with the saliva during mosquito feeding and first undergoes a round of merogony in the liver followed by multiple rounds of merogony in the erythrocytes. Gametogony begins within the erythrocytes of the vertebrate host and is completed within the mosquito where sporogony takes place. This life cycle exhibits the general features of other apicomplexan parasites characterized by asexual replication and the formation of invasive stages with typical apical organelles.

Liver Stage

Human infection is initiated when sporozoites are injected with the saliva during mosquito feeding. The sporozoites enter the circulatory system and within 30-60 minutes will invade a liver cell. Host cell entry, as in all apicomplexa, is facilitated by the apical organelles. After invading the hepatocyte, the parasite undergoes an asexual replication. This replicative stage is often called exoerythrocytic (or pre-erythrocytic) schizogony.

Schizogony refers to a replicative process in which the parasite undergoes multiple rounds of nuclear division without cytoplasmic division followed by a budding, or segmentation, to form progeny (Cox-Singh and Singh, 2008). The progeny, called merozoites, are released into the circulatory system following rupture of the host hepatocyte. In *P. vivax* and *P. ovale* some of the sporozoites do not immediately undergo asexual replication, but enter a dormant phase known as the hypnozoite. This hypnozoite can reactivate and undergo schizogony at a later time resulting in a relapse. Relapse has a specific meaning with regards to malaria and refers to the reactivation of the infection via hypnozoites. Recrudescence is used to describe the situation in which parasitemia falls below detectable levels and then later increases to a patent parasitemia. Interestingly, strains isolated from temperate regions tend to exhibit a longer latent period between the primary infection and the first relapse than strains from tropical regions with continuous transmission.

Blood Stage

Merozoites released from the infected liver cells invade erythrocytes. The merozoites recognize specific proteins on the surface of the erythrocyte and actively invade the cell in a manner similar to other apicomplexan parasites. After entering the erythrocyte the parasite undergoes a trophic period followed by an asexual replication. The young trophozoite is often called a ring form due to its morphology in Geimsa-stained blood smears. As the parasite increases in size this 'ring' morphology disappears and it is called a trophozoite. During the trophic period the parasite ingests the host cell cytoplasm and breaks down the hemoglobin into amino acids.

A by-product of the hemoglobin digestion is the malaria pigment, or hemozoin. This golden-brown to black granules has been long recognized as a distinctive feature of blood-stage parasites.

Nuclear division marks the end of the trophozoite stage and the beginning of the schizont stage. Erythrocytic schizogongy consists of 3-5 rounds (depending on species) of nuclear replication followed by a budding process. Late stage schizonts in which the individual merozoites become discernable are called segmenters. The host erythrocyte ruptures and releases the merozoites. These merozoites invade new erythrocytes and initiate another round of schizogony. The blood-stage parasites within a host usually undergo a synchronous schizogony. The simultaneous rupture of the infected erythrocytes and the concomitant release of antigens and waste products accounts for the intermittent fever paroxysms associated with malaria. Blood stage schizogony in *P. falciparum* differs from the other human malarial parasites in that trophozoite and schizont-infected erythrocytes adhere to capillary endothelial cells and are not found in the peripheral circulation. This sequestration is associated with cerebral malaria.

Sexual Stage

As an alternative to schizogony some of the parasites will undergo a sexual cycle and terminally differentiate into either micro- or macrogametocytes. The factors involved in the induction of gametocytogenesis are not known. However, commitment to the sexual stage occurs during the asexual erythrocytic cycle that immediately precedes gametocyte formations. Daughter merozoites from this schizont will develop into either all asexual

forms or all sexual forms. Gametocytes do not cause pathology in the human host and will disappear from the circulation if not taken up by a mosquito.

Gametogenesis, or the formation of micro- and macrogametes, is induced when a mosquito ingests the gametocytes. After ingestion by the mosquito, the microgametocyte undergoes three rounds of nuclear replication. These eight nuclei then become associated with flagella that emerge from the body of the microgametocyte. This process is readily observable by light microscopy due to the thrashing flagella and is called exflagellation. The macrogametocytes mature into macrogametes. However, at the morphological level this is much less dramatic than the exflagellation exhibited by the microgametocytes.

Exflagellation occurs spontaneously when infected blood is exposed to air. Critical factors involved in the induction of this gametogenesis are a decrease in temperature, a decrease in the dissolved carbon dioxide and the subsequent increase in pH to above 8.0. This somewhat mimics the environmental changes experienced by the gametocytes in that there will be a change to ambient temperature and the gut of the mosquito exhibits a pH of approximately 7.8 as compared to a pH of 7.4 for blood. In addition, a mosquito-derived exflagellation factor (MEF) has also been described and identified as xanthurenic acid, a metabolite from insects. Xanthurenic acid lowers the permissive pH for exflagellation to below 8.0 and is possibly a biological cue for the parasite to undergo gametogenesis (Billker *et al.*, 2004).

The highly mobile microgametes will seek out and fuse with a macrogamete. Within 12-24 hours the resulting zygote develops into an ookinete. The ookinete is motile invasive stages, which will transverse both the peritrophic matrix and the midgut epithelium of the

mosquito. Transversing the midgut epithelium involves invading and exiting several epithelial cells before emerging on the basal side of the epithelium. The invasion process is similar to other apicomplexa except that the ookinete does not have rhoptries and does not form a parasitophorous vacuole after invading the host cell.

Sporogony

After reaching the extracellular space between the epithelial cells and the basal lamina, the ookinete develops into an oocyst. The oocysts undergo an asexual replication, called sporogony, which culminates in the production of several thousand sporozoites. This generally takes 10-28 days depending on species and temperature. Upon maturation the oocyst ruptures and releases the sporozoites, which cross the basal lamina into the hemocoel (body cavity) of the mosquito. These sporozoites are motile and have an ability to specifically recognize the salivary glands. After finding the salivary glands the sporozoites will invade and transverse the salivary gland epithelial cells and come to lie within its lumen. Some of these sporozoites will be expelled into the vertebrate host as the mosquito takes a blood meal, and thus reinitiate the infection in the vertebrate host. Although the hemocoel and salivary gland sporozoites are morphologically similar, they are functionally distinct. Salivary gland sporozoites efficiently invade liver cells, but cannot re-invade the salivary glands, whereas the hemocoel sporozoites are inefficient at invading liver cells.

2.3 TRANSMISSION/CLINICAL MANIFESTATIONS

The most common way to contact malaria is through the natural transmission by female anopheles mosquitoe. Malaria can also be transmitted via blood transfusions or sharing syringes. Mechanical transmission of infected blood will result in a shorter incubation period since there will be no liver stage. There is also an increased risk of fatality with mechanically transmitted *P. falciparum*. The lack of the liver stage infection also precludes relapses in *P. vivax* or *P. ovale* infections. Congenital transmission has also been documented, but is believed to be relatively rare despite the heavy infection of the placenta.

The pathology and clinical manifestations associated with malaria are almost exclusively due to the asexual erythrocytic stage parasites. Tissue schizonts and gametocytes cause little, if any, pathology. *Plasmodium* infection causes an acute febrile illness, which is most notable for its periodic fever paroxysms occuring at either 48 or 72-hour intervals (Kakkilaya, 2006). The severity of the attack depends on the *Plasmodium* species as well as other circumstances such as the state of immunity and the general health and nutritional status of the infected individual. Malaria is a chronic disease, which has a tendency to relapse or recrudesce over months or even years. Symptoms of malaria usually start to appear 10-15 days after the bite of an infected mosquito. The typical prepatent and incubation periods following sporozoite inoculation vary according to species. The prepatent period is defined as the time between sporozoite inoculation and the appearance of parasites in the blood and represents the duration of the liver stage and the number of merozoites produced (Kakkilaya, 2006). Incubation periods tend to be a

little longer and are defined as the time between sporozoite inoculation and the onset of symptoms. Sometimes the incubation periods can be prolonged for several months in *P. vivax, P. ovale,* and *P. malariae*. All four species can exhibit non-specific prodromal symptoms a few days before the first febrile attack. These prodromal symptoms are generally described as 'flu-like' and include: headache, slight fever, muscle pain, anorexia, nausea and lassitude. The symptoms tend to correlate with increasing numbers of parasites (Svenson *et al.*, 1995).

These prodromal symptoms are followed by febrile attacks also known as the malarial paroxysms. These paroxysms exhibit periodicities of 48 hours for *P. vivax, P. ovale*, and *P. falciparum*, and a 72-hour periodicity for *P. malariae.* Initially the periodicity of these paroxyms may be irregular as the broods of merozoites from different exoerythrocytic schizonts synchronize (Svenson *et al.,* 1995). This is especially true in *P. falciparum*, which may not exhibit distinct paroxysms, but exhibit a continuous fever, daily attacks or irregular attacks (eg. 36-48 hour periodicity). Patients may also exhibit splenomegaly, hepatomegaly (slight jaundice), and hemolytic anemia during the period in which the malaria paroxysms occur.

The malarial paroxysm will usually last 4-8 hours and begins with a sudden onset of chills in which the patient experiences an intense feeling of cold despite having an elevated temperature. This is often referred to as the cold stage and is characterized by a vigorous shivering. Immediately following this cold stage is the hot stage. The patient feels an intense heat accompanied by severe headache. Fatigue, dizziness, anorexia, myalgia, and nausea are often associated with the hot stage. Next a period of profuse

sweating will ensue and the fever will start to decline. The patient is exhausted and weak and will usually fall asleep. Upon awakening the patient usually feels well, other than being tired, and does not exhibit symptoms until the onset of the next paroxysm.

The periodicity of these paroxysms is due to the synchronous development of the malarial parasite within the human host. In other words, all of the parasites within a host are at approximately the same stage (that is, ring, trophozoite, and schizont) as they proceed through schizogony. The malarial paroxysm corresponds to the rupture of the infected erythrocytes and the release of merozoites. The 72-hour periodicity in *P. malariae* is due to its slower growth and maturation during blood stage schizogony. Studies in *P. vivax* have demonstrated a correlation between fever and serum TNF-α (tumor necrosis factor-alpha) level. Presumably antigens or toxins are released when the infected erythrocyte ruptures and lead to the production of TNF-α and febrile attacks. The severity of the paroxysms and duration of the symptoms varies according to species. In general, the severity of the disease correlates with the average and maximum parasitemia exhibited by the various species. *P. falciparum* is capable of producing a severe and lethal infection, whereas the other species are rarely mortal. Patients infected with *P. vivax*, especially for the first time, can be quite ill. However, *P. vivax* rarely causes complications or results in death. Occassionally severe malaria involving multiple organs has also been noted in *P. vivax* infections (Kochar *et al.,* 2005). Relapses due to the activation of *P. vivax* hypnozoites can occur for several years. *P. ovale* is the most benign in that the paroxysms tend to be mild and of short duration and relapses seldom occur more than one year after the initial infection.

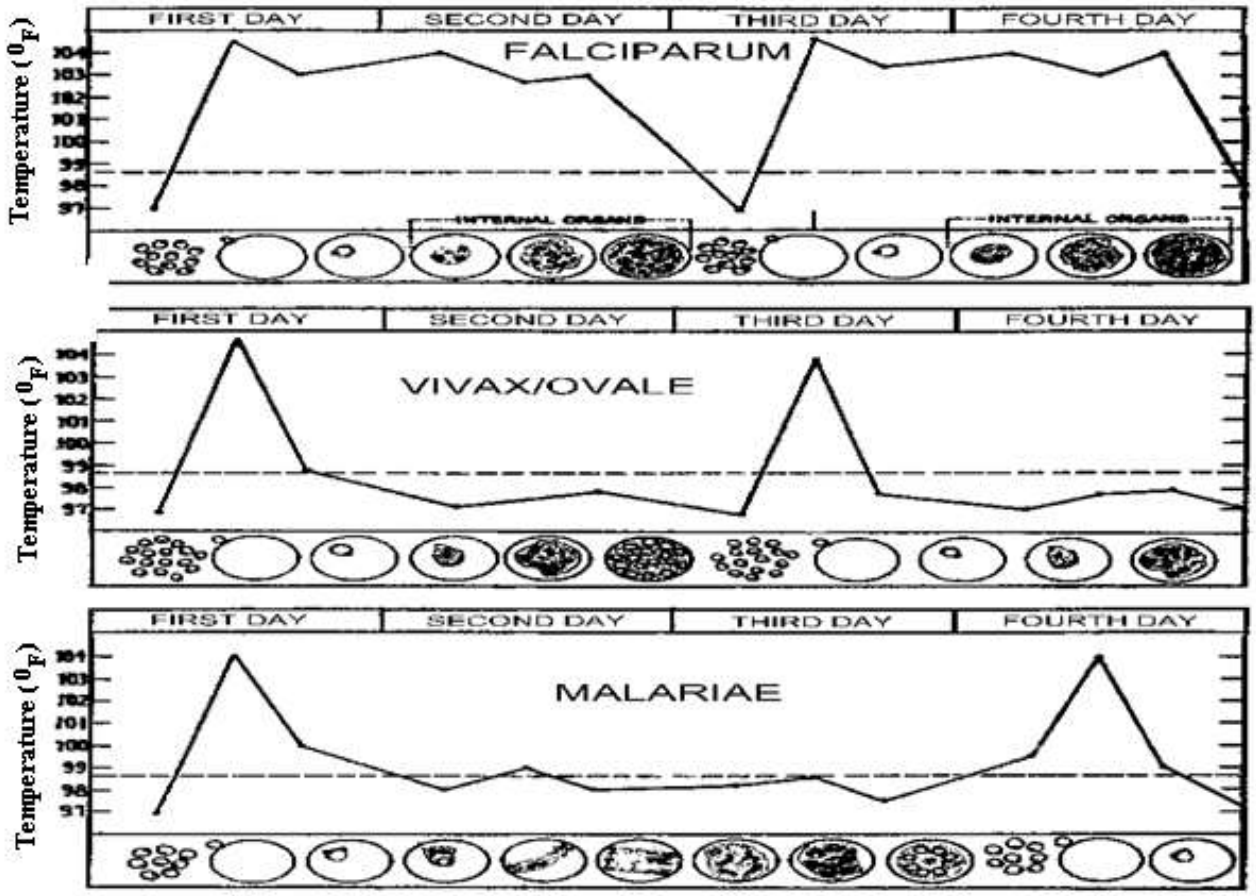


Plate 3: A typical temperature pattern (fever) in relation to blood-stage schizogony for the human malaria parasites. The fever paroxysm corresponds to the period of infected erythrocyte rupture and merozoite invasion. Adapted from Neva and Brown, (1994).

P. malariae generally produces a mild disease, but the initial paroxysms can be moderate to severe. It is the most chronic, though; recrudescences have been documented several decades after the initial infection. This chronicity is sometimes associated with renal complications, which are probably due to the deposition of antigen-antibody complexes in the glomeruli of the kidney. The malarial paroxysms will become less severe and irregular in periodicity as the host develops immunity. This immunity, however, is not a sterilizing immunity in that the infection persists longer than the symptoms and individuals can exhibit relapses or recrudescences or become reinfected. If untreated, all forms of malaria tend to be chronic. This chronicity is sometimes associated with renal complications, which are probably due to the deposition of antigen-antibody complexes in the glomeruli of the kidney.

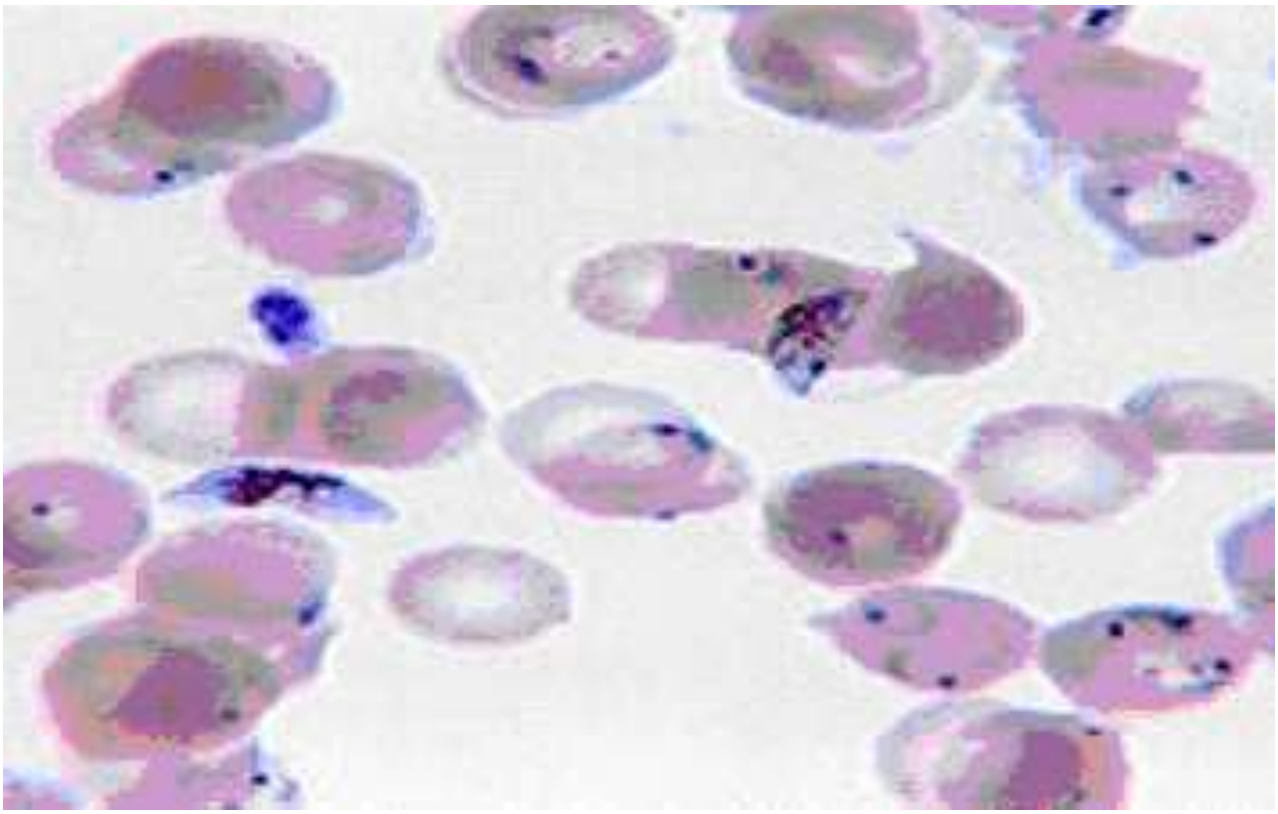


Plate 4: A thin-film Giemsa stained micrograph of ring-forms, and gametocytes of *Plasmodium falciparum* (Adapted from Florens, 2002)

In contrast to the other three species, *P. falciparum* can produce serious disease with mortal consequences. This increased morbidity and mortality is due in part to the high parasitemias associated with *P. falciparum* infections (Devarbhavi *et al.*, 2005). These potentially high parasitemias are due in part to the large number of merozoites produced and the ability of *P. falciparum* to invade all erythrocytes. In contrast, *P. vivax* and *P. ovale* prefer reticulocytes (that is, immature erythrocytes), whereas *P. malariae* prefers senescent erythrocytes. The parasitemia can also rapidly increase due to the cytoadherence and sequestration of *P. falciparum*. This sequestration in the tissues minimizes removal of infected erythrocytes by the spleen and allows for a more efficient erythrocyte invasion. The high parasitemia and sequestration result in other complications associated with falciparum malaria, the most notable being anemia and cerebral malaria. The anemia is due in part to the destruction of erythrocytes during blood-stage schizogony. Furthermore, non-infected erythrocytes are destroyed at higher rates during the infection and there is a decreased production of erythrocytes.

2.4 PATHOGENESIS OF SEVERE MALARIA

Pathology associated with all malarial species is related to the rupture of infected erythrocytes and the release of parasite material and metabolites, hemozoin (malaria pigment) and cellular debris (Claire *et al.*, 2004). In addition to the paroxysms discussed above, the deposition of hemozoin has long been known as a characteristic feature of malaria. There is an increased activity of the reticuloendothelial system, particularly in the liver and spleen and thus their enlargement, as evidenced by macrophages with ingested, infected and normal erythrocytes and hemozoin. Except for *P. falciparum*, the

pathology associated with malaria tends to be benign. Several severe complications can be associated with falciparum malaria with cerebral malaria being the most notable and a frequent cause of death (WHO, 2000).

Cerebral malaria is characterized by an impaired consciousness. The presenting symptoms are severe headache followed by drowsiness, confusion, and ultimately coma. Convulsions are also frequently associated with cerebral malaria. These neurological manifestations are believed to be due to the sequestration of the infected erythrocytes in the cerebral microvasculature. Sequestration refers to the cytoadherence of trophozoite- and schizont-infected erythrocytes to endothelial cells of deep vascular beds in vital organs, especially brain, lung, gut, heart and placenta. This sequestration provides several advantages for the parasite. The major advantage is the avoidance of the spleen and the subsequent elimination of infected erythrocytes. In addition, the low oxygen tensions in the deep tissues may provide a better metabolic environment.

Cytoadherence appears to be mediated by the electron-dense protuberances on the surface of the infected erythrocyte. These 'knobs' are expressed during the trophozoite and schizont stages and are formed as a result of parasite proteins exported to the erythrocyte membrane. Among human *Plasmodium* species, knobs are restricted to *P. falciparum* and thus suggest that the knobs play a role in cytoadherence. In addition, there is also a good correlation between animal *Plasmodium* species, which express knobs and exhibit sequestration. Electron microscopy also shows that the knobs are contact points between the infected erythrocyte and the endothelial cell.

The molecular mechanisms of cytoadherence involve receptor-ligand interactions. In other words, proteins expressed on the surface of the infected erythrocyte (ligand) will bind to proteins expressed on the surface of the endothelial cells (receptor). *Pf*EMP-1 (erythrocyte membrane protein) is a parasite protein, which has been implicated as the cytoadherence ligand. In constrast to the usually highly conserved nature of receptor-ligand interactions, *Pf*EMP-1 is a member of a highly variable (= *var*) gene family with 40-50 different genes (Smith *et al.,* 1995; Smith *et al.,* 2000). Several host proteins, which possibly function as receptors have been identified. Many of these host proteins function in cell-cell interactions and are involved in cellular adhesion. Several studies have indicated that the expression of different *Pf*EMP-1 genes is correlated with different receptor-binding phenotypes (Buffet *et al.,* 1999; Smith *et al.,* 2000). This antigenic variation associated with the surface exposed *Pf*EMP-1 allows the parasite to evade the immune system. However, the cytoadherence function is preserved through its ability to recognize multiple receptors (Smith *et al.,* 2000). This antigenic variation may also account for different disease outcomes. For example, intercellular adhesion molecule-1 (ICAM-1) is usually implicated in cerebral pathology.

Early observations of the pathology of cerebral malaria suggested a relationship between large numbers of infected erythrocytes in the microvasculature and the development of the syndrome. Initially it was assumed that the cytoadherence would lead to a mechanical blockage (that is, cerebral ischemia) and subsequently hypoxia. In addition, the parasite could also cause localized metabolic effects such as hypoglycemia and/or lactic acidosis. The hypoxia and metabolic effects would then cause the coma and subsequent death. However, there are some problems with the sequestration hypothesis:

- The coma associated with cerebral malaria is rapidly reversible upon treatment.
- A high percentage of survivors have no permanant neurological complications
- A lack of ischemic damage (rules out hypoxia as major mechanism).
- Sequestration also occurs in non-cerebral malaria.

Because of these problems others have suggested that the coma is mediated by short-lived molecules that affect cerebral function. Possible host mediators include cytokines, such as TNF-α, or nitric oxide. In this cytokine theory, malarial antigens would stimulate TNF-α, which could then induce nitric oxide or have other pathological effects. Nitric oxide is known to affect neuronal function and it could also lead to intracranial hypertension through its vasodilator activity. It is unlikely, though, that the systemic release of cytokines would cause coma and one needs to also postulate that release of these mediators in the brain would lead to high local concentrations. In addition, there is minimal lymphocyte infiltration or inflammation associated with the blocked capillaries.

The sequestration hypothesis and cytokine theory for the pathophysiology of cerebral malaria are not mutually exclusive, and both phenomenon are likely to be involved. For example, parasite exo-antigens, which are released at erythrocyte rupture, are known to stimulate macrophages to secrete TNF-α. TNF-α is known to upregulate the expression of adhesion molecules such as ICAM-1 on the surface of brain endothelial cells. This would lead to increase binding of infected erythrocytes and amplify the effects whether they are due to vascular blockage, soluble mediators, metabolic effects, or a combination.

The cytoadherence of infected erythrocytes to brain endothelial cells (BEC) and the release of exoantigen could stimulate the BEC and immune effector cells such as

macrophages to release cytokines. These cytokines, such as tumor necrosis factor-α (TNF), could lead to an increased expression of possible endothelial cell receptors (eg., ICAM-1) and promote an increase cytoadherence of infected erythrocytes. Large numbers of bound infected erythrocytes can lead to vascular blockage and hypoxia and have localized metabolic effects (for example, hypoglycemia, lactic acidosis). The increased number of infected erythrocytes and exo-antigens can also lead to higher cytokine levels. TNF-α is also known to stimulate nitric oxide (NO). Nitric oxide can affect neuronal function by interfering with neurotransmission. Nitric oxide also causes vasodilation, which can lead to the intracranial hypertension associated with cerebral malaria.

Important features in the pathogenesis of malaria are as follows;

- *P. falciparum* can cause a severe and fatal disease
- This increased pathogenecity as compared to other human malaria is related to *P. falciparum's*:
    - High reproductive capacity
    - Cytoadherence and sequestration
- Sequestration contributes to the higher reproductive capacity via spleen avoidance
- The cytoadherence to endothelial cells could also have local pathological effects and in particular is likely involved in cerebral malaria
- The pathophysiology of cerebral malaria is not completely understood, but likely involves multiple factors and complex interactions between the host and parasite.

2.5 IMMUNITY

Persons living in endemic areas do develop immunity against malaria. Almost always a person will exhibit symptoms during their initial exposures to malaria. Symptoms associated with subsequent exposures to malaria are usually less severe, though the immunity against malaria is slow to develop and requires multiple exposures. In highly endemic areas only young children are at a high risk of developing severe falciparum malaria whereas older children and adults are essentially protected from severe disease and death. However, this immunity is not a sterilizing immunity in that persons can still become infected. In addition the immunity is short lived and in the absence of repeated exposure the level of immunity decreases. For example, previously semi-immune adults will often develop severe malaria upon returning to an endemic area after being in a non-endemic area for 1-2 years. This state of partial immunity in which parasitemia is lowered, but not eliminated, and parasitemia is better tolerated is sometimes referred to as premunition. Premunition refers to immunity that is contingent upon the pathogen being present.

The immune response could be directed at either the pre-erythrocytic or erythrocytic stages of the parasite's life cycle. However, the erythrocytic stage of the life cycle is probably the most important in terms of clearing the parasite and lessening the disease. Due to the lack of HLA molecules on the surface of the parasite or the erythrocyte it is usually assumed that antibody will play a key role in blood-stage immunity. Possible effector mechanisms for antibody include: blocking erythrocyte invasion by merozoites, antibody-dependent cellular killing mediated by cytophilic antibodies, or increased

clearance of infected erythrocytes due to binding of antibodies to parasite antigens exposed on the erythrocyte surface (Ian *et al.*, 2006). All of these will result in lower parasitemia. The relative importance of these various mechanisms is not clear and probably immunity probably requires the generation of antibodies against numerous targets. This, along with antigenic variation and polymorphisms in many *Plasmodium* antigens, could explain the slow development of immunity.

The observation that asymptomatic individuals can exhibit high levels of parasitemia has led to the concept of 'anti-disease immunity'. This would be in addition to the 'anti-parasite' immunity discussed above which results in lower parasitemias. Severe malaria and death are correlated with TNF-α and other proinflammatory cytokines (Ian *et al.*, 2006). As discussed for the paraxoysms and cerebral malaria, antigens or toxins released by the infected erythrocyte could stimulate the production of proinflammatory cytokines. Antibodies against these exo-antigens could possibly neutralize their toxic effects and thus lead to an anti-disease immunity.

Because of the difficulties in controlling malaria by other means there is much interest in developing a vaccine against malaria. Currently there is no available vaccine, but there is a substantial research effort directed at identifying vaccine candidates and testing potential vaccines for safety and efficacy. The complex life cycle and biology of the parasite provide several potential targets. For example, vaccination against the sporozoite or exoerythrocytic stage could prevent infection. However, the induced immunity would need to be completely effective since the escape of a single sporozoite would lead to a blood-stage infection and disease. Vaccines targeted against merozoites or the infected

erythrocyte would lower parasitemia by interfering with merozoite invasion or increasing the elimination of infected erythrocytes. Such a vaccine could potentially alleviate much of the pathogenesis associated with malaria even if it were not completely effective. In addition, infection may serve to boost the immune response. It may be possible to vaccinate against the disease by immunizing against potentially toxic antigens. Antibodies neutralizing antigens that stimulate a proinflammatory immune response may lessen some of the pathogenesis associated with malaria. Sexual stages of the parasite such as gametocytes and gametes could also be targeted. Antibodies directed against gamete antigens can prevent infection of the mosquito and sporogony. Such a vaccine would be altruistic in that it would not protect the individual against disease, but protect others in the community by lowering the transmission (Ramachandra *et al.*, 2000).

2.5.1 Human Genetics and Innate Resistance

Certain genetic diseases and polymorphisms have been associated with decrease infection or diseases. For example, individuals who lack the Duffy blood-group antigen are refractory to *P. vivax*. A large proportion of the populations in Western Africa are Duffy negative, thus accounting for the low levels of *P. vivax* in West Africa. This innate resistance led to the identification of the Duffy antigen as the erythrocyte receptor for merozoite invasion. Several inherited erythrocyte disorders are found predominantly in malaria endemic areas and at frequencies much higher than expected. This has lead to speculation that these disorders confer some protection against malaria. For example, Southeast Asian ovalcytosis is due to a mutation in an erythrocyte membrane protein called band 3. This mutation causes the erythrocyte membrane to become more rigid and

more refractory to merozoite invasion. The mechanism by which other diseases might confer protection against malaria is not known. In most cases it is presumed or speculated that the combination of the defect and infection leads to premature lysis or clearance of the infected erythrocyte. For example, glucose-6-phosphate dehydrogenase (G6PD) deficient erythrocytes would have an impaired ability to handle oxidative stress. The additional oxidants produced as a result of parasite metabolism and the digestion of hemoglobin may overwhelm the infected erythrocyte and lead to its destruction before the parasite is able to complete schizogony. Sickle cell anemia and thalassemia are also speculated to make the infected erythrocyte more susceptible to oxidative stress.

2.6 MALARIA IN NIGERIA

Despite various declarations by African governments in the 1990s and promises made in the main content of the Roll Back Malaria declaration in Abuja in the year 2000, malaria remains a major health challenge in Nigeria. It is responsible for an estimated average annual reduction of 1.3% in economic growth for those countries with the highest burden, Nigeria inclusive (WHO, 2002). Nigeria, Democratic Republic of Congo (DRC), Ethiopia, and Uganda account for nearly 50% of global malaria deaths (United States Embassy in Nigeria, 2011).

In Nigeria, malaria accounts for more cases and deaths than any other country in the world with 97% of the Nigerian population at risk of the infection. There is an estimated 100 million malaria cases with over 300,000 deaths per year in Nigeria (WHO, 2005). This compares with 215,000 deaths per year in Nigeria from HIV/AIDS. It contributes to

11% of maternal mortality, 60% of outpatient visits and 30% of hospitalizations among children under five years of age in Nigeria (United States Embassy in Nigeria, 2011).

The seemingly intractable trend of this ancient scourge has compounded the national and household poverty due to intensive loss of productive time to the scourge. A cause for worry at the moment is the resistance of the disease to cheap first line drugs and the need for the more expensive artemisinin combination therapy (ACT). Given that malaria is endemic throughout Nigeria, and that more than half the country's population are living below poverty line, the malaria burden may constrain the ability of the country to achieve the target set out in the millennium development goals (DeLeire and Manning, 2004).

2.7 PREVENTION AND CONTROL

Strategies for preventing and controlling malaria involve three different approaches. Prevention of malaria in individuals will generally involve the reduction of human-mosquito contact through the use of bednets, repellents, etcetera. Chemoprophylaxis can also be used, especially in travelers. However chemoprophylaxis only suppresses parasitemia and does not prevent infection.

Control activities at the community level can utilize approaches, which directly reduce human-mosquito contact, as well as approaches, which reduce the total number of mosquitoes in an area. Such approaches include the reduction in mosquito breeding grounds (for example, environmental modification), targeting the larva stages with chemical or biological agents, and massive insecticide spraying for the adult mosquitoes. Biological control methods include the introduction of fish, which eat the mosquito larvae, or bacteria (for example, *Bacillus thuringiensis*), which excrete larval toxins. Case detection and

treatment is another potential control method. Identifying and treating infected persons, especially asymptomatic individuals, will reduce the size of the parasite reservoir within the human population and can lower transmission rates. However, this can be a relatively expensive approach.

These approaches are not mutually exclusive and can be combined. Many of the successful control programs include both measures to control mosquitoes and treatment of infected individuals. There is no standard method of malaria control that has proven universally effective. The epidemiologic, socioeconomic, cultural and infrastructural factors of a particular region will determine the most appropriate malaria control. Some of the factors, which need to be considered, include:

- Infrastructure of existing health care services and other resources
- Intensity and periodicity (for example, seasonality) of transmission
- Mosquito species (ecological requirements, behavioral characteristics, insecticide sensitivity, etcetera)
- Parasite species and drug sensitivities
- Cultural and social characteristics of the population
- Presence of social and ecological change

The control of malaria in tropical Africa has been particularly problematic because of the high transmission rates and the overall low socio-economic level. Several studies have shown that insecticide treated bednets (ITBN) reduce the morbidity and mortality associated with malaria. In most areas the introductions of bed nets do not require large promotional programs and their use is readily accepted. This may be in part due to the

reduction in mosquito nuisance biting. Some questions have been raised with regards to the economic sustainability of bed net programs. It is necessary to re-treat the bed net with insecticide periodically and the bednets need to be repaired and replaced as they become torn and worn out. In addition, some have raised concerns about the long-term benefits of bednets since they reduce exposure, but do not eliminate it. This reduction in exposure may delay the acquisition of immunity and simply expose the older groups to a higher rate of morbidity and mortality.

2.7.1 Diagnosis

Malaria is suspected in persons with a history of being in an endemic area and presenting symptoms consistent with malaria. These symptoms, especially in the early stages of the infection, are non-specific and often described as flu-like. As the disease progresses, the patient may exhibit an enlarged spleen and/or liver and anemia. Diagnosis is confirmed by microscopy. Thick blood smears are generally more sensitive for the detection of parasites, whereas thin smears are preferable for species identification. If parasites are not found on the first blood smear it is recommended to make additional smears every 6-12 hours for as long as 48 hours. A tentative diagnosis of *P. falciparum* (numerous and exclusively ring stages) could constitute a medical emergency, especially in a non-immune person. Rapid immunochromatographic tests (that is, dipsticks) based on antigen detection are also available.

2.8 AVAILABLE AGENTS

Two types of antimalarial drugs are to be distinguished:

- The agents used as prevention (called prophylactic drugs). This first type is taken as prevention and requires continuous administration to reduce the risk of infection.
- The second type, called therapy drugs are taken once a person is already infected.

However, strategies for combating malaria has changed rapidly, and when drugs are administered in combination, it can be impractical to identify which agents are prophylactic and which are therapeutic. Another approach for classifying antimalarials is to group them by mechanism and by chemical structure.

Quinine and related agents

Quinine has a long history stretching from Peru, the discovery of the Cinchona tree, and the potential uses of its bark, to the current day and a collection of derivatives that are still frequently used in the prevention and treatment of malaria. Quinine is an alkaloid that acts as a blood schizonticide and weak gametocide against *Plasmodium vivax* and *Plasmodium malariae*. As an alkaloid, it is accumulated in the food vacuoles of plasmodium species, especially *Plasmodium falciparum*. It acts by inhibiting the hemozoin biocrystallization, thus facilitating an aggregation of cytotoxic heme. Quinine is less effective and more toxic as a blood schizonticidal agent than Chloroquine; however it is still very effective and widely used in the treatment of acute cases of severe *P. falciparum*. It is especially useful in areas where there is known to be a high level of

resistance to Chloroquine, Mefloquine and sulfadoxine drug combinations with pyrimethamine. Quinine is also used in post-exposure treatment of individuals returning from an area where malaria is endemic.

The treatment regimen of Quinine is complex and is determined largely by the parasite's level of resistance and the reason for drug therapy (i.e. acute treatment or prophylaxis). The World Health Organization recommendation for Quinine is 8 mg/kg three times daily for 3 days (in areas where the level of adherence is questionable) and for 7 days (where parasites are sensitive to Quinine). In areas where there is an increased level of resistance to Quinine 8 mg/kg three times daily for 7 days is recommended, combined with Doxycycline, Tetracycline or Clindamycin. Doses can be given by oral, intravenous or intramuscular routes. The recommended method depends on the urgency of treatment and the available facilities (i.e. sterilised needles for IV or IM injections).

Use of Quinine is characterised by a frequently experienced syndrome called cinchonism. Tinnitus (a hearing impairment), rashes, vertigo, nausea, vomiting and abdominal pain are the most common symptoms. Neurological effects are experienced in some cases due to the drug's neurotoxic properties. These actions are mediated through the interactions of Quinine causing a decrease in the excitability of the motor neuron end plates. This often results in functional impairment of the eight cranial nerve; resulting in confusion, delirium and coma. Quinine can cause hypoglycaemia through its action of stimulating insulin secretion, this occurs in therapeutic doses and therefore it is advised that glucose levels are monitored in all patients every 4–6 hours. This effect can be exaggerated in pregnancy and therefore additional care in administering and monitoring the dosage is

essential. Repeated dosage or over-dosage can result in renal failure and death through depression of the respiratory system.

Quinimax and Quinidine are the two most commonly used alkaloids related to Quinine, in the treatment or prevention of Malaria. Quinimax is a combination of four alkaloids (namely Quinine, Quinidine, Cinchoine and Cinchonidine). This combination has been shown in several studies to be more effective than Quinine, supposedly due to a synergistic action between the four Cinchona derivatives (Plowe, 2003). Quinidine is a direct derivative of Quinine. It is a distereoisomer, thus having similar anti-malarial properties to the parent compound. Quinidine is recommended only for the treatment of severe cases of malaria.

Chloroquine

Chloroquine was until recently the most widely used anti-malarial. It was the original prototype from which most other methods of treatment were derived. It is also the least expensive, best tested and safest of all available drugs. The emergence of drug resistant parasitic strains is rapidly decreasing its effectiveness; however it is still the first-line drug of choice in most sub-Saharan African countries. It is now suggested that it is used in combination with other antimalarial drugs to extend its effective usage.

Chloroquine is a 4-aminoquinoline compound with a complicated and still unclear mechanism of action. It is believed to reach high concentrations in the vacuoles of the parasite, which, due to its alkaline nature, raises the internal pH (Trape, 2001). It controls the conversion of toxic heme to hemozoin by inhibiting the biocrystallization of

hemozoin thus poisoning the parasite through excess levels of toxicity. Other potential mechanisms through which it may act include interfering with the biosynthesis of parasitic nucleic acids, the formation of a chloroquine-haem or chloroquine-DNA complex. The most significant level of activity found is against all forms of the schizonts (with the obvious exception of chloroquine-resistant *P. falciparum* and *P. vivax* strains) and the gametocytes of *P. vivax, P. malariae, P. ovale* as well as the immature gametocytes of *P. falciparum*. Chloroquine also has a significant anti-pyretic and anti-inflammatory effect when used to treat *P. vivax* infections, thus it may still remain useful even when resistance is more widespread. According to a report, there is very little drug resistance among children infected with malaria on the island of Madagascar (Trape, 2001). A slightly different drug called nivaquine or chloroquine phosphate has also been invented. Popular drugs that make use of this compound are Chloroquine FNA, Resochin® and Dawaquin®.

Children and adults should receive 25 mg of chloroquine per kg given over 3 days. A pharmacokinetically superior regime, recommended by the WHO, involves giving an initial dose of 10 mg/kg followed 6–8 hours later by 5 mg/kg, then 5 mg/kg on the following 2 days. For chemoprophylaxis: 5 mg/kg/week (single dose) or 10 mg/kg/week divided into 6 daily doses is advised. It should be noted that chloroquine is only recommended as a prophylactic drug in regions only affected by *P. vivax* and sensitive *P. falciparum* strains. Chloroquine has been used in the treatment of malaria for many years and no abortifacient or teratogenic effects have been reported during this time, therefore it is considered very safe to use during pregnancy. However, itching can occur at intolerable level.

Amodiaquine

Amodiaquine is a 4-aminoquinoline anti-malarial drug similar in structure and mechanism of action to Chloroquine. It is most frequently used in combination with Chloroquine, but is also very effective when used alone. It is thought to be more effective in clearing parasites in uncomplicated malarial than Chloroquine, thus leading to a faster rate of recovery. However, some fatal adverse effects of the drug were noted during the 1980's, thus reducing its usage in chemoprophylaxis. The WHO's most recent advice on the subject still maintains that the drug should be used when the potential risk of not treating an infection outweighs the risk of developing side effects. It has also been suggested that it is particularly effective, and less toxic than other combination treatments in HIV positive patients. The drug should be given in doses between 25 mg/kg and 35 mg/kg over 3 days in a similar method to that used in Chloroquine administration. Adverse reactions are generally similar in severity and type to that seen in Chloroquine treatment. In addition, bradycardia, itching, nausea, vomiting and some abdominal pain have been recorded. Some blood and hepatic disorders have also been seen in a small number of patients.

Pyrimethamine

Pyrimethamine is used in the treatment of uncomplicated malaria. It is particularly useful in cases of chloroquine-resistant *P. falciparum* strains when combined with Sulfadoxine. It acts by inhibiting dihydrofolate reductase in the parasite thus preventing the biosynthesis of purines and pyrimidines. Therefore, halting the processes of DNA

synthesis, cell division and reproduction. It acts primarily on the schizonts during the hepatic and erythrocytic phases.

Sulfadoxine

The action of Sulfadoxine is focused on inhibiting the use of para-aminobenzoic acid during the synthesis of dihydropteroic acid. When combined with Pyrimethamine the two key stages in DNA synthesis in the plasmodia are prevented. It also acts on the schizonts during the hepatic and erythrocytic phases. It is mainly used for treating *P. falciparum* infections and is less active against other *Plasmodium* strains. However usage is restricted due to the long half life of the combination which exerts a potentially large selection pressure on the parasite hence encouraging the possibility of resistance development. This combination is not recommended for chemoprophylaxis because of the severe skin reactions commonly experienced. However it is used frequently for clinical episodes of the disease.

Proguanil

Proguanil (Chloroguanadine) is a biguanide; a synthetic derivative of pyrimidine. It was developed in 1945 by a British Antimalarial research group. It has many mechanisms of action but primarily is mediated through conversion to the ative metabolite cycloguanil pamoate. This inhibits the malarial dihydrofolate reductase enzyme. Its most prominent effect is on the primary tissue stages of *P. falciparum, P. vivax* and *P. ovale*. It has no known effect against hypnozoites therefore is not used in the prevention of relapse. It has a weak blood schizonticidal activity, although not recommended for therapy currently,

when combined with Atovaquone (a hydroxynaphthoquinone) it has been shown to be effective against multi-drug resistant strains of *P. falciparum*. Proguanil is used as a prophylactic treatment in combination with another drug, most frequently Chloroquine. 3 mg/kg is the advised dosage per day, (hence approximate adult dosage is 200 mg). The pharmacokinetic profile of the drugs indicates that a half dose, twice daily maintains the plasma levels with a greater level of consistency, thus giving a greater level of protection. It should be noted that the Proguanil-Chloroquine combination does not provide effective protection against resistant strains of *P. falciparum*. There are very few side effects to Proguanil, with slight hair loss and mouth ulcers being occasionally reported following prophylactic use (Plowe, 2003).

Mefloquine

Mefloquine was developed during the Vietnam War and is chemically related to quinine. It was developed to protect American troops against multi-drug resistant *P. falciparum*. It is a very potent blood schizonticide with a long half-life. It is thought to act by forming toxic heme complexes that damage parasitic food vacuoles. It is now used solely for the prevention of resistant strains of *P. falciparum* despite being effective against *P. vivax, P. ovale* and *P. marlariae*. Mefloquine is effective in prophylaxis and for acute therapy. It is now strictly used for resistant strains (and is usually combined with Artesunate). Chloroquine/Proguanil or Sulfadoxine-Pyrimethamine combinations should be used in all other plasmodial infections.

The major commercial manufacturer of mefloquine-based malaria treatment is Roche Pharmaceuticals, which markets the drug under the trade name "Lariam". A dose of 15–

25 mg/kg is recommended, depending on the prevalence of Mefloquine resistance. The increased dosage is associated with a much greater level of intolerance, most noticeably in young children; with the drug inducing vomiting and oesophagitis. The effects during pregnancy are unknown, although it has been linked with an increased number of stillbirths. It is not recommended for use during the first trimester, although considered safe during the second and third trimesters. Mefloquine frequently produces side effects, including nausea, vomiting, diarrhea, abdominal pain and dizziness. Several associations with neurological events have been made, namely affective and anxiety disorders, hallucinations, sleep disturbances, psychosis, toxic encephalopathy, convulsions and delirium. Cardiovascular effects have been recorded with bradycardia and sinus arrhythmia being consistently recorded in 68% of patients treated with Mefloquine (in one hospital-based study). Mefloquine can only be taken for a period up to 6 months (due to side effects). After this, other drugs (such as those based on paludrine/nivaquine) again need to be taken (Plowe, 2003).

Atovaquone

Atovaquone a new type of antimalarial drug is now available. It is very effective since no mosquito population have generated resistance due to exposure. Also, the drug has no side-effects such as the cardiovascular effect with mefloquine which can trigger cardiac arrhythmia. Atovaquone is available in combination with Proguanil under the name Malarone, albeit at a price higher than Lariam.

Primaquine

Primaquine is a highly active 8-aminoquinoline that is used in treating all types of malaria infection. It is most effective against gametocytes but also acts on hypnozoites, blood schizonticytes and the dormant plasmodia in *P. vivax* and *P. ovale*. It is the only known drug to cure both relapsing malaria infections and acute cases. The mechanism of action is not fully understood but it is thought to mediate some effect through creating oxygen free radicals that interfere with the plasmodial electron transport chain during respiration.

For the prevention of relapse in *P. vivax* and *P. ovale* 0.15 mg/kg should be given for 14 days. As a gametocytocidal drug in *P. falciparum* infections a single dose of 0.75 mg/kg repeated 7 days later is sufficient. This treatment method is only used in conjunction with another effective blood schizonticidal drug. There are few significant side effects although is has been shown that Primaquine may cause anorexia, nausea, vomiting, cramps, chest weakness, anaemia, some suppression of myeloid activity and abdominal pains. In cases of over-dosage granulocytopenia may occur.

Artemisinin and derivatives

Artemisinin is a Chinese herb (Qinghaosu) that has been used in the treatment of fevers for over 1,000 years (Meshnick, 2001), thus predating the use of Quinine in the western world. It is derived from the plant *Artemisia annua*. The first documentation as a successful therapeutic agent in the treatment of malaria was in 340 AD by Ge Hong in his book *Zhou Hou Bei Ji Fang* (*A Handbook of Prescriptions for Emergencies*) (Meshnick,

2001). The active compound was isolated first in 1971 and named Artemisinin. It is a sesquiterpene lactone with a chemically rare peroxide bridge linkage. It is this that is thought to be responsible for the majority of its anti-malarial action. At present it is strictly controlled under WHO, (2010) guidelines as it has proven to be effective against all forms of multi-drug resistant *P. falciparum*, thus care is taken to ensure compliance and adherence together with other behaviours associated with the development of resistance. It is only given in combination with other anti-malarials.

Artemisinin has a very rapid action and the vast majority of acute patients treated show significant improvement within 1–3 days of receiving treatment. It has demonstrated the fastest clearance of all anti-malarials currently used and acts primarily on the trophozite phase, thus preventing progression of the disease. It is converted to active metabolite dihydroartemesinin which inhibits calcium ATPase in sarcoplasmic reticulum encoded by *P. falciparum*. On the first day of treatment 20 mg/kg should be given, this dose is then reduced to 10 mg/kg per day for the following 6 days. Few side effects are associated with artemesinin use. However, headaches, nausea, vomiting, abnormal bleeding, dark urine, itching and some drug fever have been reported by a small number of patients. Some cardiac changes were reported during a clinical trial, notably non specific ST changes and a first degree atrioventricular block (these disappeared when the patients recovered from the malarial fever).

Artemether is a methyl ether derivative of dihydroartemisinin. It is similar to Artemisinin in mode of action but demonstrates a reduced ability as a hypnozoiticidal compound, instead acting more significantly to decrease gametocyte carriage. Similar restrictions are

in place, as with Artemisinin, to prevent the development of resistance, therefore it is only used in combination therapy for severe acute cases of drug-resistant *P. falciparum*. It should be administered in a 7 day course with 4 mg/kg given per day for 3 days, followed by 1.6 mg/kg for 3 days. Side effects of the drug are few but include potential neurotoxicity developing if high doses are given.

Artesunate is a hemisuccinate derivative of the active metabolite dihydroartemisinin. Currently it is the most frequently used of all the Artemisinin-type drugs. Its only effect is mediated through a reduction in the gametocyte transmission. It is used in combination therapy and is effective in cases of uncomplicated *P. falciparum*. The dosage recommended by the WHO is a 5 or 7 day course (depending on the predicted adherence level) of 4 mg/kg for 3 days (usually given in combination with Mefloquine) followed by 2 mg/kg for the remaining 2 or 4 days. In large studies carried out on over 10,000 patients in Thailand, no adverse effects have been shown (Dorsey *et al.*, 2002).

Dihydroartemisinin is the active metabolite to which Artemisinin is reduced. It is the most effective Artemisinin compound and the least stable. It has a strong blood schizonticidal action and reduces gametocyte transmission. It is used for therapeutic treatment of cases of resistant and uncomplicated *P. falciparum*. 4 mg/kg doses are recommended on the first day of therapy followed by 2 mg/kg for 6 days. As with Artesunate, no side effects to treatment have thus far been recorded.

Arteether is an ethyl ether derivative of dihydroartemisinin. It is used in combination therapy for cases of uncomplicated resistant P. falciparum. The recommended dosage is 150 mg/kg per day for 3 days given by IM injections. With the exception of a small

number of cases demonstrating neurotoxicity following parenteral administration no side effects have been recorded.

Halofantrine

Halofantrine was developed by the Walter Reed Army Institute of Research in the 1960s. It is a phenanthrene methanol, chemically related to Quinine and acts as a blood schizonticide effective against all plasmodium parasites. Its mechanism of action is similar to other anti-malarials. Cytotoxic complexes are formed with ferritoporphyrin XI that cause plasmodial membrane damage. Despite being effective against drug resistant parasites, Halofantrine is not commonly used in the treatment (prophylactic or therapeutic) of malaria due to its high cost. It has very variable bioavailability and has been shown to have potentially high levels of cardiotoxicity. It is still a useful drug and can be used in patients that are known to be free of heart disease and are suffering from severe and resistant forms of acute malaria. A popular halofantrine based drug is halfan. The level of governmental control and the prescription-only basis on which it can be used contributes to the cost, thus halofantrine is not frequently used.

A dose of 8 mg/kg of halofantrine is advised to be given in three doses at six hour intervals for the duration of the clinical episode. It is not recommended for children under 10 kg despite data supporting the use and demonstrating that it is well tolerated. The most frequently experienced side-effects include nausea, abdominal pain, diarrhoea, and itch. Severe ventricular dysrhythmias, occasionally causing death are seen when high doses are administered. This is due to prolongation of the QTc interval. Halofantrine is not recommended for use in pregnancy and lactation, in small children, or in patients that

have taken mefloquine previously. Lumefantrine is a prototype of halofantrine that is used in some combination antimalarial regimen (Van Vugt *et al.,* 1998; Van Vugt *et al.*, 2000).

Doxycycline

Doxycycline is a tetracycline compound derived from oxytetracycline. The tetracyclines were one of the earliest groups of antibiotics to be developed and are still used widely in many types of infection. It is a bacteriostatic agent that acts to inhibit the process of protein synthesis by binding to the 30S ribosomal subunit thus preventing the 50s and 30s units from bonding. Doxycycline is used primarily for chemoprophylaxis in areas where quinine resistance exists. It can be used in resistant cases of uncomplicated *P. falciparum* but has a very slow action in acute malaria; therefore it should never be used in monotherapy.

When treating acute cases and given in combination with Quinine; 100 mg/kg of doxycycline should be given per day for 7 days. In prophylactic therapy, 100 mg (adult dose) of Doxycycline should be given every day during exposure to malaria. The most commonly experienced side effects are permanent enamel hypoplasia, transient depression of bone growth, gastrointestinal disturbances and some increased levels of photosensitivity. Due to its effect on bone and tooth growth it is not used in children under 8, pregnant or lactating women and those with a known hepatic dysfunction.

Tetracycline is only used in combination for the treatment of acute cases of *P. falciparum* infections. This is due to its slow onset. Unlike Doxycycline it is not used in

chemoprophylaxis. For Tetracycline, 250 mg is the recommended adult dosage (it should not be used in children) for 5 or 7 days depending on the level of adherence and compliance expected. Oesophageal ulceration, gastrointestinal upset and interferences with the process of ossification and depression of bone growth are known to occur. The majority of side effects associated with doxycycline are also experienced.

Clindamycin

Clindamycin is a derivative of Lincomycin, with a slow action against blood schizonticytes. It is only used in combination with Quinine in the treatment of acute cases of resistant *P. falciparum* infections and not as a prophylactic. Being more expensive and toxic than the other antibiotic alternatives, it is used only in cases where the tetracyclines are contraindicated (for example in children).

Clindamycin should be given in conjunction with Quinine as a 300 mg dose (in adults) four times a day for 5 days. The only side effects recorded in patients taking clindamycin are nausea, vomiting, abdominal pains and cramps. However these can be alleviated by consuming large quantities of water and food when taking the drug. Pseudomembranous colitis (caused by *Clostridium difficile*) has also developed in some patients; this condition may be fatal in a small number of cases.

2.8.1 Chemotherapy and Drug Resistance

Several antimalarial drugs are available. Many factors are involved in deciding the best treatment for malaria. These factors include the parasite species, the severity of disease the patient's age and immune status, the parasite's susceptibility to the

drugs (that is, drug resistance), and the cost and availability of drugs. Therefore, the exact recommendations will often vary according to geographical region. In addition, the various drugs act differentially on the different life cycle stages.

Fast-acting blood schizonticides, which act upon the blood stage of the parasite, are used to treat acute infections and to quickly relieve the clinical symptoms. Chloroquine is generally the recommended treatment for patients with *P. vivax*, *P. ovale*, *P. malariae*, and uncomplicated chloroquine-sensitive *P. falciparum* infections. Chloroquine is safe and usually well tolerated. Side effects may include pruritus (that is, itching), nausea, or agitation. Patients infected with either *P. vivax* or *P. ovale* that are not at a high risk for reinfection should also be treated with primaquine (a tissue schizontocide). Primaquine is effective against the liver stage of the parasite, including hypnozoites, and will prevent future relapses. The combination of chloroquine and primaquine is often called 'radical cure'.

Severe, or complicated, falciparum malaria is a serious disease with a high mortality rate and must be regarded as life threatening, and thus requires urgent treatment. Treatment typically requires parenteral drug administrations (that is, injections) since the patients are often comatose or vomiting, and thus cannot take the drugs orally. Parenteral formulations are available for chloroquine, quinine, quinidine and artemisinin derivatives. The artemisinin derivatives are generally the preferred choice. However, in the United States quinine and quinidine are the approved drugs for severe malaria. Patients need to be continuously monitored for hematocrit, parasitemia, hydration levels, hypoglycemia, and signs of drug toxicity and other complications during the course of treatment. A

switch to oral administration should be made as soon as the patient is able. Most deaths due to severe malaria occur at or close to home in situations where the patients cannot be taken to the hospital. Artemisinin suppositories, which can be administered by village health workers, have also been developed and have proved to be safe and effective.

The efficacy of chloroquine is greatly diminished by the wide spread chloroquine resistance of *P. falciparum* and the emergence of chloroquine-resistant *P. vivax*. If chloroquine therapy is not effective, or if in an area with chloroquine-resistant malaria, common alternative treatments include: mefloquine, quinine in combination with doxycycline, or sulfadoxine-pyrimethamine combination. Derivatives of artemisinin (dihydroartemisinin, artesunate and artemether) are increasingly used in Asia and Africa and are now recommend as the first line of treatment by the World Health Organization. These drugs were originally derived from the wormwood plant (*Artemesia annua*) and have been used for a long time in China as an herbal tea called quinhaosu to treat febrile illnesses. To prevent the high recrudescence rates associated with artemisinin derivatives and to slow the development of drug resistance it is recommended that treatment be combined with an unrelated anti-malarial. Drugs used in combination with artemisinin include mefloquine, lumefantrine, Sufadoxine-Pyrimethamine, and amodiaquine.

2.8.2 Chemoprophylaxis

Chemoprophylaxis is especially important for persons from non-malarious areas who visit areas endemic for malaria. Such non-immune persons can quickly develop a serious and life-threatening disease. As in the case of treatment there is no standard recommendation and the choices for chemoprophylaxis are highly dependent upon the

conditions associated with the travel and the indivdual person. Chemoprophylaxis requires the use of non-toxic drugs since these drugs will be taken over extended periods of time. Generally the patient will start to take the drug before traveling and then continue taking the drug during the stay in the endemic area and continue taking the drug after returning. This is to insure the drug is maintained at sufficient levels throughout the visit and to protect against any infection obtained during the visit. Unfortunately, many of the effective and non-toxic drugs (for example, chloroquine, pyrimethamine, proquanil) are of limited use because of drug resistance. Another strategy is presumptive (or 'standby') treatment to be used in conjunction with prophylaxis. In this case a person either forgoes prophylaxis or takes chloroquine or another relatively non-toxic drug for prophylaxis and carries a drug like sulfadoxine-pyrimethamine, mefloquine, or quinine, which they will take if they start to exhibit symptoms associated with malaria.

The use of mefloquine for malaria chemoprophylaxis is somewhat controversial. Mefloquine is efficacious at preventing malaria with a single dose per week, thus offering advantages to drugs that need to be administered daily. At this dosage most individuals tolerate mefloquine. However, some people experience neuropsychiatric adverse affects such as sleep disturbances and nightmares. This could be exacerbated by international travel, which is a stressful event. Randomized, blinded and controlled trials indicate that neuropsychiatric adverse affects are only slightly higher with mefloquine than with other anti-malarials.

Killing the exoerythrocytic stage (that is, liver) would prevent the blood infection and is known as causal prophylaxis. This is highly desirable in that it limits the amount of time

the prophylactic drug needs to be taken before and after travel to an endemic area. The only currently available drug for causal prophylaxis is primaquine. However, primaquine is not approved for malaria prophylaxis and should only be prescribed for prophylaxis on a case-by-case basis. For example, for persons who frequently have trips of short duration to highly endemic areas and does not exhibit glucose-6-phosphate dehydrogenase deficiency. Tafenoquine is currently undergoing field evaluation for its use in causal prophylaxis.

2.8.3 Drug Resistance

Drug resistance, and in particular, chloroquine resistance is a major public health problem in the control of malaria. Drug resistance is defined by a treatment failure and can be graded into different levels depending on the timing of the recrudescence following treatment. Traditionally these levels of drug resistance have been defined as sensitive (no recrudescence), RI (delayed recrudescence), RII (early recrudescence), and RIII (minimal or no anti-parasite effect). A modified protocol based on clinical outcome was introduced in 1996 by WHO. In this protocol the level of resistance is expressed as adequate clinical response (ACR), late treatment failure (LTF), or early treatment failure (ETF) as defined by the following:

- ACR, absence of parasitemia (irrespective of fever) or absence of clinical symptoms (irrespective of parasitemia) on day 14 of follow-up
- LTF, reappearance of symptoms or the presence of parasitemia during days 4-14 of follow-up

- ETF, persistence of clinical symptoms in the presence of parasitemia during the first 3 days of follow-up

Either protocol can be used to determine drug resistance, but the clinical outcome protocol is more practical in areas of intense transmission where it may be difficult to distinguish re-infection from recrudescence and where parasitemia in the absence of clinical symptoms is common. Drug resistance by either protocol is determined with *in vivo* tests in which patients are hospitalized and monitored during and following standard drug treatment. There are also *in vitro* tests that can estimate the level of drug resistance by determining the efficacy of the drugs against *P. falciparum* grown in culture. The *in vivo* and *in vitro* tests do not always correspond since host immunity and other factors can affect the *in vivo* outcomes. The identification of specific mutations, which might be associated with drug resistance, may also lead to the development of tests based on molecular markers.

Drug resistance develops when parasites with decreased sensitivities to antimalarial drugs are selected under drug pressure. Decreased drug sensitivity can be conferred by several mechanisms and reflects genetic mutation or polymorphism in the parasite population. The drug-resistance parasites will have a selective advantage over the drug-sensitive parasites in the presence of drug and will be preferentially transmitted. Major factors in the development of drug resistance are the use of subtherapeutic doses of drugs or not completing the treatment regimen. The lower drug levels will eliminate the most susceptible parasites, but those that can tolerate the drug will recover and reproduce. Over time this will lead to a continued selection for parasites, which can tolerate even

higher doses of the drug. It is crucial to maintain an adequate concentration of the drug for a sufficient time to completely eliminate the parasites from any given individual.

2.8.4 Chloroquine Resistance

After its introduction near the end of World War II, chloroquine quickly became the drug of choice for the treatment and prevention of malaria. Not only is chloroquine an effective drug, probably due to its site of action in the food vacuole and its interference with hemozoin formation but is also relatively non-toxic and cheap. Two foci of chloroquine resistant *P. falciparum* were detected in Colombia and at the Cambodia-Thailand border during the late 1950's. During the 1960's and 1970's, resistant parasites spread through South America, Southeast Asia, and India. Resistance was first reported in east Africa in 1978 and spread throughout the continent during the 1980's. Chloroquine resistant *P. vivax* was not reported until 1989 in Papua New Guinea and is now found in several foci in Southeast Asia and perhaps South America.

The basis of chloroquine resistance is reduced chloroquine accumulation in the parasite's food vacuole. Furthermore, chloroquine resistance can be partially reversed with inhibitors of P-glycoprotein (an ABC transporter) which are responsible for multi-drug resistance (MDR) in tumor cell lines, thus suggesting a similar phenomenon may occur in *Plasmodium*. Mutations in a MDR-like gene from *P. falciparum* (*Pf*MDR1) were implicated in chloroquine resistance. However, these mutations are not predictive of chloroquine resistance in all geographical areas. *Pf*MDR1 appears to contribute to the degree of chloroquine resistance, but alone it is insufficient to confer resistance.

However, *Pf*MDR1 does appear to play a role in resistance to mefloquine and halofantrine and influences the sensitivity to artemisinin.

Another candidate for the genetic locus of chloroquine resistance was identified through a genetic cross and mapping experiment. A 400 kb region on chromosome 7 was found to segregate with chloroquine resistance and further analysis suggested that a single gene, called *Pf*crt, was responsible for chloroquine resistance. Out of a total of 10 polymorphisms identified in this gene, only a single mutation is perfectedly associated with the chloroquine resistance phenotype. This mutation results in a lysine at residue 76 being changed to a threonine (K76T). Several field studies have demonstrated an association between *Pf*crt-K76T and chloroquine resistance using both *in vivo* and *in vitro* methods. It has been recently suggested that there have been at least 4 founder mutations in the *Pf*crt gene associated with different geographical regions: Asia/Africa, Papua New Guinea, Brazil/Peru, and Colombia (Wootton *et al.*, 2002). Presumably the use of chloroquine resulted in the subsequent selection and spread of the resistant phenotype.

In Brazil, malaria remains endemic in the Amazon, with 630,000 cases per year, mostly in adults with a high mortality rate, mainly due to late diagnosis, to inadequate treatment or multi-drug resistant parasites. Being an acute disease, with typical symptoms (headache, recurrent fever, among others), individuals affected by malaria in endemic areas often self-medicate with available antimalarial drugs or with medicinal plants. During decades, chloroquine, a 4-aminoquinoline, was largely used for treatment and prophylaxis of acute malaria with an amazing impact on malaria control. The problem of

chloroquine drug-resistant parasites appeared in the early sixties, described in South East Asia and South America, and appeared much later in the African continent. Drug resistant parasites have now spread to most malaria infected countries, including Brazil. Here, the campaign of malaria eradication, based on chloroquine, launched by the World Health Organization and the Ministry of Health in the fifties, reduced transmission in most populated areas, in the Northeast, Southeast and South, but not in the North which is still endemic. As a consequence of drug resistance, the present situation is alarming and new drugs are urgently needed. There is no ideal drug to replace chloroquine or primaquine; there are no vaccines available and no way to block the mosquito transmission at present. The analyses of clinical and public health implications of antimalarial drug resistance, diagnosis and measurements of drug resistance, and treatment failure raise an interesting point about whether money should be used to develop new drugs or to improve access to existing drugs. The argument considers the meager resources allocated for malaria, its prevalence in the developing world, the lack of financial incentives and the consequent lack of interest by the pharmaceutical industry, a crude reality. Since resistance is the prime determinant of a drug's life span, protecting its effective use must be the number one priority in control programs.

2.9 CELLULAR AND MOLECULAR BIOLOGY OF PLASMODIUM

Members of the genus *Plasmodium* are eukaryotic microbes. Therefore, the cell and molecular biology of *Plasmodium* will be similar to other eukaryotes. A unique feature of the malarial parasite is its intracellular lifestyle. Because of its intracellular location the parasite has an intimate relationship with its host cell, which can be described, at the

cellular and molecular levels. In particular, the parasite must enter the host cell, and once inside, it modifies the host cell.

2.9.1 Host Cell Invasion

Malaria parasites are members of the Apicomplexa. Apicomplexa are characterized by a set of organelles found in some stages of the parasite's life cycle. These organelles, collectively known as apical organelles because of their localization at one end of the parasite, are involved in interactions between the parasite and host.

In particular, the apical organelles have been implicated in the process of host cell invasion. In the case of *Plasmodium*, three distinct invasive forms have been identified: sporozoite, merozoite, and ookinete.

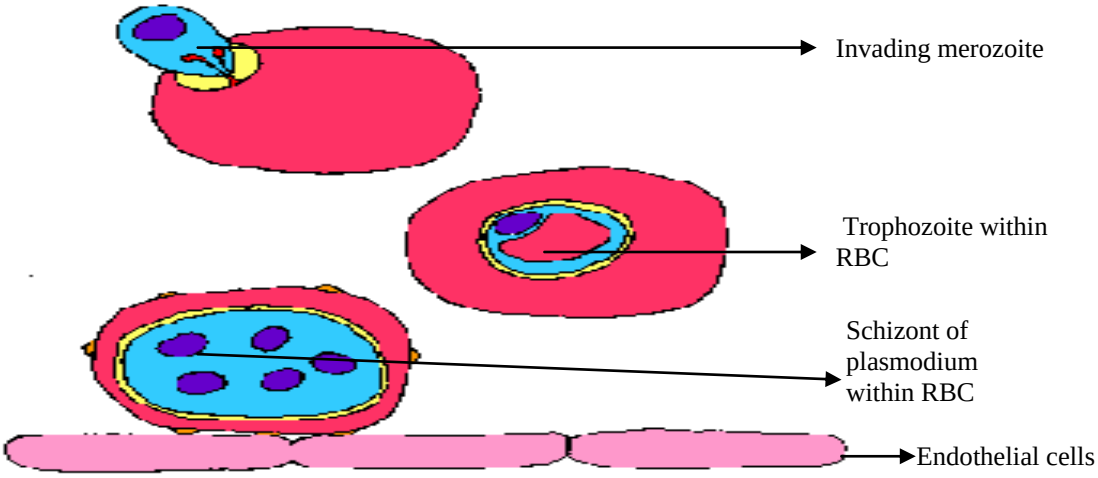


Plate 5: Parasite Invasion of Host cell (Adapted from Blackman, 2000)

Merozoites rapidly (approximately 20 seconds) and specifically enter erythrocytes. This specificity is manifested both for erythrocytes as the preferred host cell type and for a particular host species, thus implying receptor-ligand interactions. Erythrocyte invasion is a complicated process, which is only partially understood at the molecular and cellular levels (Gratzer and Dluzewski, 1993). Four distinct steps in the invasion process can be recognized:

1. Initial merozoite binding
2. Reorientation and erythrocyte deformation
3. Junction formation
4. Parasite entry.

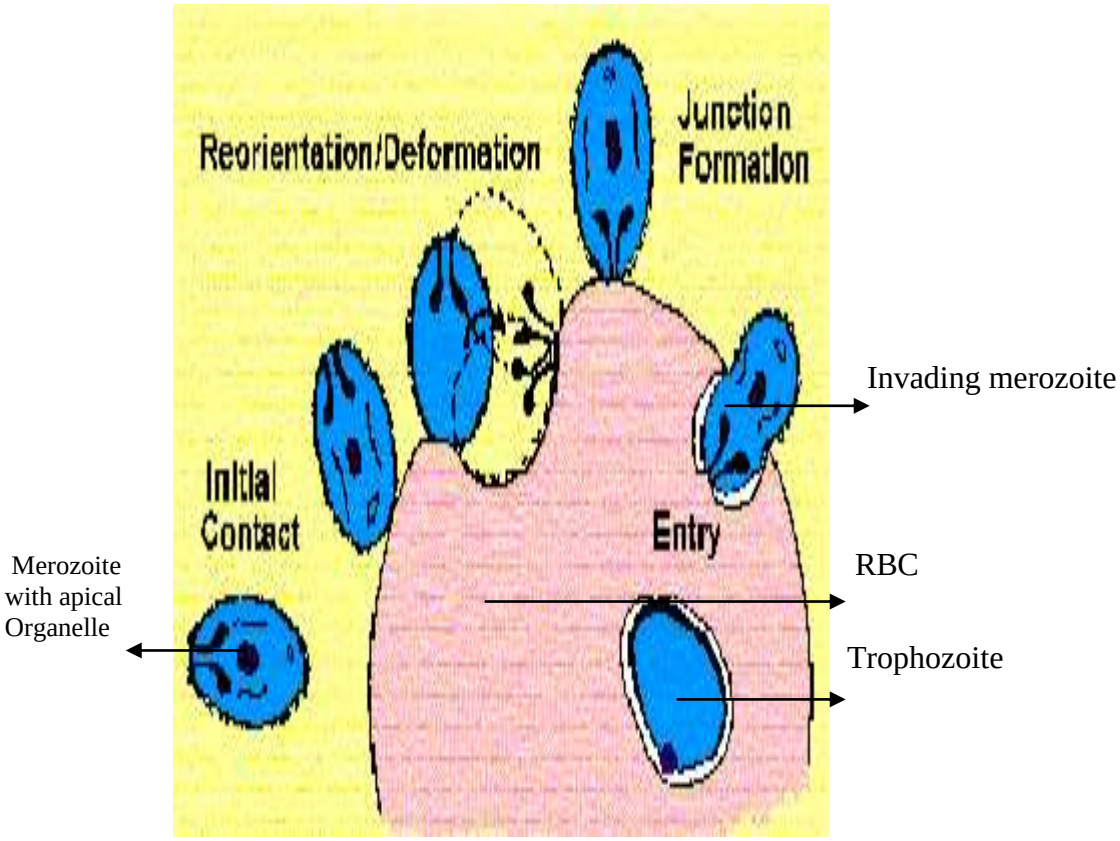


Plate 6: Stages Involved in Host Cell Invasion. (Adapted from Gratzer and Dluzewski, 1993)

2.9.2 Merozoite Surface Proteins and Host-Parasite Interactions

The initial interaction between the merozoite and the erythrocyte is probably a random collision and presumably involves reversible interactions between proteins on the merozoite surface and the host erythrocyte. Several merozoite surface proteins have been described. The best characterized is merozoite surface protein-1 (MSP-1). Circumstantial evidence implicating MSP-1 in erythrocyte invasion includes its uniform distribution over the merozoite surface and the observation that antibodies against MSP-1 inhibit invasion (Holder *et al.,* 1994). In addition, MSP-1 does bind to band 3 (Goel *et al.,* 2003). However, a role for MSP-1 in invasion has not been definitively demonstrated. Similarly, the circumsporozoite protein (CSP) probably plays a role in targeting sporozoites to hepatocytes by interacting with heparin sulfate proteoglycans (Sinnis and Sim, 1997).

Another interesting aspect of MSP-1 is the proteolytic processing that is coincident with merozoite maturation and invasion (Cooper, 1993). A primary processing occurs at the time of merozite maturation and results in the formation of several polypeptides held together in a non-covalent complex. A secondary processing occurs coincident with merozoite invasion at a site near the C-terminus. The non-covalent complex of MSP-1 polypeptide fragments is shed from the merozoite surface following proteolysis and only a small C-terminal fragment is carried into the erythrocyte. This loss of the MSP-1 complex may correlate with the loss of the 'fuzzy' coat during merozoite invasion. The C-terminal fragment is attached to the merozoite surface by a GPI anchor and consists of two EGF-like modules. EGF-like modules are found in a variety of proteins and are usually implicated in protein-protein interactions. One possibility is that the secondary

proteolytic processing, functions to expose the EGF-like modules, which strengthen the interactions between merozoite and erythrocyte. The importance of MSP-1 and its processing are implied from the following observations:

- Vaccination with the EGF-like modules can protect against malaria, and
- Inhibition of the proteolytic processing blocks merozoite invasion.

The exact role which MSP-1 and its processing play in the merozoite invasion process are not known.

2.9.3 Reorientation and Secretory Organelles

After binding to the erythrocyte, the parasite reorients itself so that the 'apical end' of the parasite is juxtaposed to the erythrocyte membrane. This merozoite reorientation also coincides with a transient erythrocyte deformation. Apical membrane antigen-1 (AMA-1) has been implicated in this reorientation (Mitchell, 2004). AMA-1 is a transmembrane protein localized at the apical end of the merozoite and binds erythrocytes. Antibodies against AMA-1 do not interfer with the initial contact between merozoite and erythrocyte thus suggesting that AMA-1 is not involved in merozoite attachement. But antibodies against AMA-1 prevent the reorientation of the merozoite and thereby block merozoite invasion.

Specialized secretory organelles are located at the apical end of the invasive stages of apicomplexan parasites. Three morphologically distinct apical organelles are detected by electron microscopy: micronemes, rhoptries, and dense granules. Dense granules are not

always included with the apical organelles and probably represent a heterogeneous population of secretory vesicles.

The contents of the apical organelles are expelled as the parasite invades, thus suggesting that these organelles play some role in invasion. Experiments in *Toxoplasma gondii* indicate that the micronemes are expelled first and occur with initial contact between the parasite and host (Carruthers and Sibley, 1997). An increase in the cytoplasmic concentration of calcium is associated with microneme discharge (Carruthers and Sibley, 1999), as is typical of regulated secretion in other eukaryotes. The rhoptries are discharged immediately after the micronemes and the release of their contents correlate with the formation of the parasitophorous vacuole. Dense granule contents are released after the parasite has completed its entry, and therefore, are usually implicated in the modification of the host cell. For example, RESA is localized to dense granules in merozoites and is transported to the host erythrocyte membrane shortly after merozoite invasion (Culvenor *et al.*, 1991).

However, subtilisin-like proteases, which are implicated in the secondary proteolytic processing of MSP-1, have also been localized to *Plasmodium* dense granules (Blackman, 2000; Blackman *et al.*, 1998; Barale *et al.*, 1999). If MSP-1 processing is catalyzed by these proteases, then at least some dense granules must be discharged at the time of invasion.

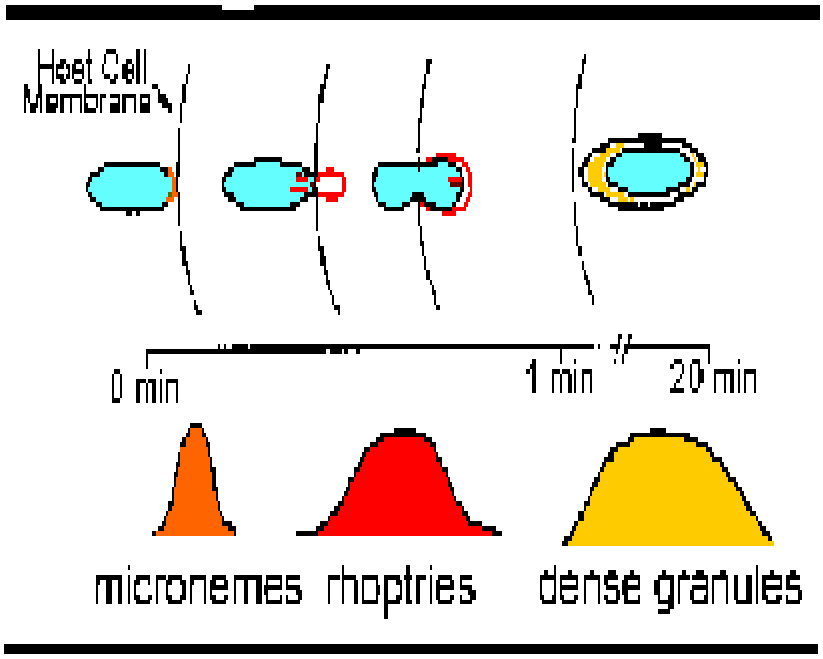


Plate 7: Kinetics of secretion in Toxoplasma (Adapted from Carruthers and Sibley, 1997)

2.9.4 Specific Interactions and Junction Formation

Following merozoite reorientation and microneme discharge, a junction forms between the parasite and host cell. Presumably, microneme proteins are important for junction formation. Proteins localized to the micromenes include:

- EBA-175, a 175 kDa 'erythrocyte binding antigen' from *P. falciparum*
- DBP, Duffy-binding protein from *P. vivax* and *P. knowlesi*
- SSP2, *Plasmodium* sporozoite surface protein-2. Also known as TRAP (thrombospondin-related adhesive protein).
- Proteins with homology to SSP2/TRAP from *Toxoplasma* (MIC2), *Eimeria* (Etp100), and *Cryptosporidium*
- CTRP, circumsporozoite and TRAP-related protein of *Plasmodium* found in the ookinete stage

Of particular note are EBA-175 and DBP, which recognize sialic acid residues of the glycophorins and the Duffy antigen, respectively. In other words, these parasite proteins are probably involved in receptor-ligand interactions with proteins exposed on the erythrocyte surface. Disruption of the EBA-175 gene results in the parasite switching from a sialic acid-dependent pathway to a sialic acid-independent pathway (Reed *et al.*, 2000), indicating that there is some redundancy in regards to the receptor-ligand interactions.

Comparison of sequences of EBA-175 and DBP reveal conserved structural features. These include transmembrane domains and receptor-binding domains (Adams *et al.*,

1992). The receptor-binding activity has been mapped to a domain in which the cysteine and aromatic amino acid residues are conserved between species. This putative binding domain is duplicated in EBA-175. The topography of the transmembrane domain is consistent with the parasite ligands being integral membrane proteins with the receptor-binding domain exposed on the merozoite surface following microneme discharge. The other microneme proteins in the 'TRAP' family have also been implicated in locomotion and/or cell invasion (Tomley and Soldati, 2001). All of these proteins have domains that are presumably involved in cell-cell adhesion, as well as N-terminal signal sequences and trans-membrane domains at their C-termini.

Key Features:

- An electron-dense junction forms between the apical end of the merozoite and host erythrocyte membrane immediately after reorientation
- Tight junction formation and microneme release occur at about the same time
- Proteins localized to the micronemes bind to receptors on the erythrocyte surface

These observations suggest that the junction represents a strong connection between the erythrocyte and the merozoite, which is mediated by receptor-ligand interactions. Junction formation may be initiated by microneme discharge, which exposes the receptor-binding domains of parasite ligands. This mechanism for initiating a tight host-parasite interaction is probably similar in other invasive stages of apicomplexan parasites.

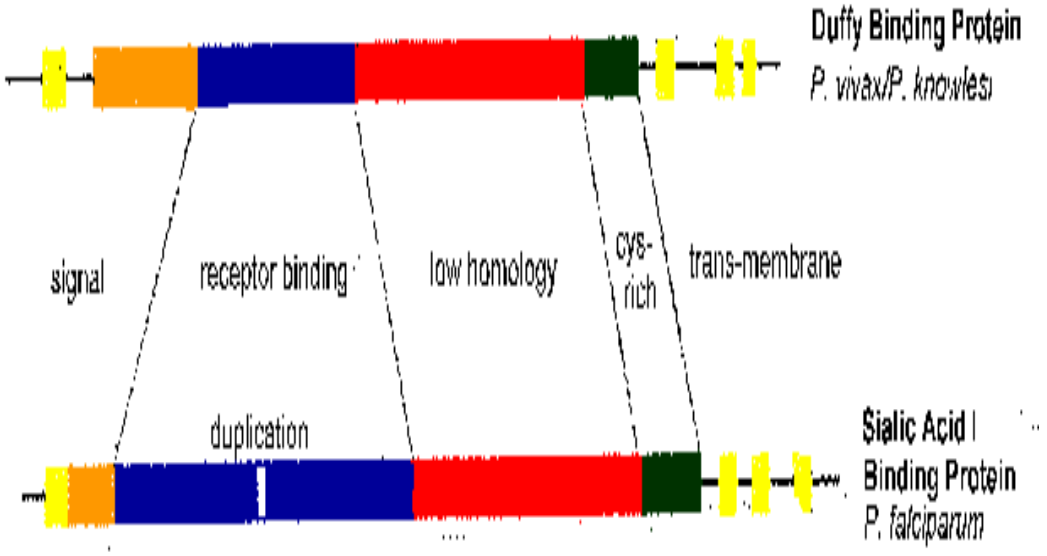


Plate 8: Transmembrane Junction formation (Adapted from Adams *et al.*, 1992)

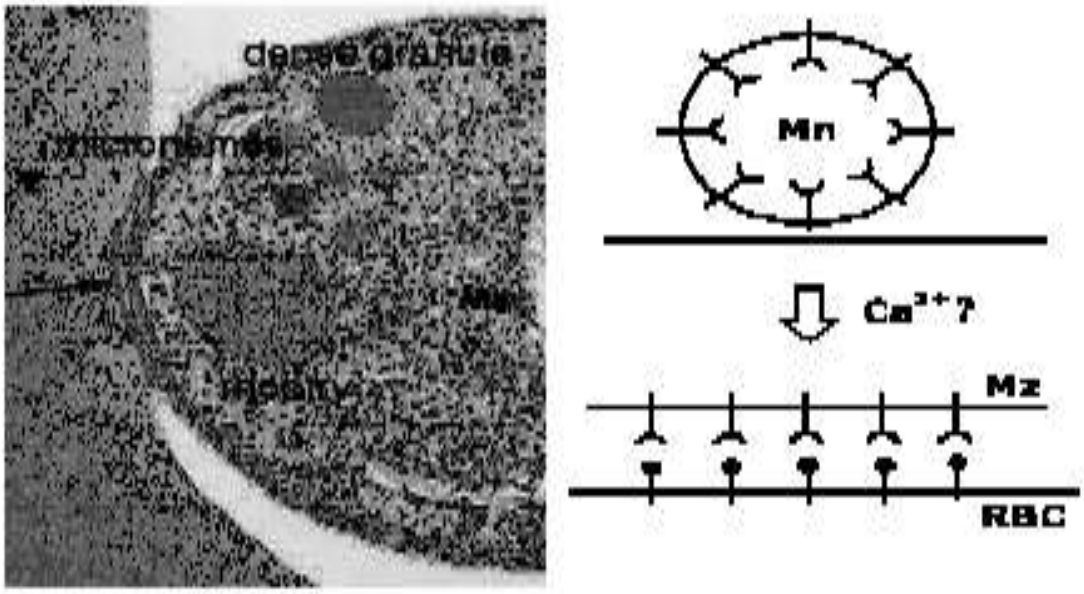


Plate 9: Micrograph of Microneme and Dense Granule (Adapted from Tomley and Soldati, 2001)

2.9.6 Parasite Entry

Apicomplexan parasites actively invade host cells and entry is not due to uptake or phagocytosis by the host cell. This is particularly evident in the case of the erythrocyte, which lacks phagocytic capability. Furthermore, the erythrocyte membrane has a 2-dimensional submembrane cytoskeleton, which precludes endocytosis. Therefore, the impetus for the formation of the parasitophorous vacuole must come from the parasite.

Erythrocyte membrane proteins are redistributed at the time of junction formation so that the contact area is free of erythrocyte membrane proteins. A merozoite serine protease, which cleaves erythrocyte band 3, has been described (Braun-Breton and Pereira da Silva, 1993). Because of the pivotal role band 3 plays in the homeostasis of the submembrane skeleton, its degradation could result in a localized disruption of the cytoskeleton.

An incipient parasitophorous vacuolar membrane (PVM) forms in the junction area. This membrane invagination is likely derived from both the host membrane and parasite components and expands as the parasite enters the erythrocyte. Connections between the rhoptries and nascent PVM are sometimes observed. In addition, the contents of the rhoptries are often lamellar (multi-layered) membranes and some rhoptry proteins are localized to the PVM following invasion, suggesting that the rhoptries function in PVM formation (Sam-Yellowe, 1996).

Ookinetes lack rhoptries and do not form a parasitophorous vacuole within the mosquito midgut epithelial cells. The ookinetes rapidly pass through the epithelial cells and cause

extensive damage as they head toward the basal lamina (Han *et al.,* 2000, Zieler and Dvorak, 2000). Similarly, sporozoites can enter and exit hepatocytes without undergoing exoerythrocytic schizogony. Those parasites, which do not undergo schizogony, are free in the host cytoplasm, whereas those undergoing schizogony are enclosed within a PVM (Mota *et al.,* 2001). These observations suggest that the PVM is needed for intracellular development and is not necessary for the process of host cell invasion. As the incipient parasitophorous vacuole is being formed, the junction (denoted with C's in figure) between the parasite and host becomes ring-like and the parasite appears to move through this annulus as it enters the expanding parasitophorous vacuole.

Apicomplexan parasites actively invade host cells and entry is not due to uptake or phagocytosis by the host cell. In addition, the zoites are often motile forms that crawl along the substratum by a type of motility referred to as ‘gliding motility’. Gliding motility, like invasion, also involves the release of micronemal adhesins, attachment to the substratum, and a capping of the adhesins at the posterior end of the zoite. One difference between gliding motility and invasion is that the micronemes must be continuously released, as the organism is moving. Thus, gliding motility does not involve this relatively small moving junction, but a continuous formation of new junctions between the zoite and the substratum. In addition, the adhesins are cleaved from the surface of the zoite as the adhesions reach the posterior of the zoite and a trail of the adhesive molecules are left behind the moving zoite on the substratum. However, the mechanism of motility and invasion are quite similar and thus, during invasion the parasite literally crawls into the host cell through the moving junction. In addition, some

apicomplexans use this type of motility to escape from cells and can traverse biological barriers by entering and exiting cells.

Cytochalasins inhibit merozoite entry, but not attachment. This inhibition suggests that the force required for parasite invasion is based upon actin-myosin cytoskeletal elements. The ability of myosin to generate force is well characterized (for example, muscle contraction). A myosin unique to the Apicomplexa has been identified and localized to the inner membrane complex (Kappe *et al.,* 2004). This myosin is part of a motor complex, which is linked to the adhesins. Members of the TRAP family and other adhesins have a conserved cytoplasmic domain. This cytoplasmic domain is linked to short actin filaments via aldolase. The actin filaments and myosin are oriented in the space between the inner membrane complex and plasma membrane so that the myosin propels the actin filaments toward the posterior of the zoite. The myosin is anchored into the inner membrane complex and does not move. Therefore, the transmembrane adhesins are pulled through the fluid lipid bilayer of the plasma membrane due to their association with the actin filaments. Thus the complex of adhesins and actin filaments is transported towards the posterior of the cell.

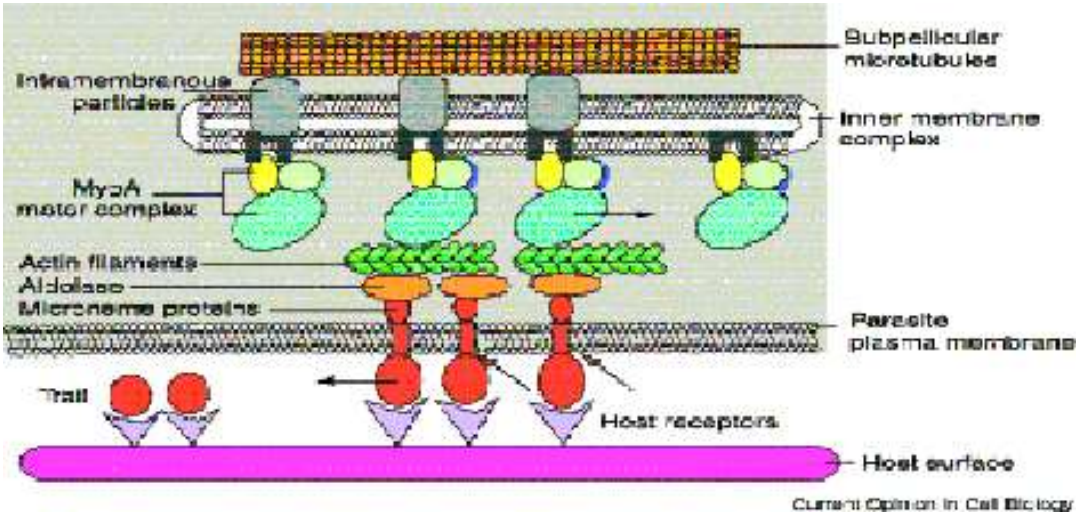


Plate 10: Current model of the motor protein complex driving gliding motility (Adapted from Tomley and Soldati, 2001)

2.9.7 Important Features of Merozoite Invasion

Merozoite invasion is a complex and ordered process. A tentative model of merozoite invasion includes:

1. Initial merozoite binding involves reversible interactions between merozoite surface proteins and the host erythrocyte. The exact roles of MSP1 and other merozoite surface proteins are not known.
2. Reorientation by an unknown mechanism results in the apical end of the merozoite being juxtaposed to the erythrocyte membrane.
3. Discharge of the micronemes is coincident with the formation of a tight junction between the host and parasite. The tight junction is mediated by receptor-ligand interactions between erythrocyte surface proteins and intergral parasite membrane proteins exposed by microneme discharge.
4. Localized clearing of the erythrocyte submembrane cytoskeleton and formation of the incipient parasitophorous vacuole. PVM formation is correlated with the discharge of the rhoptries.
5. Movement of the merozoite through the ring-shaped tight junction formed by the receptor/ligand complex. The force is generated by myosin motors associated with the trans-membrane parasite ligands moving along actin filaments within the parasite.
6. Closure of the PVM and erythrocyte membrane.

Many proteins that are involved in the invasion process have been identified. However, much still remains to be learned about the cellular and molecular biology of merozoite invasion (Iyer *et al.,* 2007; Baum *et al.,* 2008).

2.9.8 Host Erythrocyte Modification

Once inside the erythrocyte, the parasite undergoes a trophic phase followed by replicative phase. During this intraerythrocytic period, the parasite modifies the host to make it a more suitable habitat. For example, the erythrocyte membrane becomes more permeable to small molecular weight metabolites, presumably reflecting the needs of an actively growing parasite. Another modification of the host cell concerns the cytoadherence of *P. falciparum*-infected erythrocytes to endothelial cells and the resulting sequestration of the mature parasites in capillaries and post-capillary venules. This sequestration likely leads to microcirculatory alterations and metabolic dysfunctions, which could be responsible for many of the manifestations of severe falciparum malaria. The cytoadherence to endothelial cells confers at least two advantages for the parasite: 1) a microaerophilic environment which is better suited for parasite metabolism, and 2) avoidance of the spleen and subsequent destruction.

2.9.9 Knobs and Cytoadherence

Major structural alterations of the host erythrocyte are electron-dense protrusions, or 'knobs', on the erythrocyte membrane of *P. falciparum*-infected cells. The knobs are induced by the parasite and several parasite proteins are associated with the knobs (Deitsch and Wellems, 1996). Two proteins, which might participate in knob formation

or affect the host erythrocyte submembrane cytoskeleton and indirectly induce knob formation, are the knob-associated histidine rich protein (KAHRP) and erythrocyte membrane protein-2 (*Pf*EMP2), also called MESA. Neither KAHRP nor *Pf*EMP2 are exposed on the outer surface of the erythrocyte, but are localized to the cytoplasmic face of the host membrane. Their exact roles in knob formation are not known, but may involve reorganizing the submembrane cytoskeleton.

The translocation of *Pf*EMP1 to the erythrocyte surface depends in part on another erythrocyte membrane associated protein called *Pf*EMP3 (Waterkeyn *et al.*, 2000). *Pf*EMP1 probably functions as a ligand, which binds to receptors on host endothelial cells. Other proposed cytoadherence ligands include a modified band-3, called pfalhesin (Sherman *et al.,* 1995), sequestrin, rifins and clag (Craig and Scherf, 2001). *Pf*EMP1 is a member of the *var* gene family (Smith *et al.*, 2001). The 40-50 *var* genes exhibit a high degree of variability, but have a similar overall structure. *Pf*EMP1 has a large extracellular N-terminal domain, a transmembrane region and a C-terminal intracellular domain.

The C-terminal region is conserved between members of the *var* family and is believed to anchor *Pf*EMP1 to the erythrocyte submembrane cytoskeleton. In particular, this acidic C-terminal domain may interact with the basic KAHRP of the knob (Waller *et al.*, 1999) as well as spectrin and actin (Oh *et al.*, 2000).

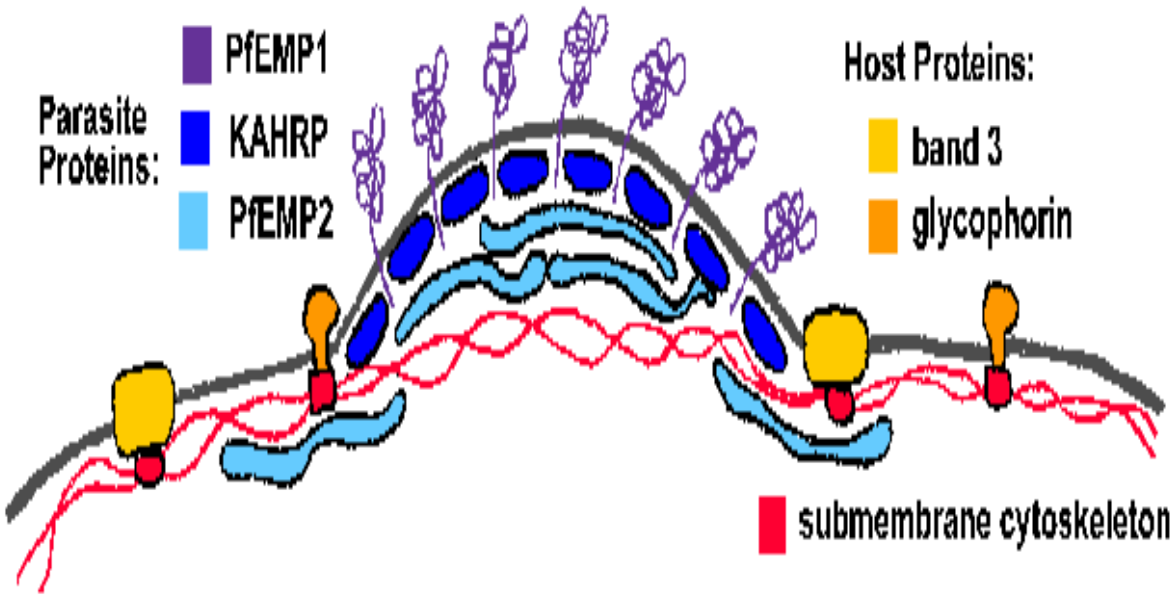


Plate 11: Structural Alteration of Host Erythrocyte (Adapted from Waterkeyn *et al.*, 2000).

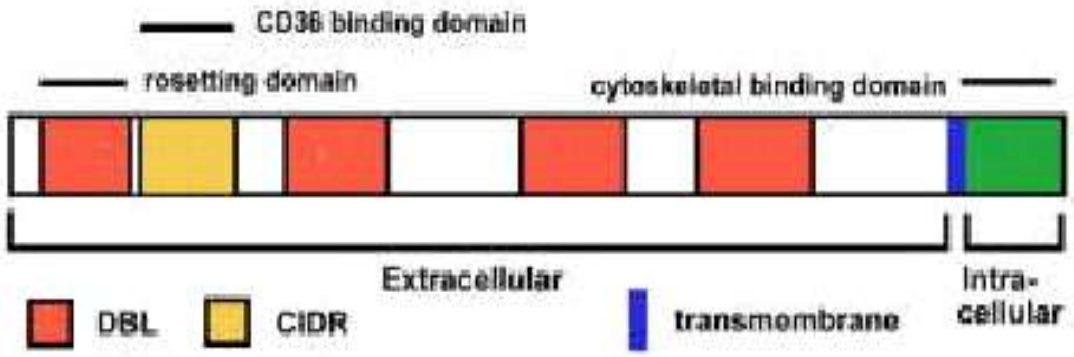


Plate 12: Structure of PfEMP1 (Adapted from Smith *et al.*, 2001).

The extracellular domain is characterized by 1-5 copies of Duffy-binding like (DBL) domains. These DBL domains are similar to the receptor-binding region of the ligands involved in merozoite invasion. The DBL domains exhibit a conserved spacing of cysteine and hydrophobic residues, but otherwise show little homology. Phylogenetic analysis indicates that there are five distinct classes (designated as α, β, γ, δ, and ε) of DBL domains (Smith *et al.*, 2001). The first DBL is always the same type (designated α) and this is followed by a cysteine-rich interdomain region (CIDR). A variable number of DBL in various orders makes up the rest of the extracellular domain of *Pf*EMP-1.

2.9.10 Endothelial Cell Receptors

Several possible endothelial receptors have been identified by testing the ability of infected erythrocytes to bind in static adherence assays (Beeson and Brown, 2002). One of the best characterized among these is CD36, an 88 kDa integral membrane protein found on monocytes, platelets and endothelial cells. Infected erythrocytes from most parasite isolates bind to CD36 and the binding domain has been mapped to the CIDR of *Pf*EMP1. However, CD36 has not been detected on endothelial cells of the cerebral blood vessels and parasites from clinical isolates tend to adhere to both CD36 and intercellular adhesion molecule-1 (ICAM1). ICAM1 is a member of the immunoglobulin superfamily and functions in cell-cell adhesion. In addition, sequestration of infected erythrocytes and ICAM1 expression has been co-localized in the brain (Turner *et al.*, 1994). Chondroitin sulfate A (CSA) has been implicated in the cytoadherence within the placenta and may contribute to the adverse affects of *P. falciparum* during pregnancy. The role of some of the other potential receptors is not clear. For example, adherence to thrombospondin

exhibits a low affinity and cannot support binding under flow conditions. Binding to VCAM1, PECAM1 and E-selectin appears to be rare and questions about their constitutive expression on endothelial cells have been raised. However, cytoadherence could involve multiple receptor/ligand interactions.

Rosetting is another adhesive phenomenon exhibited by *P. falciparum*-infected erythrocytes. Infected erythrocytes from some parasite isolates will bind mutiple-uninfected erythrocytes and *Pf*EMP1 appears to have a role in at least some rosetting. Possible receptors include complement receptor-1 (CR1), blood group A antigen, or glycosaminoglycan moieties on an unidentified proteoglycan. The different types of DBL domains and CIDR bind to different endothelial cell receptors (Smith *et al.,* 2001; Craig and Scherf, 2001). For example, DBL-α, which comprises the first domain, binds to many of the receptors associated with rosetting. The binding of the CIDR to CD36 may account for the abundance of this particular binding phenotype among parasite isolates. Sherman *et al.,* (2003), reviewed the mechanisms of cytoadherence.

2.9.11 Antigenic Variation

The encoding of the cytoadherence ligand by a highly polymorphic gene family presents a paradox in that receptor/ligand interactions are generally considered highly specific. Interestingly, selections for different cytoadherent phenotypes result in a concommitant change in the surface antigenic type (Biggs *et al.,* 1992). Similarly, examination of clonal parasite lines revealed that changes in the surface antigenic type correlated with differences in binding to CD36 and ICAM1. For example, the parental line (A4) adhered equally well to CD36 and ICAM1, whereas one of the A4-derived clones (C28) exhibited

a marked preference for CD36. Binding to ICAM1 was then reselected by panning the infected erythrocytes on ICAM1. All three parasite clones (A4, C28, C28-I) exhibited distinct antigenic types as demonstrated by agglutination with hyper-immune sera.

The expression of a particular PfEMP1 will result in a parasite with a distinct cytoadherent phenotype and this may also affect pathogenesis and disease outcome. For example, binding to ICAM-1 is usually implicated in cerebral pathology. Therefore, parasites expressing a PfEMP1, which binds to ICAM1, may be more likely to cause cerebral malaria. In fact, higher levels of transcription of particular *var* genes are found in cases of severe malaria as compared to uncomplicated malaria (Rottmann *et al.,* 2006). Similarly, a higher proportion of isolates, which bind to CSA, are obtained from the placenta as compared to the peripheral circulation of either pregnant women or children. Furthermore, placental malaria is frequently associated with higher levels of transcription of a particular var gene, which binds CSA (Duffy *et al.,* 2006). This phenomenon is not restricted to the placenta in that there is a dominant expression of particular *var* genes in the various tissues. This tissue specific expression of particular *var* genes implies that different tissues are selecting out different parasite populations based on the particular PfEMP1 being expressed on the surface of the infected erythrocyte. Although sequestration offers many advantages to the parasite, the expression of antigens on the surface of the infected erythrocyte provides a target for the host immune system. The parasite counters the host immune response by expressing antigenically distinct *Pf*EMP1 molecules on the erythrocyte surface. This allows the parasite to avoid clearance by the host immune system, but yet maintain the cytoadherent phenotype. This antigenic switching may occur as frequently as 2% per generation in the absence of immune

pressure (Roberts *et al.,* 1992). The molecular mechanism of antigenic switching is not known. Only a single var gene is expressed at a time (that is, allelic exclusion). The non-expressed genes are kept silent by proteins, which bind to the promoter region. A gene can become activated by repositioning to a particular location in the nucleus and is associated with chromatin modification. This expression spot can only accommodate a single active gene promoter. Thus the var promoter is sufficient for both the silencing and the mono-allelic transcription of a PfEMP1 allele (Voss *et al.,* 2006).

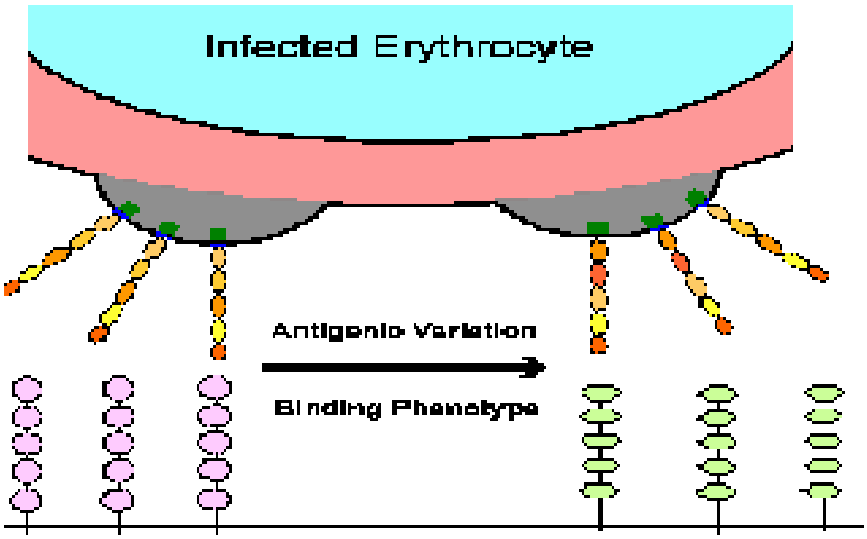


Plate 13: Endothelial Cell Cytoadherance (Adapted from Voss *et al.*, 2006).

2.9.12 Key Features of Cytoadherence

- The malarial parasite modifies the erythrocyte by exporting proteins into the host cell.
- One such modification is the expression of *Pf*EMP1 on the erythrocyte surface, which functions as the cytoadherent ligand.
- The binding of this ligand to receptors on host endothelial cells promotes sequestration and allows the infected erythrocyte to avoid the spleen.
- Numerous *Pf*EMP1 genes (the *var* gene family) provide the parasite with a means to vary the antigen expressed on the erythrocyte surface.
- This antigenic variation also correlates with different cytoadherent phenotypes.

2.10 ANTIMALARIAL DRUG DISCOVERY: OLD AND NEW APPROACHES

Malaria is one of the most important infectious diseases in the world (Breman, 2001). Unfortunately, mortality from malaria appears to be increasing in the highest risk group, African children (Snow *et al.*, 2001). A major contributor to malarial morbidity and mortality is almost certainly the increasing resistance of malaria parasites to available drugs (Olliaro and Bloland, 2001). Resistance is primarily seen in *Plasmodium falciparum*, the most virulent human malaria parasite.

Considering increasing resistance to available agents, there is broad consensus that there is a need to develop new antimalarial drugs (Ridley, 2002). Antimalarial drug development can follow several strategies, ranging from minor modifications of existing agents to the design of novel agents that act against new targets. Increasingly, available

agents are being combined to improve antimalarial regimens. Additionally, potential new targets for drug discovery have been developed (Olliaro and Yuthavong, 1999; Ridley, 2002; Rosenthal, 2001a).

2.10.1 Drug-resistant *P. falciparum* Malaria

For several decades, the gold standard for the treatment of malaria was chloroquine (CQ), a 4 aminoquinoline that was previously characterized by its efficacy, low toxicity and affordability (less than $0.2 USD for a three-day adult treatment course) (White, 1996). CQ acts by binding to haem moieties produced from proteolytically processed haemoglobin inside infected erythrocytes, thereby interfering with haem detoxification (Pagola *et al.*, 2000; Ursos and Roepe, 2002). Massive worldwide use of CQ, beginning in the late 1940s, was followed a decade later by the first reports of CQ-resistant strains of *P. falciparum* (Wellems and Plowe, 2001). Today, CQ resistance has spread to the vast majority of malaria-endemic areas, rendering this drug increasingly ineffective. However, in spite of the prevalence of CQ-resistant *P. falciparum*, this drug continues to be widely used. This is particularly problematic in sub-Saharan Africa, where resource limitations are profound and where highly immune populations often seem to respond at least partially to CQ therapy, and therefore somewhat mask the spread of resistance. CQ resistance almost certainly contributes to the recent finding that malaria-associated mortality is on the increase in Africa (Trape, 2001). Sulfadoxine-Pyrimethamine (SP), a combination antifolate drug, is the only other widely used inexpensive antimalarial, but resistance is also leading to unacceptable levels of therapeutic failure in many areas in Asia, South America and now Africa (Plowe, 2003). Despite some optimism about new drug development for the future, as noted above, the malaria endemic regions of the

world are faced with an unprecedented situation in which the only affordable treatment options are rapidly losing therapeutic efficacy.

2.10.2 The Urgent Need for New Antimalarials

New antimalarial drugs must meet the requirements of rapid efficacy, minimal toxicity and low cost. Immediate prospects for drugs to replace CQ and SP include amodiaquine (a CQ-like quinoline) and chlorproguanildapsone (LapDap, another antifolate combination that inhibits the same enzymes as SP). These replacements will probably provide a few years of efficacy, particularly in Africa, but they already suffer from some cross resistance with CQ and SP, which increases the likelihood that full-blown resistance to these drugs will emerge rapidly (Olliaro, 1996; Lang and Greenwood, 2003). High on the list of mid-term replacements are artemisinin derivatives. However, these drugs have very short half-lives, which necessitate their use in combination with a longer-acting drug. Clearly, additional new drugs are needed. If we are to avoid an ever-increasing toll of malaria on tropical areas, it is imperative to rapidly put into action strategic plans for the discovery and development of novel antimalarial compounds that are not encumbered by pre-existing mechanisms of drug resistance.

2.10.3 The Desired Profile for New Drugs

Ideally, new drugs for uncomplicated *P. falciparum* malaria should be efficacious against drug-resistant strains, provide cure within a reasonable time (ideally three days or less) to ensure good compliance, be safe, be suitable for small children and pregnant women, have appropriate formulations for oral use and, above all, be affordable (Ridley, 2002; Nwaka and Ridley, 2003). Drug development necessarily requires trade-offs among desired drug features, but for the treatment of malaria in the developing world the

provision of affordable, orally active treatments that are safe for children is, for practical purposes, mandatory. Cost drives the choice of drugs in most developing countries, especially Africa, where most people must survive on less than $15 USD per month.

Additional desirable uses include intermittent preventive treatment in pregnancy and childhood, treatment in refugee camps and other emergency situations, treatment of severe malaria, and the treatment of malaria caused by *P. vivax* (a rarely lethal, but nevertheless debilitating and widespread, agent of malaria). Of less importance to public health, but potentially offering profitability, new drugs should ideally also provide protection against malaria when used as chemoprophylaxis by advantaged non-immune populations travelling to endemic areas.

2.10.4 The Need for Drug Combinations

There is a growing consensus that drug combinations are essential to the optimal control of malaria in developing countries (Guerin, 2002). Combination therapy potentially offers a number of important advantages over monotherapies. First, they should provide improved efficacy. Appropriately chosen combinations must be at least additive in potency, and might provide synergistic activity. However, combination regimens that rely on synergy might not offer as much protection against the selection of resistance as expected, as resistance to either component of the combination could lead to a marked loss of efficacy. Indeed, the widely used synergistic combination SP acts almost as a single agent in this regard, with rapid selection of resistance (Dorsey *et al.*, 2003). Similar concerns apply to the new atovaquone/proguanil combination (Looareesuwan *et al.*, 1999). Secondly, drug combinations increase the likelihood that, in the setting of drug resistance, at least one agent will be clinically active. In East Africa, where resistance to

both amodiaquine and SP is quite prevalent, the combination of these inexpensive agents still provides good antimalarial efficacy (Staedke, 2001; Dorsey *et al.,* 2002). Third, and probably most important, drug combinations should reduce the selection of antimalarial drug resistance. Resistance has consistently been seen first in areas of relatively low endemicity, presumably due to the greater likelihood of high parasitemia and symptoms leading to treatment in relatively non-immune individuals (White and Pongtavornpinyo, 2003). In Thailand, the use of an artesunate and mefloquine combination has been accompanied by excellent efficacy and a decrease in the prevalence of mefloquine resistance in infectious isolates (Nosten, 2000). It was also recently shown that SP selected for resistance-conferring mutations and subsequent treatment failure, but that SP combined with artesunate prevented the selection of SP-resistant parasites in subsequent infections (Dorsey *et al.,* 2003). Combinations might offer additional advantages if the separate agents are active against different parasite stages and if they provide the opportunity to decrease dosages of individual agents, thereby reducing cost and/or toxicity. Ideally, combination regimens will incorporate two agents that are both new (so that parasites resistant to either agent are not already circulating), offer potent efficacy and preferably have similar pharmacokinetics profiles (to limit the exposure of single agents to resistance pressures). Unfortunately, these are challenging requirements that are not met by any combination available at present. A widely advocated strategy is to combine artemisinins, which have no resistance problem but suffer as monotherapy from late recrudescence due to their short half-lives, with longer-acting agents (White, 1999). The hope is that the potent action of artemisinin will prevent significant selection of parasites resistant to the longer acting component (for example, amodiaquine/artesunate

(Adjuik *et al.,* 2002), mefloquine/artesunate (Nosten, 2000), chlorproguanil/dapsone/artesunate (Lang and Greenwood, 2003) or lumefantrine/ artemether (Van Vugt *et al.,* 2000). However, artemisinins are natural products that are difficult to synthesize and cannot be sold at cost for less than $1–2 USD in curative combination regimens, a prohibitive price in most malaria-endemic regions. Indeed, even if widespread implementation of new artemisinin combination regimen is possible, additional new antimalarial drugs will be needed. Other regimen, offering combinations of inexpensive and available drugs for example, chlorproguanil/dapsone (Lang and Greenwood, 2003), or amodiaquine plus sulphadoxine/pyrimethamine (Staedke, 2001), might be appropriate stopgap therapies, especially in Africa, where the need is greatest and resources most limiting.

2.10.5 Target Selection and Validation

Most antimalarial drugs that are now in use were not developed on the basis of rationally identified targets, but following the serendipitous identification of the antimalarial activity of natural products (for example, quinine and artemisinin), compounds chemically related to natural products (for example, CQ and artesunate), or compounds active against other infectious pathogens (for example, antifolates and tetracyclines).

More recently, an improved understanding of the biochemistry of malaria parasites has identified many potential targets for new drugs and helped shed light on the mode(s) of action of older drugs. Targets that are shared between the parasite and human host offer opportunities for chemotherapy if structural differences can be exploited. For example, the dihydrofolate reductase inhibitors pyrimethamine and proguanil are important components of antimalarial drugs, in large part because of their relative selectivity for the

parasite enzyme. One advantage of targets that are also present in the host is that, in certain cases, the host target has already been considered as a therapeutic target for other disease indications. As a result, the cost of antimalarial drug discovery can be reduced if initial work, directed toward more profitable targets, has already been undertaken. As examples, antimalarial drug discovery efforts directed against parasite cysteine proteases (Rosenthal *et al.,* 2002) and protein farnesyl transferases (Chakrabarti *et al.,* 2002) are benefiting from industry projects directed against inhibitors of the human cysteine protease cathepsin K as treatments for osteoporosis (Rottella, 2002) and human farnesyl transferases as treatments for cancer (Gelb and Ho, 2002). Alternatively, targets can be selected from enzymes or pathways that are present in the malaria parasite but absent from humans. Here, the added difficulty of evaluating a target 'from scratch' is offset by the high degree of selectivity that should be provided by inhibitors of the parasite target. In some cases, parasite targets might be shared by other microbial organisms for which classes of inhibitors have already been generated and can be readily screened. One example is the use of prokaryotic protein synthesis inhibitors, including tetracyclines and clindamycin, which were found to have antimalarial activity. These compounds presumably act selectively against malaria parasites because of their action against prokaryote-like plasmodial organelles known as apicoplasts, which seem to have cyanobacterial origins and are related to algal plastids (Ralph *et al.,* 2001). Additional, recently identified potential selective targets for antimalarial drugs include components of type II fatty acid biosynthesis (Waller, 1998) and mevalonate-independent isoprenoid synthesis (Jomaa, 1999).

Both pathways are also targets for existing antibacterial compounds, providing initial leads for antimalarial drug discovery (Missinou, 2002; Perozzo, 2002). A 'reverse'drug discovery approach is to elucidate the nature of previously unidentified targets of existing antimalarial drugs as a basis for new drug discovery or development efforts. One germane example relates to CQ, which acts by interfering with the production of the malarial pigment haemozoin, allowing the intraparasitic build-up of toxic free haem (Ursos and Roepe, 2002; Waller *et al.,* 2004). This has defined inhibition of haemozoin formation as an attractive target for new antimalarial drugs (Biagini *et al.,* 2003). In addition to benefiting from the entire *P. falciparum* genome sequence (Gardner, 2002), investigators have access to a sophisticated database, Plasmo DB, which facilitates genome searches and analysis (Kissinger, 2002). The genomes of a number of other plasmodial species have also recently been released and are accessible through this database. These genome sequences can dramatically accelerate the early steps of drug discovery by enabling the rapid identification of putative plasmodial targets that are homologous to validated target proteins from other systems. This, however, does not obviate the need for high-quality biological studies to validate drug targets. Older approaches to target validation include the demonstration that an inhibitor has potent antimalarial activity. This approach is limited, however, by the fact that it is often difficult to determine whether an inhibitor of a particular plasmodial target is exerting its antimalarial activity specifically by the predicted mechanism of action. This problem is partially solved by the repeated demonstration of antiparasitic activity of different inhibitors of a particular target, by the identification of very potent (generally low-nanomolar) activity and, when possible, by the identification of biologically relevant

defects caused by inhibitors for example, the development of swollen food vacoules in parasites treated with cysteine protease inhibitors (Rosenthal *et al.,* 2002).

A fourth, unambiguous way of formally attributing the cellular effects of an inhibitor to the putative target is to measure its effects on a transgenic parasite in which the molecular target is resistant. If the inhibitor loses its efficacy, this demonstrates that the mutated molecule is indeed the target. This was demonstrated for the *P. falciparum* dihydrofolate reductase gene, which in its mutated form conferred resistance to pyrimethamine in transgenic *P. falciparum* (Wu *et al.,* 1996). In a related complementation strategy, transgenic expression of human dihydrofolate reductase in *P. falciparum* conferred complete protection against WR99210, proving that this compound inhibited the parasite orthologue (Fidock and Wellems, 1997). Compound screen newer technologies have greatly improved our ability to validate potential drug targets. In particular, methodologies have been developed to transfect *P. falciparum* with plasmids expressing either positive or negative selectable markers, and to thereby alter, replace or knock out genes of interest (Wu *et al.,* 1996; Van Dijk *et al.,* 1996; Sidhu *et al.,* 2002). Another promising new avenue made possible by transfection is to express genes encoding drug targets from *P. falciparum* by allelic replacement into the rodent malaria parasite *P. berghei*, for which efficient transfection technology has been developed (Waters *et al.,* 1997; Peters and Robinson, 1999). This enables evaluation of compound efficacy against the correct enzymatic target in an *in vivo* setting. An exciting extension of this approach is the introduction of a gene encoding a target in *P. vivax* — for which *in vitro* culture is unavailable and *in vivo* assays require monkeys — by allelic replacement into *P. falciparum* and *P. berghei*, thereby generating *in vitro* and *in vivo* screens against this

under studied human pathogen. Additional means of validating drug targets are made possible by new genomic and proteomic technologies. The former offer the opportunity to survey transcription across the plasmodial life cycle (Rathod *et al.,* 2002; Le Roch, 2003), and might provide insight into the transcriptional impact of target inhibition as well as highlight pathways of interest, particularly if functionally related genes share common transcriptional profiles (Le Roch, 2003; Bozdech, 2003). Proteomic approaches, which require the accurate separation of thousands of proteins, are advancing rapidly for *P. falciparum* (Lasonder, 2002; Florens, 2002) and permit more direct investigations of the biochemical impact of established drugs and potential antimalarials. In a related 'functional proteomics' approach, the inhibition of proteins that have not been biochemically characterized can be surveyed using libraries of inhibitors and competitive binding assays (Greenbaum, 2002). Proteomic studies should help investigators to identify the mechanisms of action of older drugs, confirm suspected mechanisms for new compounds and suggest novel chemotherapeutic approaches.

2.10.7 *In vitro* Screens of Potential Antimalarials

In vitro screens for compound activity, which constitute a key component of a critical path for an antimalarial drug discovery programme, are based on the ability to culture *P. falciparum in vitro* in human erythrocytes. Typically, parasites are propagated in leukocyte-free erythrocytes at 2–5% heamatocrit at 37°C under reduced oxygen (typically 3–5% O_2, 5% CO_2, 90–92% N_2) in tissue culture (RPMI 1640) media containing either human serum or Albumax (a lipid-rich bovine serum albumin).

Multiple drug-resistant and drug-sensitive isolates from around the world have now been culture-adapted. Details for one standardized protocol for culturing *P. falciparum* and

assaying susceptibility to antimalarial compounds are available. This protocol describes the measurement of the uptake of 3H-hypoxanthine (which is taken up by the parasite for purine salvage and DNA synthesis) to determine the level of *P. falciparum* growth inhibition. In most applications, parasites are cultured in the presence of different concentrations of test compound in media containing reduced concentrations of hypoxanthine, after which 3H-hypoxanthine is added for an additional incubation period before cell harvesting and measurement of radioactive counts. IC_{50} values can be determined by linear regression analyses on the linear segments of the dose–response curves. Although 3H-hypoxanthine incorporation is the most commonly used method to assay antimalarial activity *in vitro*, it is costly, radioactive and quite complex, and therefore problematic for resource-poor locations or for high-throughput screening (HTS) (Noedl *et al.*, 2003). A low-cost alternative for testing small numbers of compounds is to incubate parasites with test compounds (typically for 48 or 72 hours), and then to compare parasitaemias of treated and control parasites by counting GIEMSA-stained parasites by light microscopy.

Another established, but less standardized, assay involves the colorimetric detection of lactate dehydrogenase (Makler and Hinrichs, 1993). Flow cytometry has also been used to test candidate antimalarial compounds, and takes advantage of the fact that human erythrocytes lack DNA. In the simplest use of this technology, parasites are fixed after the appropriate period of incubation with test compounds, then either the parasitized cells are stained with hydroethidine which is metabolized to ethidium (Van der Heyde *et al.*, 1995) or the parasite nuclei are stained with DAPI (4′,6-diamidino-2-phenylindole). Counts of treated and control cultures are then obtained by flow cytometry. Appropriate

gating can also allow one to distinguish different parasite erythrocytic stages. This relatively simple assay provides quite high throughput and has replaced older methods at some centres, but requires expensive equipment. Compounds that meet an acceptable cut-off for *in vitro* activity (for example, $IC_{50} \leq 1$ μM) can then be tested for activity against a range of geographically distinct *P. falciparum* lines of differing drug-resistance phenotypes. Different research groups have incorporated a variety of modifications of the basic *in vitro* screens, which can influence the measurement of drug activity levels.

2.10.8 Unsynchronized versus Synchronized Cultures

For preliminary screening of diverse compounds, the less demanding (but less sensitive) method of using unsynchronized cultures is widely used. Synchronised cultures are used when comparing a series of compounds, establishing rank order of activities and determining potency against different parasite stages (Ter Kuile *et al.*, 1993).

2.10.9 Duration of incubation

Most assays incorporate incubation with test compounds for 48 hours, the duration of one erythrocytic cycle. Incubations can also be extended to 72 hours or longer. This can generate more reproducible IC_{50} values when working with unsynchronized cultures and is important when testing slower-acting compounds such as antibiotics.

2.10.10 Human Serum versus Albumax

In recent years many laboratories have replaced 10% human serum with the serum substitute Albumax. The latter has both clear advantages (for example, reduced batch-to-batch variation) and disadvantages (for example, a higher level of protein binding has been reported with Albumax compared with serum, such that activities of some compounds might differ depending on culture conditions (Ofulla, 1994).

2.10.11 Initial Percentage of Parasitaemia

The number of parasites present at the beginning of the drug assay can have a significant effect on *in vitro* activity known as the inoculum effect (Gluzman *et al.*, 1987). Numerous variations on these standard assays can be used to gain further insight into compound efficacy. For example, compounds can be added to synchronized cultures at different stages of development to assess which stages are the most susceptible to drug action, and inhibitors can be added for different lengths of time before removal in order to determine the minimum time of exposure needed to achieve parasite killing. To assess the effects of combining compounds, isobologram analysis (Berenbaum, 1978; Ohrt *et al.*, 2002) can be performed to assess whether two compounds are additive, synergistic or antagonist which discusses *in vitro* drug interactions as well as *in vivo* drug combinations). This is conducted using standard dose–response assays over a range of individual drug concentrations, using either a checkerboard technique (Canfield *et al.*, 1995) or fixed-ratio methods (Ohrt *et al.*, 2002). This *in vitro* analysis has been useful in identifying clinical combinations — for example, atovaquone and proguanil (Canfield *et al.*, 1995) as well as in determining the potential of 'low activity' compounds, such as azithromycin (Ohrt *et al.*, 2002).

With the notable exception of the artemisinin family of drugs, almost all antimalarial drugs developed to date are active only against asexual stage parasites and therefore do not prevent transmission of the pathogen (White, 1999). Transmission-blocking activity is nevertheless a desirable property for any new antimalarial drug. To test for this, *P. falciparum* gametocytes can be produced *in vitro* (Ponnudurai *et al.*, 1982) and compounds added to assess the impact on gametocyte development. In addition,

transmission-blocking assays can be conducted by feeding starved female *Anopheles* mosquitoes a blood meal containing infectious gametocytes via an artificial-membrane feeding apparatus. Mosquitoes can then be maintained for one week, after which the midguts are dissected. The number of midgut oocysts resulting from treated and control parasites are then compared by light microscopy (Templeton *et al.*, 2000).

2.10.12 *In vivo* Screening of Antimalarial Compounds

Plasmodium species that cause human disease are essentially unable to infect non-primate animal models (with the exception of a complex immunocompromised mouse model that has been developed to sustain *P. falciparum*-parasitized human erythrocytes *in vivo* (Moreno *et al.*, 2001). So, *in vivo* evaluation of antimalarial compounds typically begins with the use of rodent malaria parasites. Of these, *P. berghei*, *P. yoelii*, *P. chabaudi* and *P. vinckei* have been used more extensively in drug discovery and early development (Childa, 1984). Rodent models have been validated through the identification of several antimalarials — for example, mefloquine, halofantrine and more recently artemisinin derivatives (Peters, 1977; Posner, 2003). In view of their proven use in the prediction of treatment outcomes for human infections, these models remain a standard part of the drug discovery and development pathway. Individual species and strains have been well characterized, including duration of cycle, time of schizogony, synchronicity, drug sensitivity and course of infection in genetically defined mouse strains (Peters and Robinson, 1999; Sanni *et al.*, 2002).

The most widely used initial test, which uses *P. berghei* or less frequently *P. chabaudi*, is a four-day suppressive test (Peters and Robinson, 1999), in which the efficacy of four daily doses of compounds is measured by comparison of blood parasitaemia (on day four

after infection) and mouse survival time in treated and untreated mice. Rodent infection is typically initiated by needle passage from an infected to a naïve rodent via the intraperitoneal or preferably the intravenous route, often using a small inoculum (typically in the range of 10^6–10^7 infected erythrocytes). Compounds can be administered by several routes, including intraperitoneal, intravenous, subcutaneous or oral. CQ is often used as the reference drug and typically has an ED_{50} value against *P. berghei* (ANKA strain) of 1.5–1.8 mg per kg when administered subcutaneously or orally (Stocks, 2002; Stocks *et al.*, 2002). Compounds identified as being active in four-day assays can subsequently be progressed through several secondary tests, as follows. In the 'dose ranging, full four-day test', compounds are tested at a minimum of four different doses, by subcutaneous and/or oral routes, to determine ED_{50} and ED_{90} values. This test also provides useful information on relative potency and oral bioavailability. In the 'onset/recrudescence' test, mice are administered a single dose (by the subcutaneous or oral route) on day 3 post-infection and followed daily to monitor parasitaemia. Results are expressed as the rapidity of onset of activity (disappearance of parasitaemia), time to onset of recrudescence, increase of parasitaemia and survival in number of days. Compounds can also be tested for prophylactic activity by administering the compound prior to infection, followed by daily examination of smears. Additional screens have been developed to assess cross-resistance and the potential for *in vivo* selection of resistant parasites. When using rodent models, several key variables need to be considered during experimental design and interpretation. Foremost is the choice of rodent malaria species and mouse strains. As allude to before, rodent plasmodia can differ significantly in their degree of infection, lethality and synchronicity, which can dramatically affect the results.

These factors also broaden the range of possible assays for compound evaluation. For example, *P. chabaudi* and *P. vinckei* generate a high parasitaemia and produce synchronous infections, enabling studies on parasite stage specificity. Rodent malaria species can also differ significantly in sensitivity to certain classes of compounds. For example, *P. chabaudi* and *P. vinckei* are more sensitive than *P. berghei* to iron chelators and lipid biosynthesis inhibitors (Wengelnik, 2002; Peters and Robinson, 1999). The course of infection can also vary enormously depending on the mouse strain, and models exist that are amenable to studies on chronic infection or sequestration (Sanni *et al.*, 2002). For example, the *P. chabaudi* AS strain in CBA mice produces a chronic infection with a defined immune response that can be used in studies of immunomodulators. It is important to note that the drug sensitivity of a given rodent malaria species does not always mirror that of *P. falciparum* and can limit the types of investigations that can be performed. For example, cysteine proteases in rodent plasmodia show subtle active site differences to those in *P. falciparum*, leading to questions about the use of these models in lead optimization (Singh, 2002). Also, the frequently used *P. yoelii* 17X strain is intrinsically partially resistant to CQ and is therefore a poor model for studying acquisition of *P. falciparum* CQ resistance. Primate models have also had an important role in preclinical development, by providing a final confirmation of the choice of a drug candidate. Infection with certain strains of *P. falciparum* has been well characterized in both *Aotus* and *Saimiri* monkeys (Gysin, 1998). Primate models provide a clearer prediction of human efficacy and pharmacokinetics than rodent models, providing a logical transition to clinical studies (Wengelnik, 2002).

2.10.13 Approaches to Antimalarial Chemotherapy

Many different approaches to the identification of new antimalarials are being pursued at present. This includes strategies that take advantage of specific aspects of the biology of malaria parasites and/or utilize shortcuts to generate new compounds for study at relatively small cost.

2.10.14 Optimization of Therapy with Existing Agents

A first approach is to optimize therapy with existing agents. New dosing regimens or formulations may optimize activity. Combination therapies including newer agents (for example, artemisinin derivatives, atovaquone) and new combinations of older agents (for example, amodiaquine/sulfadoxine/pyrimethamine, chlorproguanil/dapsone) are under study as first-line therapies for Africa and other areas with widespread drug resistance. The use of combination antimalarial therapy offers two important potential advantages. First, the combination should improve antimalarial efficacy, providing additive or, ideally, synergistic antiparasitic activity. In the case of both the artemisinin derivatives and atovaquone, the new agents have had unacceptable failure rates when used as single agents to treat falciparum malaria but they have been highly effective in combination with other established antimalarials. Second, and probably most important, the use of combination therapy should slow the progression of parasite resistance to the new agents. This latter factor is a key consideration as attempts are being made to develop new therapies that will retain activity for a long period. Ideally, a combination regimen that prevents resistance development should include at least two agents against which parasite resistance has not yet developed and which have similar pharmacokinetics, so that low

blood levels of a single agent will not be present. No such ideal regimen is currently available, although chlorproguanil/dapsone/artesunate may prove to fit this description. Alternatively, the combination of a short-acting, highly potent compound and a longer-acting agent may prove effective, if the initial decrease in parasite burden is so great as to limit subsequent resistance development to the long-acting agent (for example, artesunate/mefloquine). As another alternative, two drugs with similar pharmacokinetics may prove effective even if resistance to each agent is present in the community (for example, amodiaquine/sulfadoxine/pyrimethamine). Considerations of combination therapy generally impact on drug development only late in the process, when individual drugs of proven efficacy are considered as components of combinations. However, it may be appropriate to consider combination therapy earlier in the drug discovery process. For example, relatively slow-acting antimalarials (for example, antibiotics) may seem to be poorly suited therapeutic agents but they may work well in combination regimens (for example, quinine and doxycycline).

Some older agents, which are now significantly limited by drug resistance, may nonetheless remain effective in combination. Presumably, the prevalence of resistance to each agent is low enough such that resistance to both drugs is unlikely. Although a combination regimen, sulfadoxine/pyrimethamine loses efficacy quickly once resistance is seen; in this case, efficacy is dependent on synergism, so is lost once resistance develops to either drug. However, the combination of sulfadoxine/pyrimethamine with amodiaquine, a chloroquine analog that remains active against many chloroquine-resistant parasites, provides two effective long-acting drugs. Importantly, this combination regimen uniquely provides two available and inexpensive drugs, although

both components are already limited by drug resistance in many areas and safety concerns (with rare but severe toxicity with long-term prophylactic use). Despite these concerns, the combination of amodiaquine and sulfadoxine/pyrimethamine showed excellent antimalarial efficacy in regions of East Africa, with fairly high levels of resistance to each individual agent (Dorsey *et al.,* 2002; Schellenberg *et al.,* 2002; Staedke *et al.,* 2001). Another intriguing possibility is the reuse of chloroquine, ideally in combination regimens, in areas where it has not been used for an extended period; chloroquine sensitivity was recently shown to reemerge in Malawi after its use was curtailed for about a decade (Kublin *et al.,* 2003).

Artemisinin analogs, in particular artesunate and artemether, have recently shown great promise as rapidly acting and potent antimalarials, but the short half-lives of these compounds lead to many late recrudescences after therapy, suggesting that combination therapies are necessary to fully exploit the potency of this class. Artesunate has been studied in combination with both sulfadoxine/pyrimethamine (Von Seidlein *et al.,* 2000) and amodiaquine (Adjuik *et al.,* 2002) in Africa, with good efficacy, although underlying resistance to the two artesunate partners may lead to unacceptable rates of late recrudescence in many areas, as seen with artesunate/sulfadoxine/pyrimethamine in Uganda (Dorsey *et al.,* 2002). Combinations of artemisinins with longer-acting drugs without underlying resistance may prove to be optimal antimalarial agents. In Thailand, where drug resistance is particularly severe, the combination of artesunate and mefloquine has proven to be highly effective, even in areas where mefloquine resistance was previously seen to be quite common (Price *et al.,* 1997). Artemether has been combined with lumefantrine, an agent related to halofantrine, to provide a highly

effective therapy (Lefevre *et al.,* 2001). Atovaquone, an agent first marketed for *Pneumocystis* pneumonia, has been combined with proguanil, an old dihydrofolate reductase (DHFR) inhibitor, to provide synergistic antimalarial activity (Canfield *et al.,* 1995) and action even against parasites resistant to individual agents in the combination (Vaidya, 2001b). Artesunate/mefloquine, artemether/lumefantrine and atovaquone/proguanil are all quite expensive, do not include components with similar pharmacokinetics, may have toxicity concerns (especially for mefloquine) and, in some cases, do not have ideal dosing regimens (for artemether/lumefantrine, twice-daily dosing with a fatty meal). Thus, these combination regimens offer promise for some indications but may not be ideal for widespread use in many areas, in particular Africa (Vaidya, 2001a).

An interesting new approach to antimalarial drug development is chlorproguanil/dapsone, which combines a close analog of proguanil with dapsone, an old dihydropteroate synthase (DHPS) inhibitor that has been widely used to treat leprosy. Chlorproguanil/dapsone has been specifically devised for the treatment of malaria in Africa, where resistance to chloroquine is very common and resistance to sulfadoxine/pyrimethamine is increasing. Although the new regimen shares the targets of sulfadoxine/pyrimethamine, it is generally effective against sulfadoxine/pyrimethamine-resistant parasites, as the common DHFR and DHPS mutations that mediate this resistance do not lead to clinical resistance to chlorproguanil/dapsone, and additional mutations that lead to higher level antifolate resistance (and resistance to chlorproguanil/dapsone) are rare in Africa (Kublin *et al.,* 2002; Mutabingwa *et al.,* 2001; Nzila *et al.,* 2000). Another key advantage of chlorproguanil/dapsone is a relatively short

half-life, which appears to be long enough to provide effective therapy with 3-day daily dosing but is not so long as to readily select for resistance. The combination of chlorproguanil/dapsone and artesunate may be ideal, combining the rapid potency of artesunate with the slower curative efficacy of chlorproguanil/dapsone, but definitive studies of this combination are still needed.

2.10.15 Development of Analogs of Existing Agents

Another approach to antimalarial chemotherapy is to improve upon existing antimalarials by chemical modifications of these compounds. This approach does not require knowledge of the mechanism of action or the biological target of the parent compound. Indeed, this approach was responsible for the development of many existing antimalarials. For example, chloroquine, primaquine and mefloquine were discovered through chemical strategies to improve upon quinine (Stocks *et al.,* 2001; Stocks *et al.,* 2002; Rosenthal 2001a). 4-aminoquinolines that are closely related to chloroquine appear to offer the antimalarial potency of the parent drug, even against chloroquine-resistant parasites (Kaschula *et al.,* 2002 ; Raynes *et al.,* 1999). A related compound, pyronaridine, was developed in China and is now undergoing extensive clinical trials in other areas (Ringwald *et al.,* 1996). An 8-aminoquinoline, tafenoquine, offers improved activity against hepatic-stage parasites over that of the parent compound, primaquine (Walsh *et al.,* 1999), and is effective for antimalarial chemoprophylaxis (Lell *et al.,* 2000). Since halofantrine use is limited by toxicity, the analog lumefantrine was developed and is now a component of the new combination artemether/lumefantrine (Van Vugt *et al.,* 2000).

New folate antagonists (Tarnchompoo *et al.,* 2002) and new endoperoxides related to artemisinin (Posner *et al.,* 2003; Vennerstrom *et al.,* 2000) are also under study.

2.10.16 Natural Products

Plant-derived compounds offer a third approach to chemotherapy. Importantly, this approach can benefit from knowledge of medicinal plants among natives of malarious regions, where the appreciation of the use of plant products to treat febrile illnesses has grown over many generations. Therefore, as a great improvement over random screening, a plant product with specific clinical activity can be the starting point for a medicinal chemistry effort. Natural products are the sources of the two most important drugs currently available to treat severe falciparum malaria, quinine and derivatives of artemisinin. In the case of artemisinin, relatively simple chemical modifications of the natural product parent compound have led to a series of highly potent antimalarials that are playing an increasingly important role in the treatment of malaria (Meshnick, 2001). However, the cost of these compounds may be limiting, and so efforts to design fully synthetic endoperoxides that are less expensive to produce are an important priority (Posner *et al.,* 2003; Vennerstrom *et al.,* 2000). Extensive evaluations of natural products as potential new therapies for many human diseases are underway (Tagboto and Townson, 2001). It is important that such trials include the evaluation of the antimalarial activity of plant extracts and potential drugs purified from these extracts. As with both the quinolines and artemisinins, it is likely that antimalarial natural products will be the parent compounds for the semi-synthetic or fully synthetic production of new drugs.

2.10.17 Compounds Active against Other Diseases

A fourth approach to antimalarial chemotherapy is to identify agents that are developed or marketed as treatments for other diseases. These compounds might act against orthologs of their targets in other systems or by different mechanisms against malaria parasites. Considering the difficulties of funding antimalarial drug discovery, the advantage of these compounds is that, whatever their mechanism, they have already been developed for a human indication, so will be quite inexpensive to develop as antimalarials. However, costs of production for drugs vary greatly, and some new agents, especially those developed for diseases of wealthy nations such as cancer, may be too expensive to produce as antimalarials, even if they do not require extensive development expenses. In many cases, however, drugs may be quite inexpensive to produce and may be available as inexpensive antimalarials, especially after patents have expired, as has been the case with some antibiotics.

Folate antagonists, tetracyclines and other antibiotics were developed for their antibacterial properties and were later found to be active against malaria parasites (Clough and Wilson, 2001a). Atovaquone was initially identified as an antimalarial, but its development was expedited by the discovery of its activity against *Pneumocystis*. More recently, it's potential as an antimalarial (as a component of the combination drug Malarone) has been re-explored, and it was found to have marked antimalarial synergy with proguanil (Canfield *et al.,* 1995). Malarone was subsequently shown to be effective in the treatment and chemoprophylaxis of malaria, and it is now approved for both of these indications (Hogh *et al.,* 2000). Iron chelators, which are used to treat iron overload

syndromes, have documented antimalarial efficacy (Loyevsky and Gordeuk, 2001). These examples suggest that it is appropriate to screen new antimicrobial agents and other available compounds as antimalarial drugs. This approach is facilitated by the presence of high-throughput assays for potential antimalarials. As suggested by the recent development of Malarone, the consideration of compounds with activity against other more economically attractive microbial targets may provide a relatively inexpensive means of identifying new antimalarials. In the case of protein farnesyl transferases, development efforts have not yet led to viable anticancer therapies, but nonetheless have expedited the consideration of these targets for antimalarial chemotherapy (Gelb *et al.*, 2003).

2.10.18 Drug Resistance Reversers

Combining previously effective agents with compounds that reverse parasite resistance to these agents offers another approach to chemotherapy. Many drugs have been shown to reverse the resistance of *P. falciparum* to chloroquine *in vitro*, most notably the antihypertensive verapamil (Martin *et al.,* 1987) and the antidepressant desipramine (Bitonti *et al.,* 1988). In many cases, unacceptably high concentrations of the resistance reversers are needed for their effects, but combinations of two or more of these agents at pharmacological concentrations may provide clinically relevant resistance reversal, as suggested by studies with verapamil, desipramine and trifluoperazine (Van Schalkwyk *et al.,* 2001). The commonly used and inexpensive antihistamine chlorpheniramine reversed resistance at safe dosing levels, although the common side-effect of drowsiness might limit acceptance of this therapy (Sowunmi *et al.,* 1997). Efforts to design new reversers of

chloroquine resistance are underway (Alibert *et al.,* 2002; Batra *et al.,* 2000). Thus, although chloroquine appears to have failed as a first-line antimalarial in most of the world, this inexpensive, rapid acting, well-tolerated antimalarial may be resurrected by combination with effective resistance reversers.

2.10.19 Cytosolic Targets

The cytosol is the location of numerous metabolic pathways, with hundreds of enzymes that are probably essential, and thus potential drug targets. However, many of these pathways are evolutionarily well conserved, such that parasite and host targets are quite similar, and so the identification of compounds that selectively inhibit parasite enzymes may be difficult.

One pathway that has proven to be a valuable target is folate metabolism, as discussed above (Plowe, 2001). Indeed, despite similarities in targets, extensive study has identified antifolates that effectively treat both bacterial and protozoan infections with minimal toxicity. This approach has also benefited from the availability of compounds developed against other diseases, in this case bacterial infections. Unfortunately, resistance to individual DHFR and DHPS inhibitors, including pyrimethamine, proguanil and sulfadoxine, leads to a marked loss in efficacy of even combination regimens (Plowe, 2001). Sulfadoxine/pyrimethamine is inexpensive and it has replaced chloroquine as first-line therapy for malaria in a number of countries in Africa. However, resistance to this agent is already common in many areas, including parts of Africa, and resistance appears to develop quickly, at least in some settings, with widespread use. The new combination of chlorproguanil/dapsone will probably be highly effective in Africa, but not some other

areas, due to differences in the *P. falciparum* DHFR and DHPS mutation patterns in different parts of the world, but it remains to be seen how quickly this new drug will select for resistance if it is used alone to treat malaria, as will probably soon be the case. Attempts are now underway to develop improved DHFR inhibitor antimalarials, including biguanides related to proguanil (Kinyanjui *et al.,* 1999). In addition, inhibitors of other folate pathway enzymes may be effective antimalarials. For example, 5-fluoroorotate exerts antimalarial activity *via* the inhibition of thymidylate synthase (Rathod *et al.,* 2002).

Glycolysis is another cytosolic pathway of interest. Malaria parasites are dependent on this pathway for energy production. *P. falciparum* lactate dehydrogenase has been characterized structurally, and its unique binding site for the NADH cofactor offers opportunities for the design of selective inhibitors (Dunn *et al.,* 1996). Selective inhibitors of *P. falciparum* lactate dehydrogenase have been identified (Deck *et al.,* 1998) and some compounds have demonstrated *in vitro* antimalarial activity (Razakantoanina *et al.,* 2000).

Purine salvage and pyrimidine synthetic pathways also offer potential drug targets. Malaria parasites cannot synthesize purines and rely on salvage of host purines for nucleic acid synthesis. The principal source of purines in *P. falciparum* appears to be hypoxanthine, and hypoxanthine-guanine phosphoribosyltransferase (HGPRT) has been considered as a potential drug target (Keough *et al.,* 1999). Other enzymes in purine salvage may offer useful targets, considering both unique features of the parasite enzymes and the essential role of purine salvage for the parasites, but not humans. In contrast to

the case with purines, malaria parasites cannot salvage pyrimidines and are thus reliant on pyrimidine synthesis. Differences between pyrimidine synthetic enzymes of parasites and humans offer potential for exploitation as drug targets. Pyrimidine synthesis also relies on mitochondrial electron transport, linking these targets to those in the mitochondrion.

2.10.20 Parasite Membrane Targets

Parasite metabolism is one potential target. Intraerythrocytic malaria parasites undergo extensive lipid synthesis to produce the membranes necessary to enclose the parasitophorous vacuole, cytosol and multiple subcellular compartments. The most abundant lipid in plasmodial membranes is phosphatidylcholine. Synthesis of phosphatidylcholine requires host choline, and blockage of choline transport has been identified as a promising therapeutic strategy (Vial and Calas, 2001). Extensive medicinal chemistry efforts have identified compounds that exert profound antimalarial effects, probably by inhibition of choline transport. A lead compound, G25, inhibited the development of cultured *P. falciparum* parasites at concentrations that were 1000-fold below those toxic to mammalian cells (Calas *et al.,* 2000; Wengelnik *et al.*, 2002). G25 was also effective *in vivo* against mice infected with rodent malaria parasites and primates infected with *Plasmodium cynomolgi* (a model for *Plasmodium vivax*) and *P. falciparum*. Importantly, the compound was remarkably potent in the primate models, with activity at doses far below 1 mg kg^{-1} day^{-1}, although potency with oral dosing was well below that with parenteral dosing.

Other membrane targets are transport pathways that are unique to malaria parasites. Intraerythrocytic parasites markedly alter erythrocyte transport pathways. Our

understanding of parasite transport mechanisms remains incomplete, but it is likely that difference between host and parasite mechanisms offer possibilities for selective antimalarial drugs (Haldar and Akompong, 2001; Kirk, 2001). One possibility is to take advantage of selective transport of cytotoxic compounds into *P. falciparum*-infected erythrocytes. A strategy currently being investigated is the use of dinucleoside phosphate dimers conjugated to antimalarial compounds to improve selective access to parasite targets (Gero *et al.,* 2003).

2.10.21 Food vacuole Targets

Malaria parasites contain acidic food vacuoles in which erythrocyte hemoglobin is hydrolyzed. In *P. falciparum* trophozoites, a single large food vacuole is present. The food vacuole appears to be the site of action of a number of existing antimalarials and also offers opportunities for therapies directed against new targets (Banerjee and Goldberg, 2001). In the food vacuole, hemoglobin is degraded into heme, which is polymerized into insoluble hemozoin pigment and globin, which is hydrolyzed to individual amino acids. Antimalarial drugs appear to act by preventing hemozoin formation, producing free radicals in the food vacuole or, in the case of experimental compounds, preventing globin hydrolysis. The 4-aminoquinoline chloroquine appears to act by blocking the formation of hemozoin from heme molecules once they are liberated from hemoglobin (Sullivan, 2002). Antiparasitic effects are presumably engendered by the toxicity of free heme, possibly by disruption of membranes. Although chloroquine use is now severely compromised by drug resistance, it is important to note that the >50-year history of successful use of this drug, with only a slow development of resistance, may be

due to its nonenzymatic mechanism of action. Many available and experimental antimalarials inhibit specific enzymes, but this approach is likely to routinely suffer from rapid selection of parasites with mutations in target enzymes that mediate drug resistance, as is the case with antifolates. With chloroquine, resistance developed only very slowly. It is now clear that resistance is due primarily to mutations in a putative transporter, PfCRT, and that, although a single mutation mediates resistance *in vitro*, multiple mutations were necessary to select for clinical resistance (Fidock *et al.*, 2000). Although chloroquine-resistant parasites are now common in almost all malarious areas, it seems reasonable to develop other compounds that attack heme polymerization. In this regard, major efforts to synthesize improved quinoline or related compounds as antimalarials are being intensified (De *et al.*, 1998; Stocks *et al.*, 2001).

The food vacuole also appears to be the target of artemisinin antimalarials. As noted above, this new class of compounds offers very potent activity. Artemisinins contain an endoperoxide bridge that is essential for antimalarial activity and that appears to undergo an iron-catalyzed decomposition into free radicals (Meshnick, 2001). The compounds apparently exert antimalarial effects *via* free-radical damage, possibly by alkylation of plasmodial proteins (Asawamahasakda *et al.*, 1994; Bhisutthibhan *et al.*, 1998), although the specific drug targets are uncertain. As is the case with other compounds active in the food vacuole, a key to the selective antimalarial toxicity of artemisinins may be the specific accumulation of the drug in this parasite organelle. Artemisinin analogs are already proven antimalarials but they are fairly expensive to produce, in part because they are semi-synthetic plant products. Extensive efforts are underway to develop fully

synthetic peroxides and related compounds as antimalarials (Borstnik *et al.,* 2002; Vennerstrom *et al.,* 2000).

Globin hydrolysis appears to be mediated by a number of classes of proteases, including food vacuole aspartic acid (Plasmepsins) (Banerjee *et al.,* 2002), cysteine (Shenai *et al.,* 2000; Sijwali *et al.,* 2001) and metalloproteases (Eggleson *et al.,* 1999), and at least one cytosolic metalloaminopeptidase (Gavigan *et al.,* 2001). These enzymes all offer potential targets for chemotherapy (Rosenthal, 2001b). In the case of the plasmepsins and falcipains, the repertoire of proteases that mediate hemoglobin hydrolysis is now known to be more complicated than originally envisioned, as biochemical studies and the availability of the full *P. falciparum* genome sequence have identified additional members of these families. Four plasmepsins are believed to participate in hemoglobin hydrolysis in the food vacuole (Banerjee *et al.,* 2002). It has been argued that plasmepsin II and perhaps additional plasmepsins mediate initial cleavages of hemoglobin, allowing additional processing by other proteases, but the specific roles of these or any other proteases in the process remain uncertain. Plasmepsin inhibitors have demonstrated antimalarial effects (Francis *et al.,* 1994; Haque *et al.,* 1999; Jiang *et al.,* 2001; Moon *et al.,* 1998; Nezami *et al.,* 2002; Noteberg *et al.,* 2003; Silva *et al.,* 1996) but these have not clearly correlated with inhibition of hemoglobin hydrolysis. Cysteine protease inhibitors appear to act by inhibiting falcipain-2 and falcipain-3, the principal cysteine protease mediators of hemoglobin hydrolysis (Shenai *et al.,* 2000; Sijwali *et al.,* 2001). Falcipain inhibitors have been shown to prevent hemoglobin hydrolysis, block parasite development and cure murine malaria (Batra *et al.,* 2003; Rosenthal 2001b; Rosenthal *et al.,* 1991, 1993, 1996, 2002; Shenai *et al.,* 2003; Singh and Rosenthal, 2001).

Importantly, the antimalarial activity of cysteine protease inhibitors is accompanied by a block in parasite hydrolysis of hemoglobin, with the accumulation of intact hemoglobin in the food vacuole. This morphological abnormality confirms the specific action of these compounds on falcipain targets. Of interest, cysteine and aspartic protease inhibitors exert synergistic antimalarial effects *in vitro* and *in vivo* (Semenov *et al.,* 1998). These results suggest that an optimal protease inhibitor antimalarial might include inhibitors of both classes of proteases.

2.10.22 Mitochondrial Targets

One new antimalarial has a mitochondrial target. Atovaquone acts against ubiquinol-cytochrome *c* oxidoreductase (complex III), inhibits electron transport and collapses mitochondrial membrane potential, which is required for a number of parasite biochemical processes (Vaidya, 2001a). The drug has potent antimalarial activity but suffers from rapid selection of resistant parasites with mutations in the target enzyme, and so is inappropriate as monotherapy. Atovaquone proved to be surprisingly effective in combination with the antifolate proguanil, and this combination is now marketed as Malarone, an effective, but very expensive, drug for both chemoprophylaxis (Hogh *et al.*, 2000) and therapy (Radloff *et al.,* 1996) of falciparum malaria. The combination probably benefits from synergistic action of atovaquone and proguanil rather than the antifolate activity of the metabolite cycloguanil, and for this reason it is more effective than would have been predicted in areas with high levels of antifolate resistance (Vaidya, 2001a).

2.10.23 Apicoplast Targets

The apicoplast has been identified as a chloroplast-like organelle of apicomplexan parasites (Kohler *et al.,* 1997). The apicoplast apparently resulted from endosymbiosis, leading to an organelle that maintains certain specific functions, probably including fatty acid, heme and amino acid metabolism. Like the mitochondrion, the apicoplast has a separate, prokaryote-like genome, and this fact probably explains the antimalarial effects of a number of antibacterial compounds that otherwise do not attack eukaryotes. However, most apicoplast proteins are encoded in the nucleus and then transported to the apicoplast by a specific mechanism involving two amino-terminal targeting sequences (Foth *et al.,* 2003; Waller *et al.,* 1998).

Apicoplast biology includes a number of biochemical pathways that are present in bacteria; plants and apicomplexan parasites but is absent in the human host and thus provides obvious opportunities for chemotherapy (Ralph *et al.,* 2001). The type II fatty acid biosynthesis pathway is absent in humans, but genes encoding homologs of bacterial enzymes from this pathway are present in the *P. falciparum* genome and contain putative apicoplast coding signals (Waller *et al.,* 1998). One type II fatty acid biosynthesis subunit, ß-ketoacyl-acyl-carrier protein synthase (FabH), is the target of the antibiotic thiolactomycin, and this antibiotic was active against cultured malaria parasites (Waller *et al.,* 1998). Another subunit, enoyl-acyl-carrier protein reductase (FabI), is also encoded by *P. falciparum* and is the target of the antibacterial triclosan (Surolia and Surolia, 2001). Triclosan demonstrated activity against cultured *P. falciparum* parasites and

against murine malaria, inhibited the target enzyme and blocked parasite fatty acid synthesis, validating this target.

A number of antibacterial compounds are effective, albeit slow-acting antimalarials (Clough and Wilson, 2001b; Vaidya, 2001b). These compounds probably act by targeting apicoplast and/or mitochondrial processes that are similar to those in bacteria (Ralph *et al.,* 2001). Tetracyclines, clindamycin, macrolides and chloramphenicol inhibit different steps of prokaryote-like protein synthesis (Clough and Wilson, 2001a). Quinolone antibiotics inhibit DNA gyrase, and rifampin inhibits RNA polymerase, again with specificity to prokaryote-like activity. It is not clear why all of these compounds appear to exert only slow antimalarial activity. It seems most likely, based in part on studies with the related protozoan *Toxoplasma*, that apicoplast toxicity primarily impacts on the life cycle after that which is initially incubated with these drugs (Fichera and Roos, 1997). In some cases, mitochondrial toxicity may also play a role. Despite the slow action of antibacterial compounds as antimalarials, some, including tetracyclines and clindamycin, are already well validated as effective antimalarial agents, and additional study of related drugs is warranted.

Isopentenyl diphosphate, the precursor for isoprenoids, is synthesized in plants and animals *via* the mevalonate pathway, but an alternative pathway, known as the 1-deoxy-D-xylulose 5-phosphate (DOXP) or non-mevalonate pathway is present in bacteria and chloroplasts. Genes encoding two enzymes in this pathway, DOXP reductoisomerase and DOXP synthase, are encoded by *P. falciparum* and contain putative apicoplast targeting signals (Jomaa *et al.,* 1999). The antibiotic fosmidomycin inhibited the activity of

recombinant DOXP reductoisomerase, inhibited the growth of cultured *P. falciparum* parasites and cured murine malaria (Jomaa *et al.,* 1999). Fosmidomycin was previously developed as an antibacterial, so it could quite quickly be brought to human trials for malaria. In an initial trial of safety and efficacy for uncomplicated malaria, fosmidomycin was well tolerated and demonstrated 100% initial cure rates (Lell *et al.,* 2003). However, the use of fosmidomycin as monotherapy will be limited by the apparent need for frequent and prolonged dosing and the common occurrence of recrudescence after therapy. The inclusion of this compound or other inhibitors of apicoplast processes in combination antimalarial regimens may be appropriate.

The DOXP pathway provides precursors for protein farnesylation. Inhibitors of protein farnesyltransferases have been studied as potential cancer therapies. Plasmodial farnesyl transferase activity has unique biochemical features (Chakrabarti *et al.,* 2002) and inhibitors of this process have *in vitro* antimalarial activity (Chakrabarti *et al.,* 2002; Ohkanda *et al.,* 2001).

Antimalarial drugs are designed to prevent or cure malaria. Some antimalarial agents, particularly chloroquine and hydroxychloroquine, are also used in the treatment of rheumatoid arthritis and lupus associated arthritis. There are many of these drugs currently in the market. Quinine is the oldest and most famous anti-malarial.

2.10.24 Resistance to Antimalarials

Antimalarial resistance is common (White, 2008). Anti-malarial drug resistance is defined as: "the ability of a parasite to survive and/or multiply despite the administration

and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject. The drug in question must gain access to the parasite or the infected red blood cell for the duration of the time necessary for its normal action. Thus excluding all cases where anti-malarial prophylaxis has failed. In order for a case to be defined as resistant, the patient under question must have received a known and observed anti-malarial therapy whilst the serum drug and metabolite concentrations are monitored concurrently. The techniques used to demonstrate this are: *in vivo*, *in vitro*, animal model testing and the most recently developed molecular techniques.

Drug resistant parasites are often used to explain malaria treatment failure. However, they are two potentially different clinical scenarios. The failure to clear parasitemia and recover from an acute clinical episode when a suitable treatment has been given shows anti-malarial resistance in its true form. Drug resistance may lead to treatment failure, but treatment failure is not necessarily caused by drug resistance despite assisting with its development. A multitude of factors can be involved in the processes including problems with non-compliance and adherence, poor drug quality, interactions with other pharmaceuticals, poor absorption, misdiagnosis and incorrect doses being given. The majority of these factors also contribute to the development of drug resistance.

The generation of resistance can be complicated and varies between plasmodium species. It is generally accepted to be initiated primarily through a spontaneous mutation that provides some evolutionary benefit. Thus, anti-malarial use is associated with a reduced level of sensitivity. This can be caused by a single point mutation or multiple mutations.

In most instances a mutation will be fatal for the parasite or the drug pressure will remove parasites that remain susceptible. However, some resistant parasites will survive. Resistance can become firmly established within a parasite population, existing for long period of time.

The first type of resistance to be acknowledged was to Chloroquine in Thailand in 1957 (White, 2008). The biological mechanism behind this resistance was subsequently discovered to be related to the development of an efflux mechanism that expels chloroquine from the parasite before the level required to effectively inhibiting the process of haem polymerization (that is necessary to prevent build up of the toxic by products formed by haemoglobin digestion). This theory has been supported by evidence showing that resistance can be effectively reversed on the addition of substances which halt the efflux. The resistance of other quinoline anti-malarials such as amiodiaquine, mefloquine, halofantrine and quinine are thought to have occurred by similar mechanisms.

Plasmodium have developed resistance against antifolate combination drugs, the most commonly used being sulfadoxine and pyrimethamine. Two gene mutations are thought to be responsible, allowing synergistic blockages of two enzymes involved in folate synthesis. Regional variations of specific mutations give differing levels of resistance.

Atovaquone is recommended to be used only in combination with another anti-malarial compound as the selection of resistant parasites occurs very quickly when used in mono-therapy. Resistance is thought to originate from a single-point mutation in the gene coding for cytochrome-b.

2.10.25 Non-Artemesinin Based Combinations

- Sulfadoxine-Pyrimethamine (SP)–This combination has been used for many years and has wide-spread resistance. It has serious adverse effects but is cheap and is available as a single dose, thus decreasing problems associated with adherence and compliance. The recommended dose is 25 mg/kg of sulfadoxine and 1.25 mg/kg of pyrimethamine.

- Sulfadoxine-pyrimethamine plus Chloroquine: This is another cost-effective combination, which benefits from the drugs having similar pharmacokinetic profiles but different biochemical parasitic targets. There is already some level of parasite resistance present and numerous side-effects are associated with the use of SP. Chloroquine is recommended at 25 mg/kg over 3 days with a single dose of SP as described above.

- Sulfadoxine-pyrimethamine plus Amodiaquine–This combination has been shown to produce a faster rate of clinical recovery than SP and Chloroquine, however there are serious adverse reactions associated with use that have limited its distribution. It is thought to have a longer therapeutic lifetime than other combinations and may be a more cost-effective option to introduce in areas where resistance is likely to develop. This is unlikely to occur until more information regarding its safety has been obtained. The recommended dose is 10 mg/kg of Amodiaquine per day for 3 days with a single standard dose of SP.

- Sulfadoxine-Pyrimethamine plus Mefloquine–This is produced as a single dose pill and has obvious advantages over some of the more complex regimen. This combination of drugs has very different pharmokinetic properties with no synergistic action. This characteristic is potentially thought to delay the development of resistance; however it is counteracted by the very long half life of Mefloquine which could exert a high selection pressure in areas where intensive malaria transmission occurs. It is also an expensive combination and has not been recommended for use since 1990 due to Mefloquine resistance.

- Tetracycline or Doxycycline plus Quinine–Despite the increasing levels of resistance to Quinine this combination have proven to be particularly efficacious. The longer half-life of the Tetracycline component ensures a high cure rate. Problems with this regimen include the relatively complicated drug regimen, where Quinine must be taken every 8 hours for 7 days. Additionally, there are severe side effects to both drugs (Cinchonism in Quinine) and Tetracyclines are contraindicated in children and pregnant women. For these reasons this combination is not recommended as first-line therapy but can be used for non-responders who are able to take oral medication. Quinine should be taken in 10 mg/kg doses every 8 hours and Tetracycline in 4 mg/kg doses every 6 hours for 7 days.

2.10.26 Experimental Drugs

In 1996, Geoff McFadden became aware of the work of the biologist Ian Wilson, who had discovered that the plasmodia responsible for causing malaria retained parts of chloroplast, an organelle usually found in plants, complete with their own functioning genomes. This led Professor McFadden to the realisation that herbicides may be useful lead compounds for the development of drugs against malaria (Khöler and Sabine, 1997). These "apicoplasts" are thought to have originated through the endosymbiosis of algae (Khöler and Sabine, 1997) and play a crucial role in fatty acid bio-synthesis in plasmodia (Gardner and Malcom, 1998). To date, 466 proteins have been found to be produced by apicoplast (Foth, 2003) and these are now being looked at as possible targets for novel anti-malarial drugs.

2.11 PlASMODIUM BERGHEI BERGHEI

Plasmodium berghei was probably first described in 1946 by Vincke in blood films of the stomach contents of *Anopheles dureni.* In 1948, it was subsequently found in blood films of *Grammomys surdaster* (thicket rat) collected in Kisanga, Katanga Congo; blood was passaged to white rats and became the K173 strain made widely available by the Institute for Tropical Medicine in Antwerp. A trio of fascinating papers describing the discovery and early analysis of the biology of *P. berghei* (Amino *et al.,* 2005) give an early indication of the natural and catholic host range of this parasite, a property that possibly underlies the successful transmission of the parasite to a variety of laboratory hosts. Although the natural vector of *P. berghei* is *A. dureni*, early laboratory studies (Amino *et al.,* 2005; Franke-Fayard *et al.,* 2006) showed that a wide range of colonized mosquitoes

would successfully transmit the parasite. Plasmodium berghei is a unicellular parasite (protozoan) and is one of the many species of malaria parasites that infect mammals other than humans. *P. berghei* is one of the four *Plasmodium* species that have been described in African murine rodents. It is found in the forests of Central Africa, where its natural cyclic hosts are the thicket rat (*Grammomys surdaster*) and the mosquito (*Anopheles dureni*). Other Plasmodium species in African murine rodents are; Plasmodium *chabaudi*, Plasmodium *vinckei* and Plasmodium *yoelii.* These are not of direct practical concern to man or his domestic animals. The interest of these parasites is that they are practical model organisms in the laboratory for the experimental study of human malaria.

2.11.1 Research

Rodent malaria parasites are used in many research institutes for studies aiming at the development of new drugs or a vaccine against malaria. In the laboratory the natural hosts have been replaced by a number of commercially available laboratory mouse strains, and the mosquito *Anopheles stephensi*, which is comparatively easily reared and maintained under defined laboratory conditions (Franke-Fayard *et al.,* 2006). Rodent parasites are recognised as valuable model organisms for the investigation of human malaria because they are similar in most essential aspects of morphology, physiology and life cycle and the manipulation of the complete lifecycle of these parasites, including mosquito infections, is simple and safe (Amino *et al.,* 2005). Like all malaria parasites of mammals, including the four human malaria parasites, *Anopheles mosquitoes transmit P. berghei* and it infects the liver after being injected into the bloodstream by a bite of an infected female mosquito. After a short period (a few days) of development and

multiplication, these parasites leave the liver and invade erythrocytes (red blood cells). The multiplication of the parasite in the blood causes the pathology such as anaemia and damage of essential organs of the host such as lungs, liver and spleen. *P. berghei* infections may also affect the brain and can be the cause of cerebral complications in laboratory mice. These symptoms are to a certain degree comparable to symptoms of cerebral malaria in patients infected with the human malaria parasite *Plasmodium falciparum*. The complete genome of *P. berghei* has been sequenced and it shows a high similarity, both in structure and gene content, with the genome of the human malaria parasite *Plasmodium falciparum* (Franke-Fayard *et al.*, 2006).

2.12 NUTRITIONAL MODULATION OF MALARIA MORBIDITY AND MORTALITY

Before 1950, it was widely accepted that malnutrition led to greater susceptibility to malaria. The Indian Famine Commission in 1898 reported that malaria was more frequent and fatal in persons with poor diets (Gill, 2000), and historical accounts from the late nineteenth and early twentieth centuries indicated that famines in north India and Sri Lanka tended to precipitate malaria epidemics (Christopher, 1911; Gill, 2000). Reports from 1920 to 1940 in Corsica (Roubaud, 1921), Algeria (Roubaud, 1921), Turkey (Eckstein, 1943), and Ghana (Williams, 1940) stated that malaria was more frequent and severe among those who were undernourished. In 1954, Garnham a prominent malariologist of the time concluded that the clinical effects and mortality from malaria were more severe in malnourished children. There was, however, anecdotal evidence to the contrary, such as the failed 1897 attempt by an Italian industrialist to protect farmers

in the malaria-infested Pontine Marshes with generous provisions of food and quinine (Hackett, 1937). Other reports found no association between nutritional status and malaria morbidity (James, 1926), and some suggested that post-famine increases in food consumption exacerbated malaria (Edington, 1954). Unfortunately, most of these reports were based on limited clinical and epidemiologic observations or even anecdotal information. Little, if any, quantitative data or methodologic information was published to substantiate the conclusions. By the early 1950s, scientists had begun to improve quantification of interactions between nutrition and malaria. Three studies from Ghana and Nigeria published between 1954 and 1971 (Edington, 1954; Hendrickse *et al.*, 1971) were particularly influential and strongly promoted the notion that malnutrition was in fact protective for malaria. This idea was reinforced by a series of studies by Murray *et al.*, (Murray *et al.*, 1978a) from 1975 to 1980 on refeeding and malaria in famine victims in Niger and Sudan. Animal studies appeared to support these reported malaria suppressive effects of a poor diet, leading to the perception that malnourished children are less susceptible to malaria infection, morbidity, and mortality (Mc Gregor, 1982; Latham, 1982). Careful review of these studies plus more recent epidemiologic data strongly indicate that the relationship between malaria and nutritional status is more complex and that general malnutrition is in fact an important risk factor for increased malaria morbidity and mortality. Additional field studies and results from experimental malaria studies indicate that multiple specific nutrients, such as vitamin A, zinc, iron, thiamine, riboflavin, and vitamin E, strongly affect the course of malaria infection and pathology.

2.12.1 Protein Energy Malnutrition (PEM)

Several studies examined the association between malnutrition, usually PEM, and malaria morbidity and mortality. The early studies generally assessed nutritional status by subjective clinical evaluation, while later studies utilized anthropometric indicators and reference standards to determine poor nutritional status based on low weight-for-age (underweight), low height-for-age (stunting), or low weight-for-height (wasting). Some studies were of clinic outpatients; others were hospital admissions or community-based cross-sectional studies. Most were case-control studies, but some were longitudinal surveillance of cohorts. In most, the malaria-related outcomes pertained to prevalence of infection or frequency of clinical attacks of *Plasmodium falciparum,* although some had data for *Plasmodium vivax.* Multiple experimental animal studies of PEM or protein deficiency were also conducted: These ranged from the initial avian malaria studies with *Plasmodium lophurae* and *Plasmodium gallinaceum* to the primate models of *Plasmodium knowlesi* and *Plasmodium cynomolgi* and rat and murine models using primarily *Plasmodium berghei* but also *Plasmodium yoelii* and *Plasmodium vinckei.* The interpretation of this body of data has generally been that PEM or protein deficiency protects the host against malaria morbidity and mortality (Mc Gregor, 1982; Latham, 1982). However, a reappraisal of the data presented herein indicates that the effect of PEM on the host is more complex and in many cases predisposes to excess morbidity and mortality.

2.12.2 Clinically Based Studies

Among the first study of PEM and malaria was a large-scale clinic-based study in Uganda (Gongora and Mc Fie, 1959), which concluded there was no association between

nutritional status and malaria mortality. This study was less than ideal as nutritional status was based on qualitative and subjective indicators (for example, thin or pale hair or being "very" thin), malaria diagnosis was based only on spleen enlargement, and mortality risk was estimated by the presence or absence of sibling mortality. Another smaller clinic-based study in India reported progressively increasing parasite density with improved nutritional status (Ahmad *et al.,* 1985), suggesting that malnutrition was protective. However, two additional studies, one in Brazil (Periera *et al.,* 1995) and one in Soviet army personnel (Liashenko, 1997), reported greater frequency or more severe malaria in those who were malnourished.

2.12.3 Early Studies of Hospital Admissions for Severe Malaria

Early hospital-based studies strongly influenced current perceptions of malaria and malnutrition. In 1954, an autopsy report by Edington (Edington, 1954) indicated that 4 Ghanian children who died of cerebral malaria were well nourished. Other accounts from South Africa in 1960 (Walt, 1960) reported that malaria was rare in malnourished children. This was followed in 1967 by another qualitative report from Edington (1967), who noted that children dying of cerebral malaria in Nigeria were usually well nourished and that cerebral malaria was rare in children with kwashiorkor. Case-control studies from Nigeria by Hendrickse and colleagues in 1967 (Hendrickse, 1967) and 1971 (Hendrickse *et al.,* 1971) concluded that children with malaria were less likely to be malnourished or have convulsions.

Hendrickse, (1967) also reaffirmed the apparent protection due to kwashiorkor. A subsequent autopsy report from Nigeria of 25 malnourished children indicated that only 2 had died of malaria (Purtilo and Conner, 1975). Although these reports appeared

convincing of a protective effect of malnutrition on malaria, several characteristics of the studies weaken the conclusions. Most importantly, the study populations comprised clinic cases or malnourished children and comparisons were of those with or without malaria. In the absence of healthy community controls, one could only conclude that malaria is less exacerbated by malnutrition than other conditions. The overall prevalence of malnutrition among malaria cases in these studies was remarkably high, suggesting possible synergy with malnutrition. A more informative analysis would have included the relationship between the degree of malnutrition among malaria cases and the risk of malaria mortality. Unfortunately, such analyses were not done, partly because malnutrition was categorized by relatively nonstandardized qualitative descriptors rather than by quantitative assessment. In some studies, incomplete analyses of the data were made. For example, Hendrickse reported decreased risk of convulsions in malnourished malaria patients, but the same decline in convulsion risk was also observed for malnourished nonmalaria patients. There was also a lack of information on socioeconomic status or residence of the cases. Well-nourished cases may tend to come from urban areas and have less acquired immunity, whereas, malnourished cases could come from out-lying areas of higher transmission leading to greater immunity.

Indeed, Edington, (1967) reported that the children with cerebral malaria tended to have less hookworm, an observation possibly related to socioeconomic status. Lastly, the conclusions based on patients with kwashiorkor may not be generalizable to overall malnutrition because aflatoxins, a causative agent of kwashiorkor, are toxic to malaria parasites *in vitro* and *in vivo* (Hendrickse, 1997; Hendrickse *et al.,* 1986).

2.12.4 Recent Studies of Hospital Admissions for Severe Malaria

In the last 10 years, studies have been completed on the relationship between malnutrition and malaria. These larger studies more carefully documented nutritional status by reference standards (that is, height, weight, and age) and evaluated malnutrition as a risk factor for malaria mortality among hospital admissions. Five studies conducted in Madagascar (Razanamparany *et al.,* 1993), Nigeria (Olumese *et al.,* 1997), Chad (Renaudin, 1997), The Gambia (Man *et al.,* 1998), and Senegal (Faye *et al.,* 1998) indicated that malnourished patients are 1.3–3.5 times more likely to die or have permanent neurologic sequelae than normally nourished malaria patients. In addition, in the study from The Gambia, malaria patients typically weighed 350 g less than healthy control children (Man *et al.,* 1998). In all studies, as seen by Hendrickse, malnourished hospital patients were less likely to have malaria than other infections, suggesting that although malaria may be exacerbated by malnutrition, other diseases may be more adversely affected. Indeed, additional analyses in The Gambia study (Man *et al.,* 1998) confirm the greater impact of malnutrition on risk of death for diarrhea and pneumonia. In contrast to these reports, one study of 60 hospital patients in India reported that parasitemia tended to increase with improving nutritional status; however, no data on clinical outcomes were presented (Goyal, 1991).

2.12.5 Cross-sectional Studies of Malariometric Indicators

Several cross-sectional surveys also favor a synergistic relationship between malnutrition and malaria. Studies in Malawi (Burgess *et al.,* 1975), Zambia (Wenlock, 1979), Papua New Guinea (Sharp and Harvey, 1980), Sudan (El Samani *et al.,* 1987), Tanzania (Mbago 1991), Chad (Renaudin and Lombart, 1994), and Zaire (Tshikuka *et al.,* 1997)

indicate greater risk for infection (Burgess *et al.,* 1975; Mbago 1991; Tshikuka *et al.,* 1997), malaria illness (El Samani *et al.,* 1987), or spleen enlargement (Sharp and Harvey, 1980) among malnourished children. A study in Colombia found that malnourished children had lower anti-malaria antibody levels (Dominguez-Vazquez and Alzate-Sanclez, 1990). This could be interpreted as a synergistic effect if malnutrition suppresses antibody response to malaria or possibly antagonistic if malnutrition protects against infection such that antibodies are not produced. In Tanzania, there was no effect of nutritional status on anti-parasite antibody levels (Carswell *et al.,* 1981). A study in Burkina Faso found no association between parasite prevalence and nutritional status (Monjour *et al.,* 1982).

2.12.6 Longitudinal Cohort Studies and Effects of Nutrition on Drug Resistant Malaria

Longitudinal cohort studies in Tanzania (Tanner *et al.,* 1987), Vanuatu (Williams *et al.,* 1997) and Congo (Tonglet *et al.,* 1999) indicate that malnutrition predisposes children to malaria illness. Another report from The Gambia (Snow *et al.,* 1991; Snow *et al.,* 1997) indicates little effect of malnutrition on malaria attacks, while one report from Papua New Guinea suggests that stunted children may be more resistant to malaria attacks (Genton *et al.,* 1998). However, this protection was not seen in underweight children. Of interest, the stunted children also exhibited increased immune responses to malaria antigens, whereas the wasted children had suppressed responses. One additional semi longitudinal study (multiple cross-sectional cohort surveys) in Zaire (Van den Broeck *et al.,* 1993) found no association between nutritional status and mortality. Because malaria was the primary cause of death in that study, the authors inferred there was no association

between nutrition and malaria mortality. However, analysis of the association between nutritional status and malaria deaths, as determined by verbal autopsy, were not presented. Further evidence supporting an exacerbative role of malnutrition on malaria can be seen in several drug-resistance studies. Malnourished Rwandan refugees had slower parasite clearance, higher parasite titers at presentation, and more severe drug resistance (Wolday *et al.,* 1995). Likewise, in the Solomon Islands, malnourished children were 3.6 times more likely to have drug-resistant malaria (Hers *et al.,* 1996; Hers *et al.,* 1997).

2.12.7 Studies during Famine Relief

The 1945 report of the Bengal Famine Commission noted that refeeding tended to precipitate malaria disease in persons with low-grade infections (Edington, 1954). The Murray family subsequently examined the presence of malaria in famine victims during nutritional rehabilitation in a series of studies. During the Sahelian famine in Niger when victims were hospitalized for refeeding, many patients developed *P. falciparum* malaria within a few days (Murray *et al.,* 1976), often resulting in cerebral pathology. Because there was no malaria transmission at the hospital, it was believed that feeding provided essential nutrients for sequestered parasites, leading to recrudescent infection (Murray *et al.,* 1975; Murray *et al.,* 1976). In another study, famine victims were given either grain or milk for rehabilitation and it was observed that those given grain were more likely to experience recrudescent cerebral malaria (Murray *et al.,* 1978a). These studies suggest that both quality and quantity of the diet is an important determinant of malaria morbidity. The Murrays concluded that the interaction between poor diet and malaria is part of an ecologic balance between humans and malaria, which they interpreted as a

beneficial aspect of malnutrition. Of note, however, the eco-biology of nutritional rehabilitation of famine-stricken persons is likely to be distinct from that of the more common condition of chronic malnutrition (Snow *et al.,* 1993).

2.12.8 Studies of PEM in Animals

A variety of animal experiments have also contributed to the idea that PEM reduces malaria morbidity. Early work showed that monkeys maintained on a low-protein diet had lower parasitemia (Geiman and McKee, 1948; Tatke and Bazaz-Malik, 1989). However, the animals were either unable to clear the infection, resulting in multiple recrudescences (Ray, 1957), or parasitemia appeared earlier and lasted longer (Bazaz-Malik and Tatke, 1982). Immune responses were also suppressed (Bazaz-Malik and Tatke, 1982). However, for monkeys with cerebral malaria, protein-deprived animals had fewer parasitized erythrocytes in the cerebral capillaries and did not develop the disrupted endothelium seen in normally fed monkeys. Still, cerebral and pulmonary edema was present in all animals regardless of dietary regimen (Tatke and Malik, 1990). These primate experiments were complemented by a variety of informative data from studies of rodent malaria. A comprehensive series of investigations by Ramakrishnan and colleagues (Ramakrishnan *et al.,* 1953; Platt *et al.,* 1960) in the early 1950s indicated that malaria parasitemia was less severe in protein-deprived rats and that survival was enhanced. They also showed that methionine and *p*-aminobenzoic acid promoted the infection in starving rats (Platt *et al.,* 1960). Of importance, it was clear that protein-deprived animals were unable to clear the infection (Ramakrishnan, 1954) and that protein restriction in young rats exacerbated malaria parasitemia and mortality (Ramakrishnan, 1954). Moreover, parasite densities were higher and more lethal during

relapses in protein-deprived animals (Ramakrishnan, 1954). Lastly, starved animals experienced strong relapse infections when food was given (Ramakrishnan, 1953). Additional studies by Edirisinghe and colleagues (Edirisinghe *et al.,* 1981a; Edirisinghe *et al.,* 1981b; Fern, 1984) documented that acute and chronic protein deprivation depressed peak parasitemia more than 75% and prevented death.

However, as shown in previous work, the animals were unable to clear the infection (Edirisinge *et al.,* 1982) and antibodies preventing parasite growth did not adequately develop. Elegant work by Fern *et al.,* 1984 demonstrated that readdition of threonine to low-protein diet restored susceptibility and that this effect was enhanced by valine, isoleucine, and methionine. However, phenylalanine, tyrosine, lysine, histidine, and tryptophan did not appear to have this promoting effect. Of interest, the amino acids that had the greatest modulatory effect on the infection tended to be those least abundant in hemoglobin, the main amino acid source for the parasite, indicating the potential importance of non-hemoglobin amino acids in parasite survival. Subsequent studies in rats and mice confirmed that low-protein diets suppressed parasitemia (Bhatia *et al.,* 1983; Van Doorne *et al.,* 1998) and inhibited cell mediated immunity (Bhatia *et al.,* 1983) and that effects were reversible by addition of para-amino benzoic acid (Bhatia and Vinayak, 1991). Effects on mortality were less consistent. In some cases, low-protein diets suppressed parasitemia but mortality was higher, albeit delayed (Bhatia *et al.,* 1983; Van Doorne *et al.,* 1998). Addition of threonine and methionine to the low-protein diet decreased mortality (Van Doorne *et al.,* 1998), although either alone had no effect when added to the deficient diet. Others observed no effect on mortality in moderately malnourished mice but noted increased deaths in severely malnourished animals

(Fagbenro-Beyioku and Oyerinde, 1990). Protein-deficient diets were, however, consistently protective for rodent cerebral malaria (Bakker *et al.,* 1992; Van Doorne *et al.,* 1998; Hunt *et al.,* 1990).

2.12.9 Synthesis of Data Regarding the Effect of PEM on Malaria

The considerable data from humans and animals, although complex, provide ample evidence to draw some conclusions regarding the interaction between protein-calorie malnutrition and malaria. Although it is frequently mentioned that PEM is protective for malaria (Mc Gregor 1982; Latham, 1982), more recent data and careful reexamination of the results of human and animal studies indicate that malnutrition is associated with increased infection rates, clinical malaria attacks, and considerably higher likelihood of malaria mortality in humans. The hospital-based studies that suggested a protective effect of malnutrition are inconclusive due to the many methodologic and design issues discussed above. Similarly, the animal-based data that are often cited as supportive evidence that malnutrition is protective are less clear when carefully examined. For instance, although parasitemia tended to be lower in poorly fed animals, they were unable to clear the infection, and immune responses to the parasite were suppressed. This led to more chronic infections and more severe relapses. Also, malnutrition was particularly deleterious in younger animals (Ramakrishnan *et al.,* 1953), an observation consistent with some age-related data from human studies (Tonglet *et al.,* 1999). The multiple studies in rodents indicating that certain amino acids and PABA have distinct parasite-promoting effects are important, but not necessarily incompatible with the apparently deleterious effects of general malnutrition on malaria. Additional data concerning the impact of selected human diets richer or poorer in certain amino acids are needed. It is

notable that for cerebral malaria in animals, poor diets are consistently protective, whereas data from humans clearly indicate that malnourished children are more likely to die of cerebral malaria. This discrepancy may be rooted in differences in the etiology of cerebral pathology in animals and humans. It should also be appreciated that certain age-dependent relationships may underlie some inconsistencies linking malnutrition and malaria in humans. Severe clinical malaria is more frequent in young children (<3 years) and tends toward severe malaria anemia. In contrast, for older children (>13 years), cerebral malaria is more frequently encountered. Likewise, various indicators of nutritional status have age-dependent distributions. Wasting (low weight for height) is seen more frequently in young children than in older children for whom stunting (low height for age) is generally more prevalent. Thus, if such age-based differences were not taken into account, a clinic-based study of severe malaria cases could erroneously conclude that wasting was protective for cerebral malaria. Famine or starvation presents a special case. It is consistently observed in humans and animals that refeeding an infected starved host reactivate low-grade infections. This may suggest that increases in parasite replication following refeeding are sufficiently rapid to temporarily outpace development of adequate immunity. Given that famines caused by political and/or meteorologic causes are not unusual, special attention should be given to malaria during nutritional rehabilitation of famine victims. This should include malaria prophylaxis and careful surveillance. In addition, it may be possible to develop refeeding regimens that minimize parasite recrudescence and hasten development of immunity.

Given that PEM appears to exacerbate malaria and that PEM is frequently accompanied by deficiencies in other nutrients, it is conceivable that multiple specific nutrients may

influence malaria infection and pathology. Several studies have examined the effects of deficiencies of multiple minerals and vitamins on malaria in humans and experimental animal models.

2.12.10 Vitamin A

Vitamin A is essential for normal immune function (Semba, 1998), and several studies suggest it could play a role in potentiating resistance to malaria. Early work in vitamin A deficient ducks indicated that vitamin A deficiency exacerbated malaria (Roos *et al.*, 1946). Further studies in vitamin A–deficient rats and mice showed an increased susceptibility to malaria that was readily reversed by supplementation (Krishnan *et al.*, 1976; Stolzfus *et al.,* 1989). More recently, a genetic locus, which includes cellular retinol binding protein 1, was shown to modulate malaria mortality and parasitemia in mice (Foote *et al.,* 1997). *In vitro,* addition of free retinol to *P. falciparum* cultures reduced parasite replication in one study (Davis *et al.,* 1998) but not in another (Samba *et al.,* 1992). In humans, cross-sectional studies in preschool children and adults have reported inverse associations between plasma vitamin A levels and *P. falciparum* parasitemia (Thurnham and Singkamani, 1991; Friis *et al.,* 1997). In addition, low plasma vitamin A levels were associated with ocular pathology during severe malaria (Lewallen *et al.,* 1998). However, these associations may have been due to an acute-phase response (Thurnham and Singkamani 1991; Filteau *et al.,* 1993; Friis *et al.,* 1997). Still, selective depletion of plasma borne pro-vitamin A carotenoids during acute malaria attacks has been described (Thurnham and Singkamani, 1991), suggesting higher utilization of vitamin A during clinical malaria episodes. The vitamin A–malaria link is further strengthened by a study in which low baseline vitamin A status was associated with

increased risk of parasitemia, although confounding by age could not be excluded (Sturchler *et al.,* 1987). In contrast, a sub-study of a vitamin A trial in pre-school children in Ghana found no statistically significant effects of vitamin A on *P. falciparum* morbidity or mortality (Binka *et al.,* 1995). However, no longitudinal surveillance of slide-confirmed malaria morbidity was done (Shankar, 1995).

The most definitive study to date of the effects of vitamin A on malaria was recently completed in Papua New Guinea (Shanker *et al.,* 1999). In this double-blind placebo-controlled trial, vitamin A supplementation reduced the frequency of *P. falciparum* episodes by 30% (95% confidence interval) among preschool children. At the end of the study, geometric mean parasite density was 36% lower in the vitamin A group than in the placebo group, and the proportion of children with spleen enlargement was reduced by 11%, although neither difference was significant. It was clear that children aged 12–36 months benefited most: They had 35% (95% Confidence interval) fewer malaria attacks, 26% fewer enlarged spleens, and a 68% reduction in parasite density. Overall, given the considerable and relatively consistent data in humans and animals, it appears that vitamin A deficiency exacerbates malaria illness.

2.12.11 Zinc

Zinc is required for normal immune function (Shanker and Prasad, 1998) and reduces the incidence of diarrhea and pneumonia (Black, 1998). Indeed, zinc is essential for a variety of lymphocyte functions implicated in resistance to malaria, including production of IgG, interferon-γ, and tumor necrosis factor-α and microbicidal activity of macrophages (Shankar and Prasad, 1998). Cross-sectional studies among school-age children in Papua New Guinea (Shanker and Prasad, 1998) and in pregnant women in Malawi (Gibson and

Huddle, 1998) revealed inverse associations between zinc status and *P. falciparum* parasitemia. In addition, a placebo-controlled trial of zinc supplementation in preschool children in The Gambia documented a 30% reduction in health center attendance due to *P. falciparum* (Bates *et al.,* 1993), although this was not statistically significant. In mice, zinc supplements decreased markers of oxidative stress during infection with *P. berghei* (Arif *et al.,* 1987). Additional murine studies indicated that moderate zinc deficiency resulted in 40% mortality from the normally nonlethal rodent malaria *P. yoelii* 17X-NL (Shanker *et al.,* 1995). A recently completed placebo-controlled trial of zinc supplementation of preschool children in Papua New Guinea provides additional evidence for the role of zinc in malaria (Shanker *et al.,* 2000; Augustin *et al.,* 2008). In this study, zinc supplementation reduced the frequency of health center attendance due to *P. falciparum* malaria by 38%. Moreover, a 69% reduction was observed for malaria episodes accompanied by high levels of parasitemia (>100,000 parasites/mL), suggesting that zinc may preferentially protect against more severe malaria episodes. Although these effects are encouraging, additional trials are needed to document the geographic regions and conditions of malaria transmission in which zinc might be effective and to determine the potential effect on severe malaria. As for vitamin A, concordance between animal and human studies strongly suggests a significant exacerbative effect of even mild zinc deficiency on malaria.

2.14.12 Iron

Iron deficiency affects nearly 2 billion people worldwide and results in over 500 million cases of anemia (DeMaeyer *et al.,* 1989). Additional sequelae include poor neurologic development, lower work capacity, low birth weight, and increased maternal and infant

mortality (Walter 1992; Fairbanks, 1988). The burden of both iron deficiency and malaria falls primarily on preschool children and pregnant women (Gibson, 1990; Wyler, 1993), and iron supplementation of these groups is the primary means of preventing and treating anemia. Multiple studies have attempted to evaluate the benefit of iron supplementation in malaria-endemic areas (Murray *et al.,* 1978b; Oppenheimer *et al.,* 1986; Masawe *et al.,* 1974). Some studies reported that iron supplementation increased the risk of developing or reactivating malarial illness (Murray *et al.,* 1978a; Oppenheimer *et al.,* 1986; Smith *et al.,* 1989; Stolzfus *et al.,* 2000), while others reported no significant adverse effects (Harvey *et al.,* 1989; Fleming *et al.,* 1986; Harvey *et al.,* 1985). Experiments with rodent malaria have also yielded conflicting results (Harvey *et al.,* 1985; Goma *et al.,* 1996). To clarify this issue, a systematic review and meta-analysis of placebo-controlled trials of iron supplementation in humans was recently completed (Shanker *et al.,* 2000) data from 13 trials involving 5230 subjects, were pooled to obtain composite effects of iron supplements on malaria attack rates, parasite prevalence, parasite density, prevalence of enlarged spleens, hemoglobin levels, and anemia (Murray *et al.,* 1978b; Oppenheimer *et al.,* 1986; Smith *et al.,* 1989; Lawless *et al.,* 1994; Harvey *et al.,* 1989; Fleming *et al.,* 1986; Bates *et al.,* 1987). Iron supplementation resulted in a nonsignificant 9% (relative risk [RR] p1.09; 95% CI) increase in the risk of a malaria attack. End-of-trial cross-sectional data indicated a 17% greater risk of infection in those given iron. For trials providing baseline data, the absolute increase in infection rates was 5.7%, which was not significant. Iron supplements were also associated with a nonsignificant 12% (RRp1.12; 95% CI, 0.99–1.26; *n*p6) increase in risk of spleen enlargement. Qualitative assessment of parasite density suggested a tendency toward higher levels in persons receiving iron. A

subanalysis of trials that implemented iron supplementation regimens in accord with international dosing recommendations revealed no evidence for increased infection or morbidity. Overall, hemoglobin levels improved by 1.2 g/dL (95% CI, 1.2–1.3; *n*p11) following iron supplementation, and the risk of anemia was reduced by 50% (RRp0.50; 95% CI, 0.45–0.54; *n*p4).

The data indicate that prophylactic iron supplementation was associated with increases in certain malariometric indices. However, these tended to be relatively small effects. In contrast, improvements in hematologic status following iron supplementation were substantial and with clear public health benefit. It should be noted that the meta-analysis was unable to address the effects of oral iron supplements on severe malaria. In addition, although some evidence suggested greater adverse effects for persons residing in areas with moderate levels of malaria transmission, the specific effects of iron supplementation in certain settings or populations could not be determined. Overall, the meta-analysis suggests that iron supplementation programs in accord with international dosing guidelines should be advocated for iron-deficient populations residing in malaria-endemic areas. Ideally, malaria surveillance and control activities should be continued in such areas or integrated into those programs.

2.12.13 Folate

Folate is a crucial nutrient for cellular growth and is considered important for erythrocyte production. For these reasons and for its role in preventing neural tube defects, folate supplementation is part of prenatal care programs in most countries. Given that folate metabolism of the parasite is also a target for several antimalarial drugs, the interactions between host folate status and supplementation in malaria-endemic areas is of interest.

Folate deficiency was first seen to enhance susceptibility to avian malaria (Seeler and Ott, 1945). In contrast, primate malaria species were unable to survive in severely folate-deficient rhesus monkeys (Das *et al.,* 1992). This protective effect of folate deficiency differs from observations in humans. Low infection rates were reported in pregnant women who were consuming a diet high in folates (Hamilton *et al.,* 2000), and greater infection rates were also reported in those suffering from megaloblastic anemia (Fleming and Werblinska, 1982). However, malaria itself may induce folate deficiency (Fleming and Werblinska 1982; Fleming *et al.,* 1968), and there is some evidence of improper red cell utilization of folates during malaria infection (Brabin *et al.,* 1986). A trial of prophylactic folate supplementation in preschool children in The Gambia (Fuller *et al.,* 1988) showed no adverse effects for malaria. Also, a trial of folate supplements in pregnant women (Gail and Herms, 1969) showed no adverse effect on parasitemia, even though reticulocyte counts increased. As mentioned, there has been concern over the possible interference of folate supplements with antifolate antimalarial drugs (for example, pyrimethamine). Several studies have attempted to resolve this issue. Two separate trials reported that development of *P. falciparum in vivo* was not affected by folate supplements given with pyrimethamine (Hurley 1959, Tong *et al.,* 1970). In one study, the folates were even given in doses sufficient to reverse the side effects of high-dose pyrimethamine (Tong *et al.,* 1970, Topley, 1975). However, a recent trial found greater treatment failure for pyrimethamine when folate supplements were given (Van Hensbroek *et al.,* 1995). Overall, these data suggest that folate deficiency may exacerbate malaria morbidity. Although the routine use of folate supplements in malarious areas has been advocated to prevent or treat anemia (Fleming and Werblinska, 1982), additional

studies may be warranted despite strong evidence suggesting no adverse effects of folate supplements on drug efficacy.

2.12.14 Riboflavin

Riboflavin (vitamin B_2) has an influence on malaria morbidity. The relationship appears to be one of antagonism such that deficiency confers a degree of protection. In Papua New Guinea, riboflavin-deficient infants are less likely to be infected with malaria (Oppenheimer *et al.,* 1983; Thurnham *et al.,* 1983). Similar observations were made in India (Dutta *et al.,* 1985) and The Gambia (Bates *et al.,* 1986). In India, malaria parasitemia was less severe in riboflavin-deficient persons (Das *et al.,* 1988), although the course of clinical illness appeared worse. Because riboflavin is an essential factor for glutathione peroxidase, an antioxidative enzyme, it has been proposed that deficiency promotes an oxidative environment that leads to destruction of the parasite. Indeed, lipid peroxidation was increased in riboflavin-deficient children with malaria infection (Das *et al.,* 1990), and reduced glutathione peroxidase activity was observed in red cells from riboflavin-deficient infected persons (Barraviera *et al.,* 1988). Reduced glutathione peroxidase activity persists in some populations in malarious areas despite adequate riboflavin intake (Anderson *et al.,* 1993), suggesting that isoforms with reduced activity confer resistance to malaria. There is evidence for other mechanisms as well. *P. falciparum*– infected erythrocytes have an increased requirement for riboflavin (Dutta, 1991). Moreover, riboflavin analogues inhibit the growth of parasites *in vitro* and *in vivo* in experimental murine malaria (Cowden and Clark, 1987). In some cases these activities also correlated with reduced activity of glutathione reductase (Cowden and Clark, 1987). Riboflavin-deficient rats are also more resistant to malaria (Kaikai and Thurnham, 1983).

However, the studies in rats also suggested that the protective effect was not due to increased susceptibility of erythrocytes to oxidative damage, hemolysis, or erythropoiesis (Dutta *et al.,* 1988). In contrast to the rodent studies, riboflavin-deficient chicks were more susceptible to *P. gallinaceum* (Seeler and Ott, 1944). Despite the human and animal-based evidence that riboflavin deficiency is protective, recent *in vitro* experiments suggest that high doses of riboflavin suppress parasite growth by preventing the oxidation of hemoglobin needed for digestion by the parasite (Akompong *et al.*, 2000). Thus, although low riboflavin status may be protective, high-dose riboflavin therapy may prove beneficial for malaria patients. Paradoxically, the protective and exacerbative functions would be based on different sites of action for the same antioxidant properties of riboflavin. This again emphasizes the complex pathways through which nutrients influence malaria parasites and host morbidity.

2.12.15 Thiamine and Pyridoxine

A recent report from Thailand suggests that deficiency of thiamine (vitamin B_1) is associated with greater risk of severe malaria and with simple clinical malaria (Krishna *et al.,* 1999). This is consistent with early experiments in which thiamine-deficient ducks were more susceptible to avian malaria (Ramo Rao and Sirsi, 1956). There are also reports that acute cerebral ataxia following malaria can be treated with thiamine (Adamolekun and Eniola, 1993), suggesting that disrupted thiamine metabolism may be a pathologic feature of malaria. The role of pyridoxine (vitamin B_6) is less clear. In the sole experimental study of rats infected with *P. berghei,* pyridoxine deficiency attenuated the course of parasitemia (Ramakrishnan, 1954a; Ramakrishnan, 1954b; Ramakrishnan, 1954c; Ramakrishnan, 1954d). Of interest, erythrocyte pyridoxine metabolism is down

regulated in humans with β-thalassemia (Anderson *et al.,* 1979), possibly indicating a protective effect of B_6 deficiency. Additional studies are needed to clarify the role of B vitamins in malaria.

2.12.16 Vitamin E and Other Antioxidants

Several reports indicate that deficiencies of vitamin E and other antioxidants tend to protect against malaria infection (Levander and Ager, 1993). As discussed above, the explanation most frequently given is that the absence of antioxidants makes the parasite more vulnerable to damage by oxygen radicals produced by the immune system. In humans, it was initially proposed that the exacerbative effects on cerebral malaria following refeeding of famine victims with grain was due to the vitamin E content of the grain (Dorsey, 2001)—a notion supported by the absence of cerebral malaria among famine victims given milk, which normally lacks vitamin E (Kretschmar, 1966). Other reports suggest that persons with lower plasma vitamin E levels recover more quickly from clinical malaria (Eaton *et al.,* 1976; Davis *et al.,* 1994). The exacerbative effect of vitamin E on experimental animal malaria was first described in 1957 by Godfrey, who demonstrated that the antimalarial effects of cod liver oil in mice were reversible by giving vitamin E. Multiple studies in rodent systems confirm a protective effect of vitamin E deficiency (Eckman *et al.,* 1976; Taylor *et al.,* 1997; Levander *et al.,* 1990; Levander *et al.,* 1991) and the ability of vitamin E to abrogate the protective effects of pro-oxidant compounds, such as peroxidizable fatty acids, on malaria (Levander *et al.,* 1989a; Levander *et al.,* 1989b; Taylor *et al.,* 1997). Of interest, vitamin E deficiency is also protective against murine cerebral malaria (Levander *et al.,* 1997) in which oxidative damage plays a significant pathologic role. Studies of avian malaria in the duck also

showed a protective effect of vitamin E deficiency (Yarrington *et al.,* 1973). Other reports suggest that persons with lower plasma vitamin E levels recover more quickly from clinical malaria (Akpotuzor, 2007). A more recent report revealed that intraperitoneal administration of vitamin E negatively impacted on the course of *P. berghei* development in mice (Ibrahim *et al.,* 2012) and that vitamin C deficiency in a L-gulono-γ-lactone oxidase gene knockout mice might not affect the development of malaria parasite in mice (Herbas and Suzuki, 2010). No human studies have addressed the role of selenium in malaria. A few animal studies indicate that selenium has little role in modulating rodent malaria (Levander 1992; Levander *et al.,* 1989). However, selenium-deficient ducks were more susceptible to avian malaria (Yarrington *et al.,* 1973). Human data are clearly needed to clarify the influence of selenium on malaria. Vitamin C has also been studied in animals but there have been few human studies. In monkeys, vitamin C deficiency exacerbated malaria (McKee and Geiman, 1946); however, in mice, results have been mixed. Godfrey, 1957 reported that large doses of vitamin C, as with vitamin E, abrogate the protective effect of cod liver oil. This was not the case, however, when lower doses were used in conjunction with vitamin E–deficient mice (Levander and Ager, 1993). Vitamin C supplements did not modify the course of parasitemia in normal mice. However, given that mice can synthesize vitamin C from precursors, the conclusions from such experiments are unclear. In contrast, there are also reports that highlight the role that vitamin C can play as a catalyst for generation of malariacidal free radicals in conjunction with iron or copper (Levander and Ager, 1993; Higson *et al.,* 1988). It has therefore been suggested that vitamin C supplementation may have a role in case management of malaria (Marva *et al.,* 1992; Marva *et al.,* 1989).

Another antioxidant, b-carotene, has strong free-radical quenching properties and is nontoxic even at high doses. Indeed, b-carotene may have a protective role in murine cerebral malaria (Laniyan *et al.,* 1989) in which oxygen radical–mediated damage plays a role, and some human studies have indicated specific modulation of plasma b-carotene levels during infection (Thurnham *et al.,* 1983; Das *et al.,* 1996; Njoku *et al.,* 1995). There are also anecdotal accounts of protective effects from hypercarotenemia (Goodall *et al.,* 1986) due to high consumption of palm oil, a rich source of b-carotene. Overall, the data concerning antioxidants indicate that although antioxidant nutrients may have an exacerbative role under some conditions, it is not possible to predict the effect of a nutrient on malaria morbidity or mortality based on its antioxidant properties alone. Clearly, as seen with riboflavin and vitamin C, more detailed knowledge of the site of action of antioxidants on the parasite and host may prove useful. In addition, examination of the broader biologic functions of compounds referred to as antioxidants may provide insight. Perhaps most importantly, there is a need for data from clinical trials in humans concerning the effects of antioxidant nutrients on malaria morbidity and mortality. Given that oxygen and nitrogen radical production is strongly activated during malaria illness and has been implicated in associated pathologies (Kremsner *et al.,* 2000), there may be a role for antioxidants as adjunct for management of clinical malaria.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 *IN VIVO* STUDIES

3.1.1 Materials for the Study

Animals

Two hundred and fifty Swiss albino mice of either sex weighing 18-25g and aged 12-16 weeks were obtained from the Animal House of the Nigerian Institute of Medical Research, Yaba Lagos State.

Malaria Parasites

Plasmodial berghei: NK 65 strain and ANKA strain (chloroquine resistant) were obtained from the laboratory of Dr O.O Aina of the Malaria Research Unit, Department of Biochemistry, Nigerian Institute of Medical Research, Yaba, Lagos State Nigeria.

Chemicals and Equipments

Heparinised capillary tubes, Light Microscope (Olympus, Japan), EDTA bottles, Feeding trochars, Syringes (1ml, 5mls), Cotton wool, Microscopic slides (Olympus, China), Hand gloves, Giemsa stain (Sigma), 98% Methanol (Sigma) and Tween 80 (sigma).

Drugs

Vitamin A (Clarion Medical Pharmaceuticals, Nigeria), Vitamin E (Clarion Medical Pharmaceuticals, Nigeria), Vitamin C (Emzor Pharmaceuticals, Nigeria), Zinc gluconate (Mason Vitamins Incorporated USA), Selenium-organic (Mason Vitamins Incorporated USA), Chloroquine (Emzor Pharmaceuticals, Nigeria), Pyrimethamine (Glaxo Smith Klime, Nigeria) and Artesunate (Emzor Pharmaceuticals, Nigeria).

3.1.2 Preparation of Animals

The animals were housed in stainless steel cages with wire screen top and maintained on commercial feeds (pelleted feeds from Vital Feeds Ltd, Jos, Plateau State, Nigeria) and tap water *ad libitum* for the entire duration of the study. The mice were allowed to acclimatize for 1 week in the laboratory environment under a controlled temperature and good hygiene was maintained by constant cleaning and removal of faeces and spilled feeds from the cages daily (Obernier and Baldwin, 2007). The animals were weighed and the average weight noted before dividing into groups of 5 mice each. In the first stage of this study, a 4-day suppressive test was performed using the methods of (Peter *et al.*, 1975; Agbaje and Onabanjo, 1991; David *et al.,* 2004; Tekalign *et al.,* 2010). Certain parameters were assessed during the 4-day suppression test; these included blood % parasitemia on day 4 after infection, the base line parasitemic profile and average survival time in the control group. Chloroquine sulphate at a dose of 25mg/kg was used as a reference drug (Tekalign *et al.,* 2010). The antioxidant micronutrients were administered orally as follows; vitamin A (60 mg/kg), vitamin E (100 mg/kg), vitamin C (200 mg/kg), zinc (100 mg/kg), selenium (1mg/kg) using doses based on LD_{50} values as reported by Gerhard, (2000); Oncu *et al.*, (2002); Oreagba and Ashorobi, (2006). Tween-80 (0.2 ml) was used, as vehicle for fat-soluble vitamins A and E. Agents, which demonstrated activity in the 4-day assay, went through a secondary test as follows:

a) Curative Test (Rane Test). In this test, mice were administered a single daily dose of test agent by oral route on day 3 (72 hours) post-infection followed by daily monitoring of parasitemia. Results were expressed as rapidity of onset of activity

(disappearance of parasitemia i.e. clearance time), time to onset of recrudescence (reappearance of parasite after an initial clearance of parasitemia), % chemosuppression and average survival time in number of days.

b) Test for chemoprophylactic activity (Repository Test) by administering the compound prior to infection followed by daily examination of smears.

c) Test for synergistic activity using different combination of antioxidant micronutrients and standard antimalarials after the establishment of infection in mice.

3.1.3 Stage 1

Preparation of Inoculum of chloroquine sensitive strain of *Plasmodium berghei*

Plasmodium berghei NK 65 strain maintained in the laboratory of Nigerian Institute of Medical Research, Yaba by serial blood passage from mouse to mouse was used for the study. Donor mouse with a rising parasitaemia of 20 -30% confirmed by thin and thick blood film microscopy was used. Blood (0.2 ml) was collected in a heparinized tube from the auxiliary plexus of veins in the donor mouse using heparinized capillary tubes. The blood was diluted with 5 ml of Phosphate buffer solution (PBS) pH 7.2 so that each 0.2 ml contained approximately 1×10^7 infected red cells (Peter *et al.,* 1975; David *et al.,* 2004). Each animal received inocula of about 10 million parasites per kilogram body weight, which is expected to produce a steadily rising infection in mice. The infection of the recipient mice was initiated by needle passage of the above mentioned parasite preparation, from the donor to healthy test animals via an intraperitoneal route (Adzu *et al.,* 2007). *Plasmodium berghei* infected red blood cells were intraperitoneally injected into the mice with the donor blood diluted with PBS medium so that each 0.2 ml had approximately 10^7 infected red cells (parasite per kg of body weight).

The infected mice were randomly divided into standard drug group (A), control group (B), vehicle group (C) and treatment groups (D, E, F, G and H). The treatment group, sub-divided into sub groups D, E, F, G and H, received vitamin A (60 mg/kg), vitamin E (100 mg/kg), selenium (1 mg/kg), zinc (100 mg/kg) and vitamin C (200 mg/kg) respectively. Group C received the vehicle (0.2 ml of tween-80); while group A received chloroquine 25 mg/kg, daily for 4 days, starting on the same day as that of the parasite inoculation. The drug/micronutrients were administered through intra gastric route using a feeding trochar coupled to a 1ml syringe. All were given daily for 4 consecutive days starting 3 hours after infection, i.e. from day 0 (D0) to day 3(D3), receiving a total of 4 intra gastric doses. Thin smears of blood films were obtained from the peripheral blood by nipping the tail of each mouse on day four after infection. The smears were placed on microscopic slides, fixed with methanol and stained with 3% Giemsa at pH 7.2, for parasitaemia. The numbers of parasitized erythrocytes in each of the 10 fields were counted and the average was calculated to give the % Parasitemia. Parasitemia was monitored over 15 days, percentage suppression was calculated by using the formula

% Suppression = Parasitemia in negative control-Parasitemia in study group / Parasitemia in negative control × 100 (Peter *et al.*, 1977; Peter and Anatoli, 1998; David *et al.*, 2004; Adzu *et al.*, 2007).

3.1.4 Stage 2

The 4 day curative test was done using the method of Ryley and Peters, (1970); Agbaje and Onabanjo, (1994); David *et al.*, (2004); Adzu *et al.*, (2007). The animals were weighed and divided into 7 groups (A, B, C, D, E, F and G) of 5 mice each. The animals were all inoculated intraperitoneally with 1×10^7 *Plasmodium berghei* infected red blood

cells on day 0. Prior to this, the level of parasitaemia in the donor mouse was assessed microscopically (thin and thick blood films) to ascertain parasitemia of up to 20%-30%. On day 3 (72 hours after inoculation) a thin blood smear was made from the nipped tail of all the animals to ascertain established infection. The animals in group A were then administered a single oral dose of 25 mg/kg chloroquine. Group B, which served as the negative control group was treated with 0.2 ml of distilled water. Group C animals were treated with 0.2 ml of the vehicle tween 80, group D animals were treated with a single oral dose of vitamin A (60 mg/kg), group E animals with a single oral dose of vitamin E (100 mg/kg), group F animals with a single oral dose of selenium (1mg/kg), and group G animals with a single oral dose of zinc (100 mg/kg) administered daily for 4 days consecutively. On day four post infection, daily smears for thin blood film were made to ascertain % chemosuppression, parasite clearance time (PCT), recrudescence time (RT) and mean survival time.

3.1.5 Stage 3

In this stage, test for chemoprophylaxis (repository test) was conducted using the method of David *et al.,* (2004). Animals were divided into eight groups of five mice each (A, B, C, D, E, F, G and H) and treated with various test agents. Swiss albino mice in group A were dosed daily with chloroquine (25 mg/kg) via the oral route using a feeding trochar from day 0 to 5. Mice in group B were dosed daily with 0.2 ml of distilled water, Group C animals were dosed with 0.2 ml vehicle (tween 80) via same route and duration. Animals in group D were dosed daily with pyrimethamine (1.25 mg/kg), group E animals were dosed with 60 mg/kg of vitamin A, animals in group F were dosed daily with

vitamin E (100 mg/kg), animals in group G were dosed daily with zinc (100 mg/kg) while animals in group H were dosed with selenium (1mg/kg) all from day 0 to 5 prior to inoculation respectively. On day 6, all the animals were inoculated intraperitoneally after preparing the inoculum from donor mice. After 72 hours post inoculation, thin blood smears were made from the nipped tail of all the animals (Abatan and Makinde, 1986; David *et al.,* 2004) to ascertain the % chemosuppression (a measure of repository activity of the drugs/micronutrients). Subsequently, animals were monitored to ascertain the mouse survival time.

3.1.6 Stage 4

In this stage of the study, the curative effect of different antioxidant micronutrient combinations was conducted using the 4 day curative test as described in previous shedule. The animals were weighed and divided into 9 groups (A, B, C, D, E, F, G, H and I) of 5 animals each, and thereafter inoculated. Animals in group A were administered a single oral dose of 25 mg/kg chloroquine. Group B animals were administered 0.2 ml of distilled water orally, group C animals with 0.2 ml of the vehicle tween 80, group D animals were treated with a single oral dose of vitamin A (60 mg/kg) and single oral dose of vitamin E (100 mg/kg), group E animals with a single oral dose of vitamin A (60 mg/kg) and selenium (1mg/kg), group F animals were treated with a single oral dose of vitamin A (60 mg/kg) and zinc (100 mg/kg), group G animals recieved vitamin E (100 mg/kg) and zinc (100 mg/kg), group H were administered oral doses of vitamin E (100 mg/kg) and selenium (1mg/kg) and group I recieved oral doses of zinc (100 mg/kg) and selenium 1mg/kg respectively for 4 days. On day four post infection, daily smears for

thin blood film were made to ascertain % chemosuppression, parasite clearance time (PCT), recrudescence time (RT) and mean survival time.

3.1.7 Stage 5

In this stage, the synergistic or additive antimalarial effects of different combinations of selected antioxidant micronutrients and some standard antimalarial drugs were assessed. The inoculum containing the chloroquine resistant strain of *Plasmodium berghei* (ANKA strain) was used. However, preparation of inoculum was similar to previously described preparation. Swiss albino mice were placed in 9 groups (A, B, C, D, E, F, G, H and I) of 5 animals each. Animals in all the groups were inoculated intraperitoneally on day 0 after ascertaining that donor mice had at least 20-30% parasitemia. On day 3 post infection, thin blood smears were made from all the animals to ascertain the establishment of parasitemia. Subsequently, the animals were dosed with varying antimalarial and micronutrient combination. Group A animals were dosed orally with a single dose of 25mg/kg chloroquine, group B animals were dosed orally with 4 mg/kg artesunate, group C animals were dosed orally with distilled water (0.2 ml), group D animals were dosed orally with tween 80 (0.2 ml), group E animals recieved artesunate (4 mg/kg) + chloroquine (25 mg/kg), group F animals were administered oral doses of artesunate (4 mg/kg) and selenium (1 mg/kg), group G animals were dosed with artesunate (4 mg/kg) and zinc (100 mg/kg), group H animals were dosed with artesunate (4 mg/kg) and vitamin A (60 mg/kg), while group I animals were dosed with artesunate (4 mg/kg) and vitamin E (100 mg/kg) daily for 4 days respectively. From day 4 post inoculation, daily thin blood film were made to assess the level of parasitaemia, % chemosuppression, parasite clearance time, recrudescence time and mouse survival time.

3.1.8 Stage 6: Determination of Possible Mechanism of Action of Antioxidant Micronutrients

A four day curative test was done according to previous schedule using inocula containing chloroquine sensitive NK 65 strain of *P. berghei*. Mice were divided into nine groups of 5 each as follows;

- Group A = uninfected mice (control group), no drugs administered
- Group B= parasitized mice (negative control group) treated with distilled water
- Group C= parasitized mice (vehicle control group) treated with tween-80
- Group D= parasitized mice (positive control) treated with chloroquine 25 mg/kg
- Group E= parasitized mice (positive control) treated with artesunate 4 mg/kg
- Group F= parasitized mice (test group) treated with vitamin A 60 mg/kg
- Group G= parasitized mice (test group) treated with vitamin E 100 mg/kg
- Group H=parasitized mice (test group) treated with selenium 1mg/kg
- Group I=parasitized mice (test group) treated with zinc 100 mg/kg

At the end of the 4 day curative treatment (day 5 post treatment) blood samples were collected via the auxiliary vein into EDTA and lithium heparin specimen bottles for hematological and biochemical laboratory analysis.

3.1.9 Laboratory Analysis

3.1.9.1 Osmotic Fragility Test

Erythrocyte osmotic fragility was determined by adding 0.02 ml of blood to tubes containing increasing concentration of phosphate-buffered sodium chloride (NaCl) solution at pH 7.4 (0, 0.1, 0.3, 0.5, 0.7, 0.8 and 0.9%) as described by Azeez and Oyewale, (2010); Alanazi, (2010). The tubes were mixed and incubated at room

temperature (29°C) for 30 min. The content of each tube was then centrifuged at 3500 rev min^{-1} for 10 min. The relative amount of hemoglobin released into the supernatant was determined with the use of a spectrophotometer (SPECTRONIC 20, Labtech – Digital Blood Analyzer®) at maximum wavelength (max) of 540 nm. Absorbance were obtained and multiplied by a factor of 100. The range of values represented the percentage of erythrocyte lysis at each saline concentration. The corresponding concentration of saline solution (NaCl g/dl) that caused haemolysis in each tube was expressed as a percentage, taking haemolysis in distilled water (0% NaCl) as 100%. The quotient of absorbance of the content of individual test tubes that caused 50% lysis of red blood cells was the mean corpuscular fragility index (MCFI) (Dewey *et al.,* 1982; Krogmerer *et al.,* 1993; Chikezie, 2007).

The MCFI values were interpolated from the cumulative erythrocyte osmotic fragility curves obtained by plotting the percentage lysis against saline concentrations. The relative capacity of the antimalarials and antioxidant micronutrients to stabilize or destabilize red blood cell membrane was evaluated as percentage of the quotient of the difference between the MCFI values of the test and control samples (Chikezie, 2007).

Thus % Stability or Distability = MCFI Test – MCFI control ÷ MCFI control x 100.

3.1.9.2 Estimation of Lipid Peroxidative Indices

Lipid peroxidation as evidenced by the formation of malondialdehyde, a marker of free radical damage was measured using the method of Niehaus and Samuelsson, (1968) and Jiang *et al.,* (1992) as adopted by Rajagopalan *et al.,*(2004). 0.1ml of tissue homogenate (Tris-Hcl buffer, pH 7.5) was treated with 2ml of (1:1:1 ratio) TBA-TCA-HCL reagent (thiobarbituric acid 0.37%, 0.25 N HCL and 15% TCA) and placed in water bath for 15

minutes, cooled and centrifuged at room temperature for 10 min at 1,000 rpm. The absorbance of clear supernatant was measured against reference blank at 535nm.

3.1.9.3 Determination of Superoxide Dismutase, Catalase and Gluthathione Peroxidase Activity

Superoxide dismutase (SOD) was assayed using the method of Kakkar *et al.,* (1984) as adopted by Rajagopalan *et al.,* (2004). A single unit of enzyme was expressed as 50% inhibition of NBT (Nitroblue tetrazolium) reduction/min/mg protein.

Catalase (CAT) was assayed calorimetrically at 620nm and expressed as µmol of hydrogen peroxide consumed/min/mg protein as described by Sinha, (1972) and adopted by Rajagopalan *et al.,* (2004). Glutathione peroxidase (GPx) activity was measured by the method described by Ellman, (1959) and adopted by Rajagopalan *et al.,* (2004). Total protein was estimated by using the protein and albumin kit from Qualigens Chemicals, Worli, Mumbai.

3.1.9.4 Full Blood Count Estimation

Full blood count was estimated using an automated analyzer (Cell-Dyn®, Abbott, Santa Clara, California) that identifies cell types via flow cytometry. Differential white blood cell counts were estimated by manual count to identify the number of different white cells in a blood film. This was expressed in percentages.

3.2 CLINICAL STUDIES

3.2.1 Study Area

This study was conducted in Ekpoma, Esan West Local Government Area of Edo State Nigeria. The community is a semi-urban community that lies between latitudes 6^0 43′ South and 6^0 45′ North of the Equator and longitudes 6^0 6′ West and 6^0 8′ East of the Greenwich Meridian (Aziegbe, 2006) with an estimated population of over 125,842 inhabitants (NPC, 2006). The community is located in the rain forest belt endemic for *Plasmodium falciparum* malaria transmitted by the female Anopheles mosquito. It has a rainy period that spans from April to November which is when the bite of the mosquito is more rampant. The inhabitants comprise of workers in the private sector, civil servants, students, self employed, traders etc. Patients were recruited from two Medical Centers; Central Primary Health Center and Faith-Dome Medical Center both in Esan West Local Government Area of Edo State.

3.2.2 Sample Size Estimation

Sample size was estimated using the formulae $M = 2 \times [Z_{(1-\alpha/2)} + Z_{(1-\beta)}]^2 \div \Delta^2$ and $\Delta = P_1-P_2/\sqrt{p \times (1-p)}$ (Donner, 1984; Campbell *et al.*, 1995). Where $p = P_1+P_2/2$, $Z_{(1-\alpha/2)} = 5\% = 0.05 = 1.96$, $Z_{(1-\beta)}$ = power at 80%=0.8=0.8416 and Δ= standardized difference = 2.1053. Therefore, the minimum sample size is approximately 5 patients per group (total of 75 participants) at 5% significance and 80% power. However, the calculated sampling size was doubled to make room for lost to follow up.

3.2.3 Study Participants

A total of 150 participants were recruited for the study. The participants were drawn from early child hood (6months-5yrs of age). They were all recruited from the outpatient clinic

of the two selected Medical Centers (Faithdome Medical Centre and Central Primary Health Centre) in the study area.

3.2.4 Study Design

The study is a randomized controlled clinical trial with consecutive recruitment of eligible patients until the total sampling size was achieved. Specifically designed medical record forms were used to elicit biodata and clinical data from participants. Participants were admitted into the study after meeting the following inclusion criteria:

- age of $\geq$ 6months $\leq$ 5 years,
- asexual parasitemia of between 1,000 and 200,000/µl,
- acute manifestation of malaria (e.g., history of fever in the preceding 24 hours, a temperature of >37.5°C at baseline),
- body weight between 5 and 30 kg,
- ability to tolerate oral therapy,
- informed consent was sought and obtained from the parent/guardian of the subject
- resident in the study area for duration of at least 4 weeks.

The exclusion criteria for the study were as follows:

- adequate antimalarial treatment within the previous 7 days,
- use of micronutrients in the last 2 weeks,
- use of herbal medications in the last 2 weeks
- antibiotic treatment for a concurrent infection
- haemoglobin level of <7 g/dl,

- haematocrit of <25%,
- leukocyte count of >15,000/µl,
- mixed plasmodial infection,
- severe malaria, any other severe underlying disease,
- concomitant disease masking assessment of the treatment response,
- Inflammatory bowel disease and any other disease causing fever.

3.2.5 Ethical Issues/Considerations

The trial was registered with clinical trials.gov (registration number NCT01152931) and was conducted in accordance with the principles of the Helsinki Declaration and its Hong Kong amendment and according to the principles of good clinical practice. The following ethical issues were considered in the course of the study;

- Ethical permission was obtained from the Ethical Review Board of the Edo State Ministry of Health after submitting the research proposal for the study.
- Informed consent was procured from the Parent's and Guardian of study participants. Additionally, co-operation was sought from the child depending on their age group.
- Permission was obtained directly from the Medical Directors of Health Institutions involved in the study.
- Participants on micronutrient combinations without an appropriate response were scheduled to be given adequate treatment after 72 hours of commencing micronutrient therapy.

- Information elicited from the study participants were treated with utmost confidentiality.

3.2.6 Study Drugs and Administration

Drugs were administered daily at different doses depending on the age and weight of the participants. The intervention was based on the use of standard antimalarial therapy for uncomplicated malaria according to WHO recommendation (WHO, 2003). Micronutrient dosage was based on dietary reference intake values (DRI) adapted from Food and Nutrition Board (FNB: IOM), (2010). The study participants were randomly grouped into 15 cohorts (A-O) of 10 patients each after appropriate age and sex matching. Envelopes containing the letters (A1-10, B1-10, C1-10, D1-10, E1-10, F1-10, G1-10, H1-10, I1-10, J1-10, K1-10, L1-10, M1-10, N1-10 and O1-10) were placed in a basket. The content of the envelope picked after balloting, determined the arm of the study the participants were allotted to. The treatment groups were as follows (Table 1);

- Group A = Amodiaquine + Artesunate (active comparator or control group)
- Group B = Lumefantrine + Artemether fixed combination-Coartem (active comparator or control group)
- Group C = Artesunate + vitamin A (test group)
- Group D = Artesunate + Vitamin E (test group)
- Group E = Artesunate + Zinc (test group)
- Group F = Artesunate + Selenium (test group)
- Group G = Amodiaquine + Vitamin A (test group)

- Group H = Amodiaquine + Vitamin E (test group)
- Group I = Amodiaquine + Zinc (test group)
- Group J = Amodiaquine + Selenium (test group)
- Group K = Artesunate + Vitamin A + Vitamin E (test group)
- Group L = Artesunate + Vitamin A + Zinc (test group)
- Group M = Artesunate + Vitamin A + Selenium (test group)
- Group N = Artesunate + Vitamin E + Zinc
- Group O = Artesunate + Vitamin E + Selenium

Table 1: Drug Administration in the Study Population:

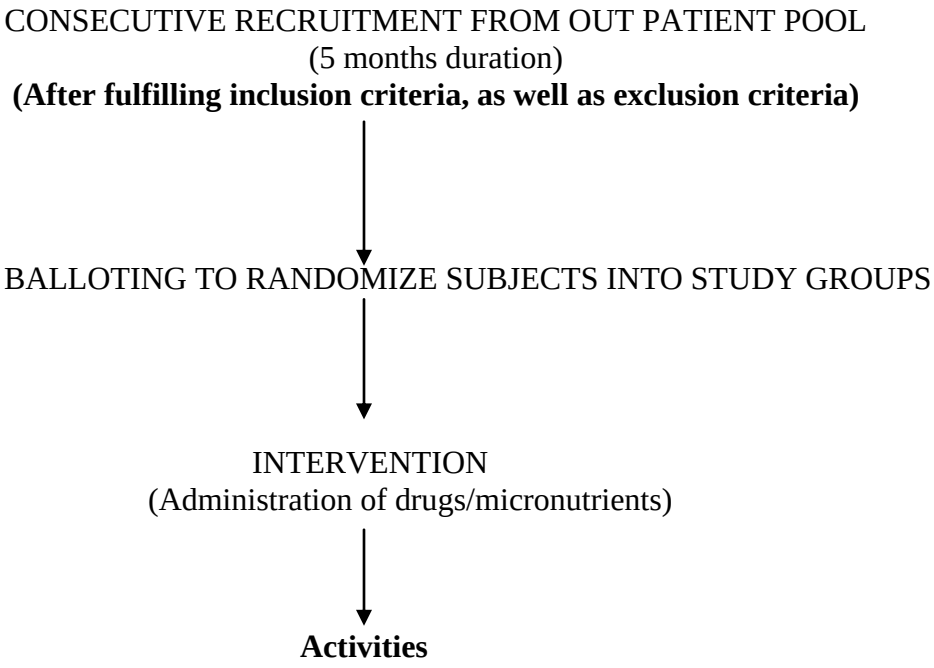
Groups	Drugs (Tablets/Gels)	Administration (Oral route)
A	**Amodiaquine + Artesunate**	Amodiaquine 10mg/kg dly x 3 days Artesunate 4mg/kg daily x 3days
B	**Lumefantrine +Artemether**	Fixed combination 120/20mg daily x 3days
C	**Artesunate + Vitamin A**	Artesunate 4mg/kg daily for 4 days + Vitamin A 5000IU dly x 4 days if ≤ 1yr. 10,000IU dly if > 1 yr x 4 days
D	**Artesunate + Vitamin E**	Artesunate 4mg/kg daily for 4 days + vitamin E 100mg dly x 4 days
E	**Artesunate + Zinc**	Artesunate 4mg/kg daily for 4 days + zinc 50mg dly x 4 days if > 1 yr. 25mg dly x 4 days if ≤ 1yr
F	**Artesunate + Selenium**	Artesunate same dose as above + selenium 100ug dly x 4days if > 1 yr. 50ug dly x 4 days if ≤ 1yr
G	**Amodiaquine +Vitamin A**	Amodiaquine 10mg/kg dly x 3 days + vitamin A same schedule as above for 4 days
H	**Amodiaquine+ Vitamin E**	Amodiaquine 10mg/kg dly x 3 days+ vitamin E 100mg daily for 4 days
I	**Amodiaquine+Zinc**	Amodiaquine 10mg/kg dly x 3 days + zinc same schedule as above for 4 days.
J	**Amodiaquine+Selenium**	Amodiaquine 10mg/kg dly x 3 days + selenium same schedule as above for 4 days.
K	**Artesunate+ Vit A+ Vit E**	Artesunate 4mg/kg daily for 4 days + Vitamin A + E same schedule as above for 4 days
L	**Artesunate+ Vit A+ Zinc**	Artesunate same schedule as above + Vitamin A + zinc same schedule as above for 4 days
M	**Artesunate+ Vit A+ Selenium**	Artesunate same schedule as above + Vitamin A + selenium same schedule as above for 4 days
N	**Artesunate+ Vit E+ Zinc**	Artesunate same schedule as above + Vitamin E + Zinc same schedule as above for 4 days
O	**Artesunate+ Vit E+Selenium**	Artesunate same schedule as above + Vitamin E + selenium same schedule as above for 4 days.

Each study drug administration was supervised by at least one dedicated study physician. For patients aged 6months to 2 years, tablets were crushed and mixed with water or a suitable beverage to facilitate drug uptake. Subjects who vomited or rejected the study drug within 30 min were made to repeat a full dose. Vomiting or rejection of the second dose leads to withdrawal from the study and administration of a rescue treatment.

3.2.7 Study Flow and Procedures

Patients were recruited consecutively for over 5 months until the sampling size was achieved. The study is a single blind study with concealment of the interventional groups using the sealed envelope system (David and Chris, 1999). The patients were followed up consecutively by a study physician at 24-hour intervals after each drug administration and/or until two consecutive negative blood smears occurred and subsequently on days 7, 14, 21, and 28 post-treatment or as otherwise indicated. At each visit during the treatment and follow-up phases, the medical history was taken, vital signs were checked, axillary temperature were measured with a standard mercury thermometer, and a thick blood smear was prepared from the finger prick for microscopic examination. Adverse events were monitored and documented accordingly during the follow-up period. Venipunctures were performed on study days 0 and 28 to monitor the hemoglobin level, hematocrit, differential white blood cell count. Serum vitamin A, E, ascorbic acid, selenium and zinc levels were also assessed in twenty apparently healthy children as well as participants in the interventional groups on day 0 of treatment and day 28 of follow up.

FLOW CHART OF ACTIVITY



D0 = administration of study drugs/micronutrients under direct observation of physician
DI = follow up activities (history, physical examination, preparation of blood smears, adverse event monitoring etc)/administration of drugs/micronutrients
D2= same activity as above
D3 = same activity as above
D4 = 5th day of event = history taking, physical examination, preparation of blood smears, adverse event monitoring etc.
7th day = same activity as above
14th day = same activity as above
21st day = same activity as above
28th day = same activity as above----- End of follow up activity

Note: Recruitment, intervention and follow up was done consecutively for the subjects over 5 months although each patient was followed up for 4 weeks. This implies that subjects had different D0-D4 and different 7th, 14th, 21st and 28th day of follow up.
Loss to follow up occurred predominantly on day 2. Reason presented by the parents/guardian of subject is the pain of subjecting their wards to repeated finger prick for blood smear preparation.
Key: D0-D4 = Days of drug/micronutrient administration

3.2.8 Therapeutic End points

The primary efficacy end point for the study was the 7-day cure rate. Cure was defined as initial and sustained parasite and symptom clearance with no increase in asexual parasitemia 48 hours after the initiation of treatment and the absence of microscopically detected asexual parasitemia within 120 hours of the commencement of treatment until day 7 (WHO, 2003). The primary safety end point in the study was the emergence of adverse events after the start of treatment. Secondary end points were the parasite and fever clearance time, recrudescence time and the 28 day cure rate.

3.2.9 Laboratory procedures

Dried thick blood smears were stained with 10% Giemsa solution at pH 7.2 for 10 minutes. Parasite species were identified using standard morphological characteristics, and the parasite density was calculated using standard procedure in which parasite were counted per 200 WBC multiplied by a standard count of 8,000 leukocytes/µl (Trape, 1985; WHO, 1991; WHO, 2000; O'Meara *et al.*, 2007).

3.2.9.1 Sample Collection/Determination of Serum Antioxidants (Vitamins A, C, E, Trace Metals; Zinc and Selenium)

Five milliliters of venous blood was collected by venepuncture into a plain plastic container. The samples were spun in a bucket centrifuge at a speed of 2500 revolution per minute to separate serum from red cells. The serum obtained was stored in a chest freezer at a temperature of −20ºC. Vitamin A (retinol) was estimated by a modification of the spectrophotometric method described by Neild and Pearson, (1967) as adopted by Bilbis

et al., (2010). In this method, the conjugated double bonds of vitamin A were made to react with trifluoroacetic acid (TFA), forming a faint, short-lived, blue compound that can be read spectrophotometrically.

Serum vitamin C (ascorbic acid) was determined using the method of Roe and Kuether, (1942). The principle is to prepare a protein free filtrate of the sample using trichloroacetic acid. Acid washed charcoal was added to the portion of the supernatant to facilitate the rapid oxidation of ascorbic acid to dehydroascorbic which then reacts with 2, 4-dinitrophenylhydrazone to form 2, 4-dinitrophenylosazone of dihydroascorbic acid which forms a red colour in an acidic pH. The emerging colour was read spectrophotometrically at 540nm. Vitamin E (tocopherol) was estimated using the method of Emmerie-Engel, (1939). The principle was to oxidize tocopherol to tocopheryl quinone using ferric chloride ($FeCl_3$). The resultant ferrous compound coupled with α, α-dipyridyl forms a red colour which is measured spectrophotometrically at 540nm.

Serum levels of zinc and selenium were determined by atomic absorption spectrophotometry (AAS) (Spectrophotometer 23D, England) based on the direct method described by Kaneko, (1999). The method is based on the principle that atoms of the elements when aspirated into AAS vaporized and absorbed light of the same wavelength as emitted by the element when in the excited state.

3.2.10 Data management and Statistical Analysis

Data were captured using specifically designed concise medical record forms which were subsequently entered into an electronic database. Data was then validated by complete manual review. Statistical analyses of the data were performed using statistical software

package SPSS version 17.0. Cure rates were calculated from the number of patients with clinical and parasitological cure by day 7, 14, or 28 divided by the total number of patients who could be evaluated (per protocol population) (WHO, 2003). Fever clearance time (FCT) was calculated from the start of treatment until the first of two consecutive temperature measurements remained below 37.5°C (WHO, 1996; WHO, 2003; O'Meara *et al.,* 2007). The time required for parasite clearance (PCT) was calculated as the time between the beginning of treatment and the time when no asexual forms were found in the blood film. The parasite reduction ratio or rate was calculated as the rate between the parasite density before treatment and that at 48 hours, as described by (White, 1997; O'Meara *et al.,* 2007).

The safety analysis includes abnormal laboratory data and adverse events for all subjects who received at least one dose of the study drug (intention-to-treat population). Student's *t* test and one way ANOVA were used to compare the mean of laboratory data between groups. Bonferroni correction was done for multiple comparisons. Pearson's correlation test was also used to establish the relationship between variables such as parasite density and packed cell volume, haemoglobin concentration, white blood cell count etc. The statistical significance level was set at 95% confidence interval and P value < 0.05 was considered significant.

CHAPTER 4

4.0 RESULTS

4.1 *IN VIVO* STUDIES

As shown in Figure 1, the parasitemic profile in the control group during the 4 day suppressive test peaked at 54.3% prior to the death of inoculated mice with an average mean survival of (16.00 ± 1.48 days).

As shown in Figure 2, antioxidant micronutrients in this study exhibited significant shizonticidal activity in the early phase of *Plasmodium berghei* infection ($p < 0.05$), however, this was more marked in selenium treated group (82.01%) and insignificant in the vitamin C treated group when compared with control ($p > 0.05$).

Additionally, mean % parasitemia was markedly reduced in the selenium treated group (0.86±0.70%) when compared to the other micronutrient groups after 4 days suppressive treatment of *P. berghei* infection (Figure 3). This was significant ($p < 0.05$) when compared to control.

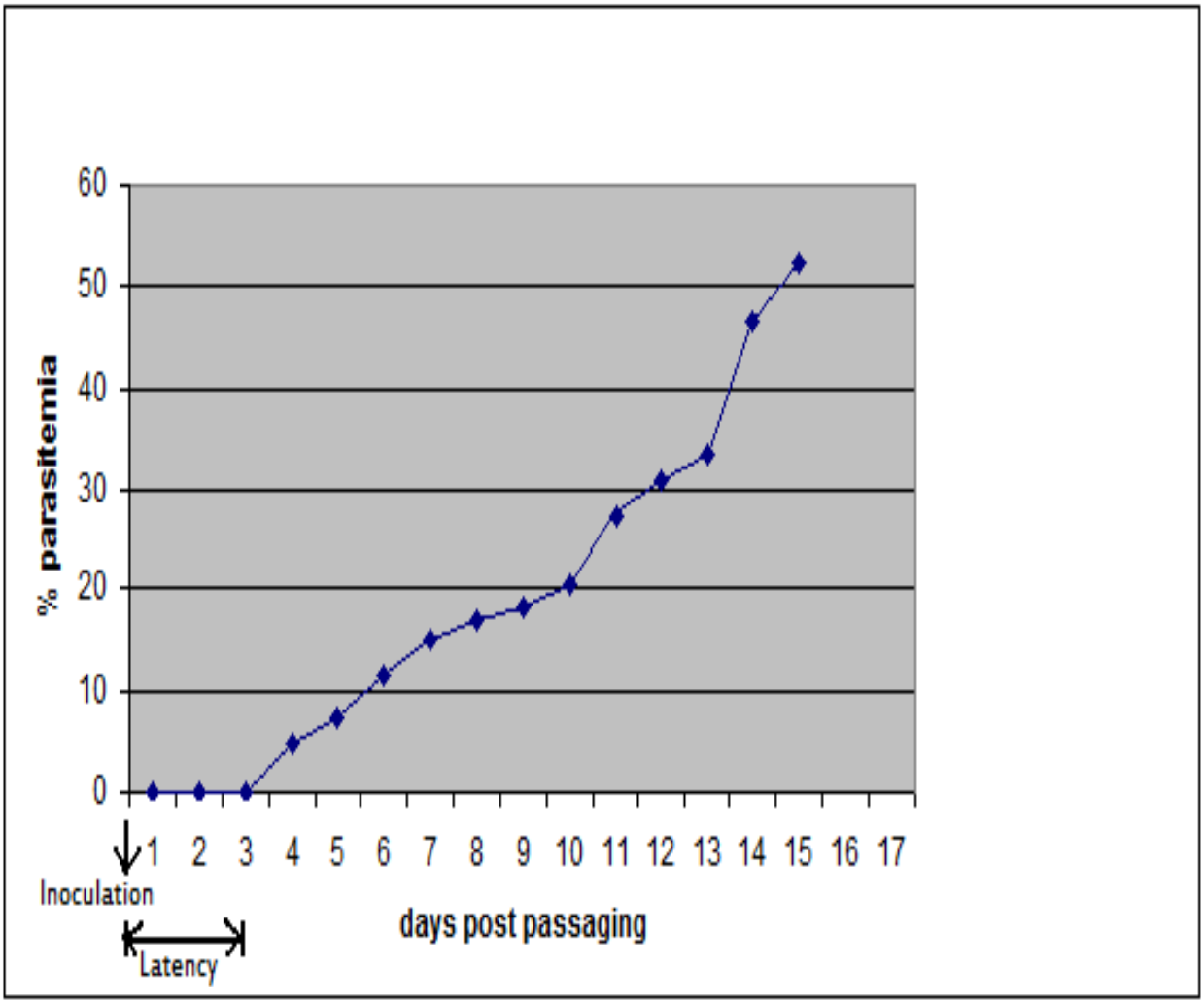


Figure 1: Parasitemic profile in negative control group 15 days post-passaging (4 Day Suppressive Test)

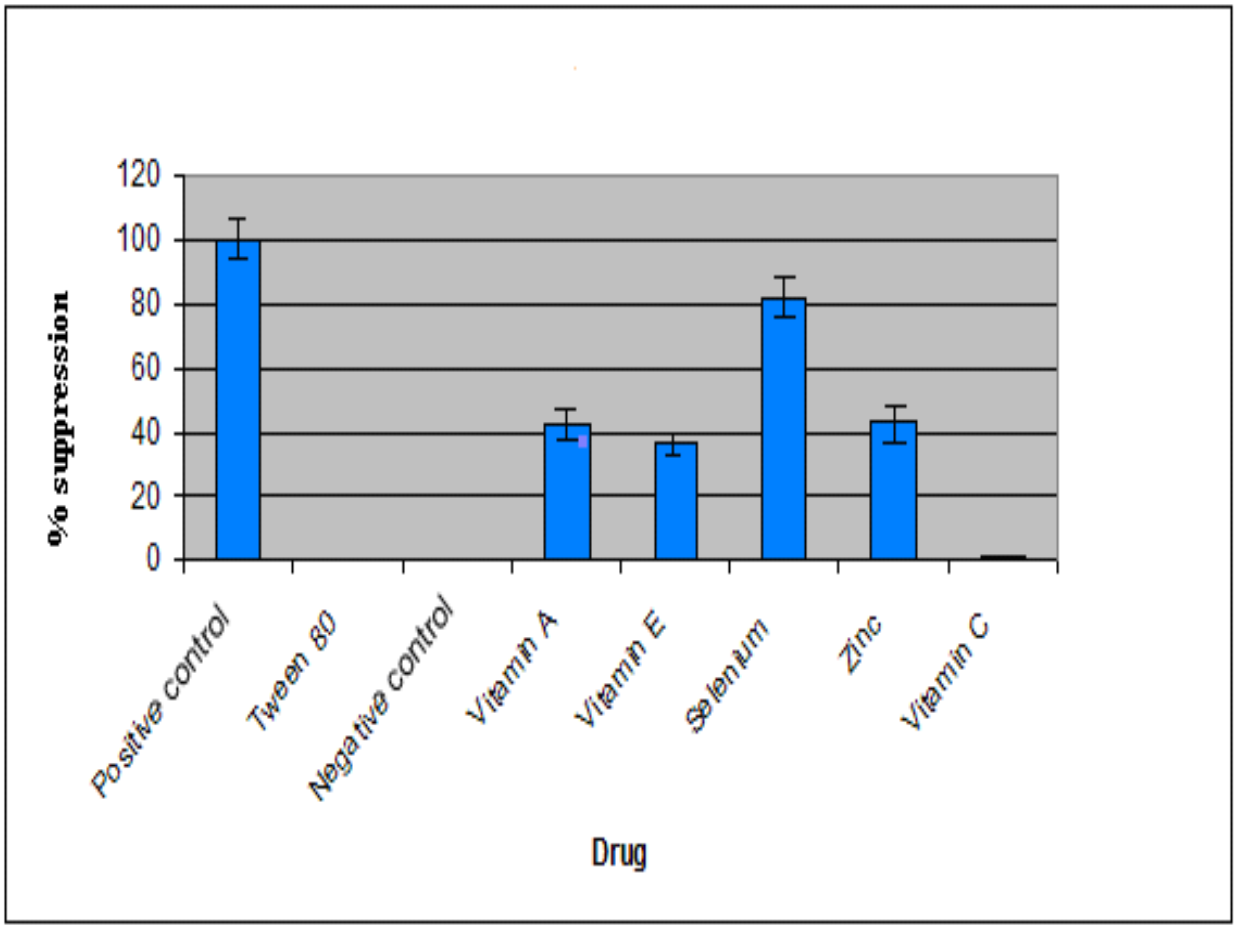


Figure 2: % suppression of different test agents in 4-day suppressive test

Key: Positive control = chloroquine, Negative control = distilled water

Mean parasitemic levels after 4 days curative treatment was 5.82± 1.48% in the selenium treated group and 9.95 ± 2.81% in the vitamin A treated group respectively, when compared with the control group (24.42 ± 3.84%).

This was significant when compared with negative control ($p< 0.05$) and significant between micronutrient treated groups ($F = 7.04$; $p< 0.05$), though chemosuppression was not as marked when compared to the chloroquine treated group (100%) as shown in Table 2. Additionally, the mean survival time was significantly prolonged in the entire micronutrient treated group when compared to control ($p< 0.05$).

Synergistic schizonticidal activity was more marked with the vitamin A + E combination therapy (94.52%) when compared with any other micronutrient combination after 4 days treatment of established infection.

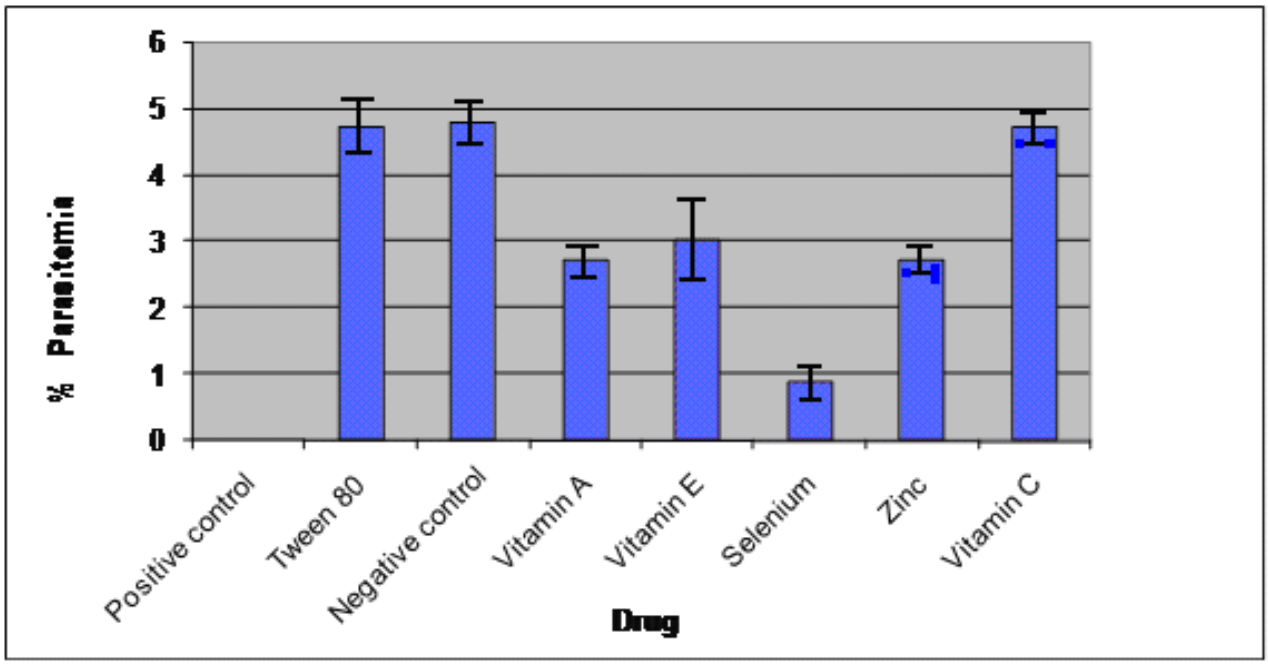


Figure 3: % parasitemia of different test agents in 4 day suppressive test

Key: Positive control = chloroquine, Negative control = distilled water

This was closely followed by the vitamin A + selenium combination (94.43%), though the mean difference in parasitemic levels was significant between groups ($F = 2.59$; $P < 0.05$) as shown in Table 3. In addition, the vitamin A + E combination group had a more prolonged mean survival time (27.80 ± 0.37 days) when compared with other micronutrient combination groups. This was however, significant between groups ($F = 1359.70$; $P < 0.05$).

As revealed in Table 4, all the antioxidant micronutrients demonstrated significant chemoprohylactic activity ($p < 0.05$) when compared with negative control, with significant reduction in mean parasitic levels between groups ($F = 1663.01$; $P < 0.05$). However, selenium demonstrated a more marked repository activity (94.94%) which is comparable to pyrimethamine (95.32%).

Table 2: Mean Parasitemic Levels of Established *P. berghei* Infection after 4 Days of Treatment (Curative Test). n = 5 mice per group.

Group	Dose (mg/kg)	Av % Parasitemia	% Suppression	MST (days)
Positive Control (Chloroquine)	25	0.00 ± 0.00	100	*55.00± 3.16
Negative Control (Distilled H_2 0)	0.20ml	24.42 ± 3.84	-	16.00±1.48
Tween-80	0.2ml	24.15 ± 2.08	0.70	15.80±1.20
Vitamin A	60	*9.95 ± 2.81	59.19	*22.70±0.86
Vitamin E	100	*15.98 ± 2.60	34.56	*21.60±1.17
Selenium	1	*5.82 ± 1.48	76.16	*24.00±0.63
Zinc	100	*15.33 ± 2.63	37.22	*22.40±0.81
ONE WAY ANOVA		**F = 7.04 P = < 0.05**		**F = 374.31 P = < 0.05**

Results are expressed as mean ± SEM. df 4, *p < 0.05 is significant when compared with control

Table 3: Mean Parasitemic Levels of Established *P. berghei* Infection after 4 Days of Treatment (Curative Synergistic Test). n= 5 mice per group.

Groups	Dose (mg/kg)	Av % Parasitaemia	% Suppression	MST (days)
Positive Control (Chloroqiune)	25	0.00 ± 0.00	100	*56.00±2.45
NegativeControl (Distilled H_2 0)	0.2ml	3.26 ± 0.94	-	15.80±1.46
Tween-80 (Vehicle)	0.2ml	32.98 ± 1.09	0.84	16.00±1.14
Vitamin A+E	60/100	*1.82 ± 0.10	94.52	*27.80±0.37
Vit A+Selenium	60/1	*1.85 ± 0.43	94.43	*27.20±0.58
Vit A+Zinc	60/100	*2.72 ± 0.79	91.82	*26.50±0.37
Vit E+Zinc	100/100	*6.60 ± 0.85	80.16	*24.40±0.51
Vit E +Selenium	100/1	*3.66 ± 1.48	89.00	*25.60±0.58
Zinc +Selenium	100/1	*3.27 ± 0.19	90.17	*26.20±0.51
ONE WAY ANOVA		**F =2. 59 P < 0.05**		**F=1359.70 P < 0.05**

Results are expressed as mean ± SEM. df 4, $^{*}p < 0.05$ is significant when compared with control

The parasitemic profile of *Plasmodium berghei* in micronutrient treated mice during the curative monotherapy test showed an initial decline in parasitemia after the first day of treatment in the selenium group. However, this effect was not sustained when compared to the chloroquine treated group as there was a steady rise in parasitemia after the fourth day of treatment (Figure 4).

A similar pattern was also observed during the curative synergistic study; however, the decline in parasitemia was sustained in the vitamin A + E treated group till the 4^{th} day post treatment when compared to the chloroquine treated group. In the other micronutrient combination groups there was a steady decline in parasitemia till the 2^{nd} day post treatment; this was not sustained afterwards. However, peak parasitemia was lower on day 17 post treatment in all the micronutrient combination groups when compared with control (Figure 5).

Table 4: Chemoprophylactic Effect of Test Agents against *P. berghei* Infection (n=5 mice per group).

Groups	Dose (mg/Kg)	Av % Parasitaemia	% Suppression
Chloroquine	25	1.45 ± 0.81	81.17
Distilled $H_2 0$	0.20	7.70 ± 1.96	-
Tween 80 (Vehicle)	0.20	7.63 ± 1.45	0.90
Pyrimethamine (Std)	1.25	0.36 ± 0.25	95.32
Vitamin A	60	*1.81 ± 0.96	76.49
Vitamin E	100	*1.69 ± 0.49	78.05
Zinc	100	*0.64 ± 0.18	91.61
Selenium	1	*0.39 ± 0.22	94.94
ONE WAY ANOVA		**F = 1663.01**	
		P < 0.05	

Results are expressed as mean ± SEM. df 4, *p < 0.05 is significant when compared to control

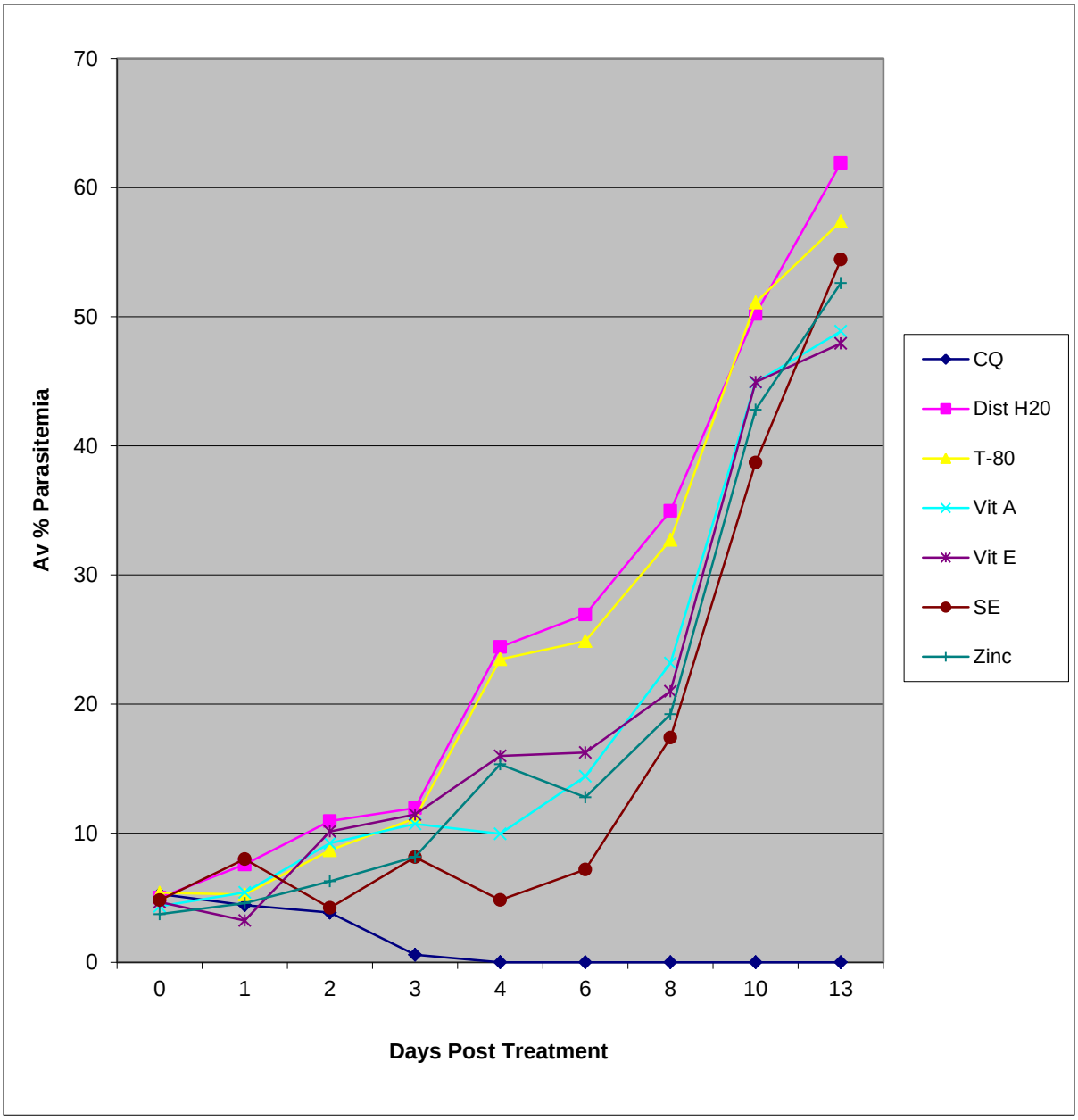


Figure 4: Parasitemic Profile of *P. berghei* 13 days Post-treatment in Curative Test

In the 4 day curative synergistic test using chloroquine resistant ANKA strain, there was a markedly significant chemosuppression in all the micronutrient and standard drug groups when compared with negative control ($P < 0.05$). Additionally, parasite clearance was complete in artesunate + zinc and artesunate + selenium group when compared to the artesunate + vitamin A and artesunate + vitamin E group; however, the mean difference in parasitemic level was significant ($F = 13.57$; $P < 0.05$) as shown in Table 5.

Parasite clearance was more rapid in the artesunate + selenium treated group (2.40±0.40 days) when compared with the artesunate group (3.00±0.32 days), artesunate + chloroquine group (2.60±0.24 days) and artesunate + zinc group (2.60±0.40 days) respectively. Comparatively this was significant $P < 0.05$ when compared with the artesunate group and artesunate + vitamin A groups and insignificant when compared with the artesunate + chloroquine and artesunate + zinc group respectively (Table 5).

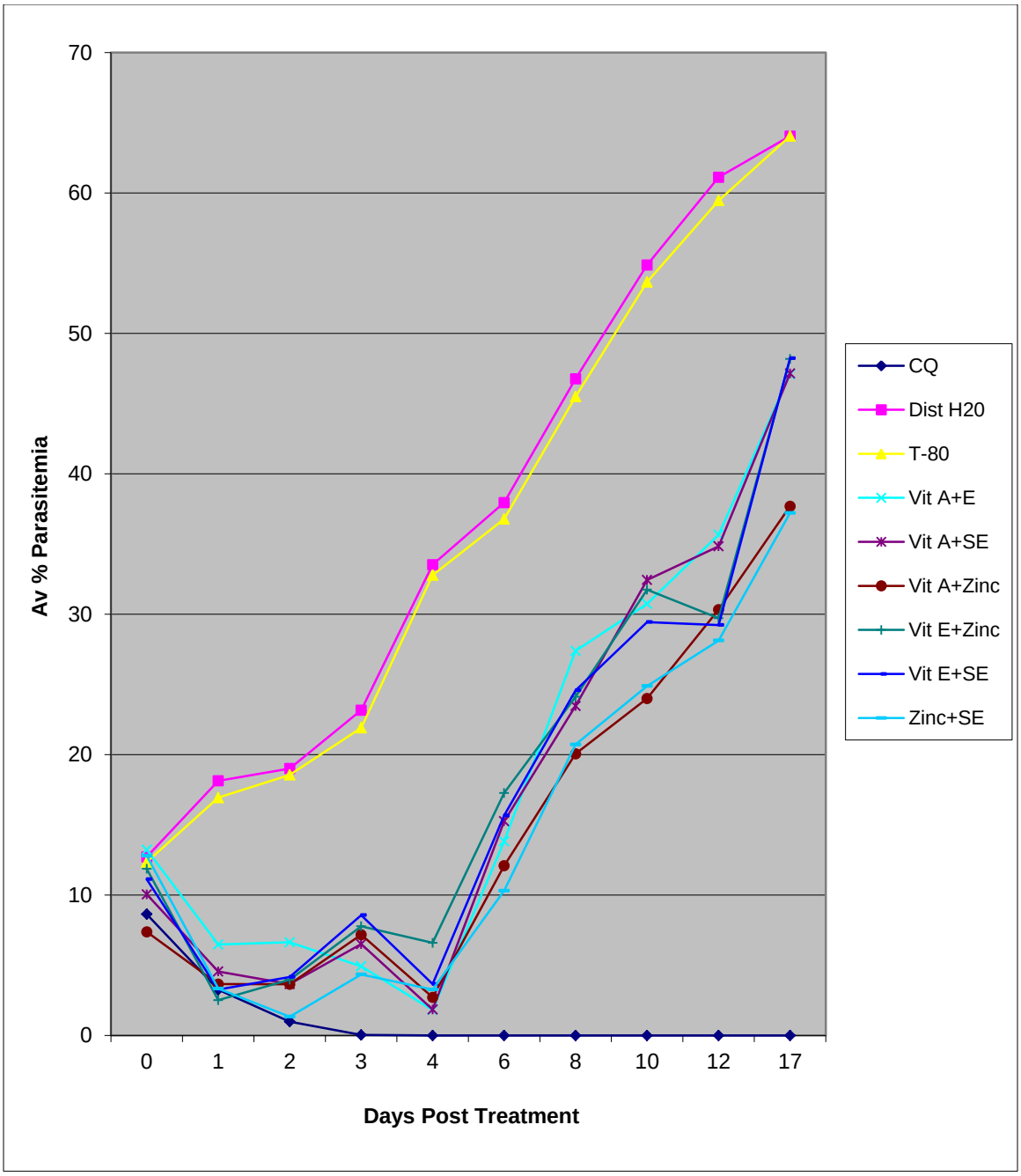


Figure 5: Parasitemic Profile of *P. berghei* 17 days Post-treatment in Curative Synergistic Test

The mean survival time (MST) was significantly prolonged in all the treated groups when compared with negative control and vehicle group. Additionally, it was more prolonged in the artesunate + chloroquine group, followed by the artesunate + selenium group. Though the mean difference between the groups was significant ($F = 230.71$; $P < 0.05$) , the average mouse survival time between the artesunate + chloroquine, artesunate + selenium, artesunate + zinc, artesunate + vitamin A and artesunate + vitamin E groups was not statistically significant.

Worthy of note was the occurrence of recrudescence on the 21st day post inoculation in the artesunate (n = 2), artesunate + vitamin A (n = 2) and artesunate + vitamin E (n =1) groups. However, no recrudescence was observed in other treated groups after initial complete parasite clearance.

Results of the present study on the possible mechanism of action revealed a similar pattern of chemosuppression as observed in earlier studies after a 4 day curative treatment of established *P. berghei* infection in mice (Table 6).

Table 7, reveals the mean % hemolysis in the treated groups at varying saline concentration (0.0-0.9g/dl). The osmotic fragility curve showed a similar sigmoidal pattern in all the groups after a 4 day curative treatment of established *Plasmodium berghei* infection in mice (Figure 11-19).

Table 5: Mean Parasitemic Levels of Established *P. berghei* (chloroquine resistant ANKA strain) Infection after 4 Days of Treatment in Curative Synergistic Test. n = 5 mice per group.

Groups	Dose mg/kg	% Parasitemia	% Suppression	PCT (days)	MST (days)
Chloroquine	25mg/kg	23.23±1.51	60.30	0.00±0.00	25.00±1.22
Artesunate	4mg/kg	0.00±0.00	100	3.00±0.32	53.00±1.84
Distilled H_20	0.2 ml	58.51±3.85	0	0.00±0.00	9.80±0.84
Tween 80	0.2 ml	57.55±3.63	1.64	0.00±0.00	10.20±0.37
Artesunate + Chloroquine	4mg/kg + 25mg/kg	*0.00±0.00	100	*2.60±0.24	*55.60±1.34
Artesunate + Selenium	4mg/kg + 1mg/kg	*0.00±0.00	100	*2.40±0.40	*54.80±2.34
Artesunate + Zinc	4mg/kg + 100mg/kg	*0.00±0.00	100	*2.60±0.40	*53.60±4.62
Artesunate + vitamin A	4mg/kg + 60mg/kg	*1.51±0.95	97.42	*3.60±0.87	*51.00±2.92
Artesunate + vitamin E	4mg/kg + 100mg/kg	*1.77±1.04	96.97	*2.80±0.37	*52.00±1.64
ONE WAY ANOVA		**F = 13.57** **P < 0.05**		**F=13.83** **P < 0.05**	**F = 230.71** **P < 0.05**

Results are expressed as mean ± SEM. df = 4 *p< 0.05 is significant when compared with control

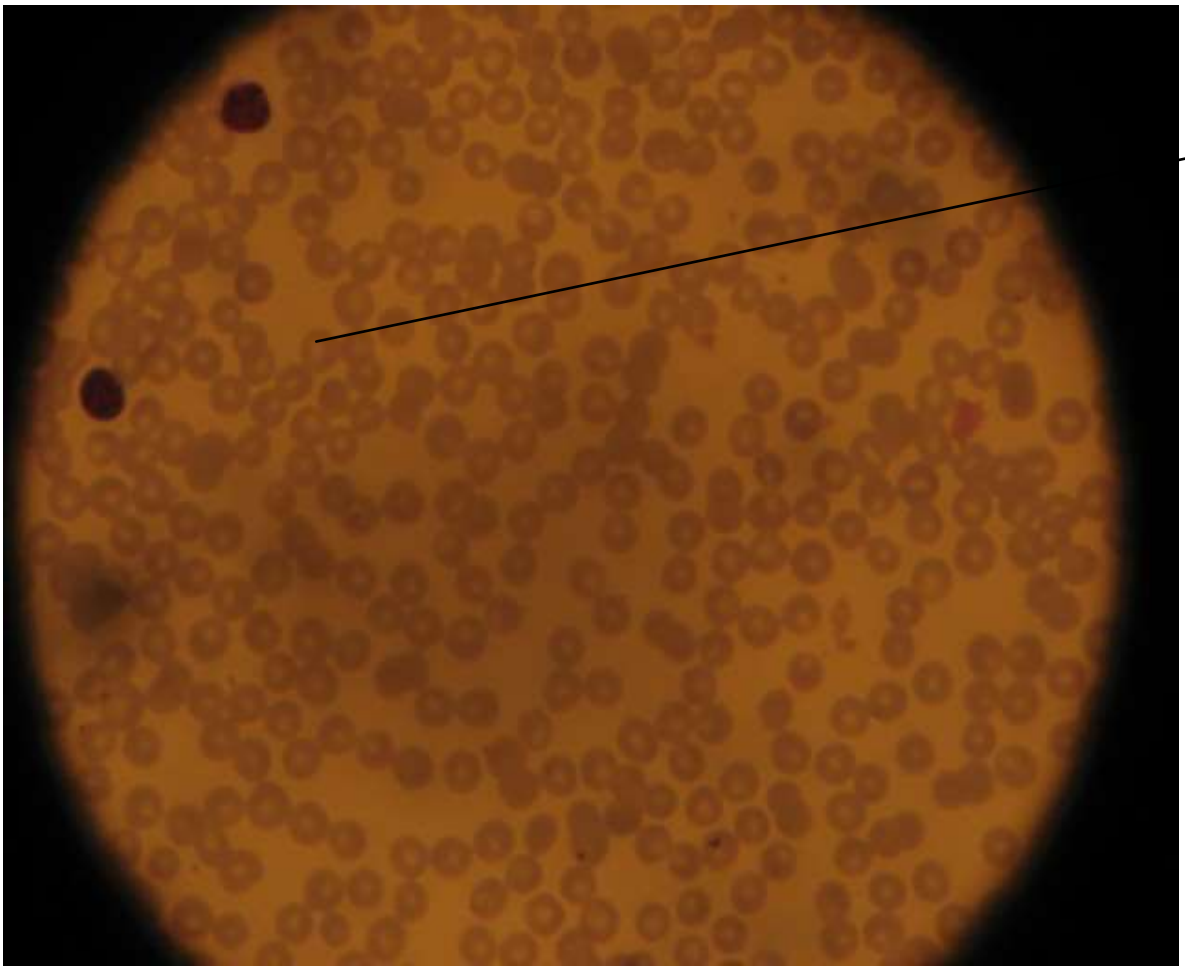


Figure 6: Normal Thin Blood Film Micrograph in Swiss Albino Mice (X 100 magnification)

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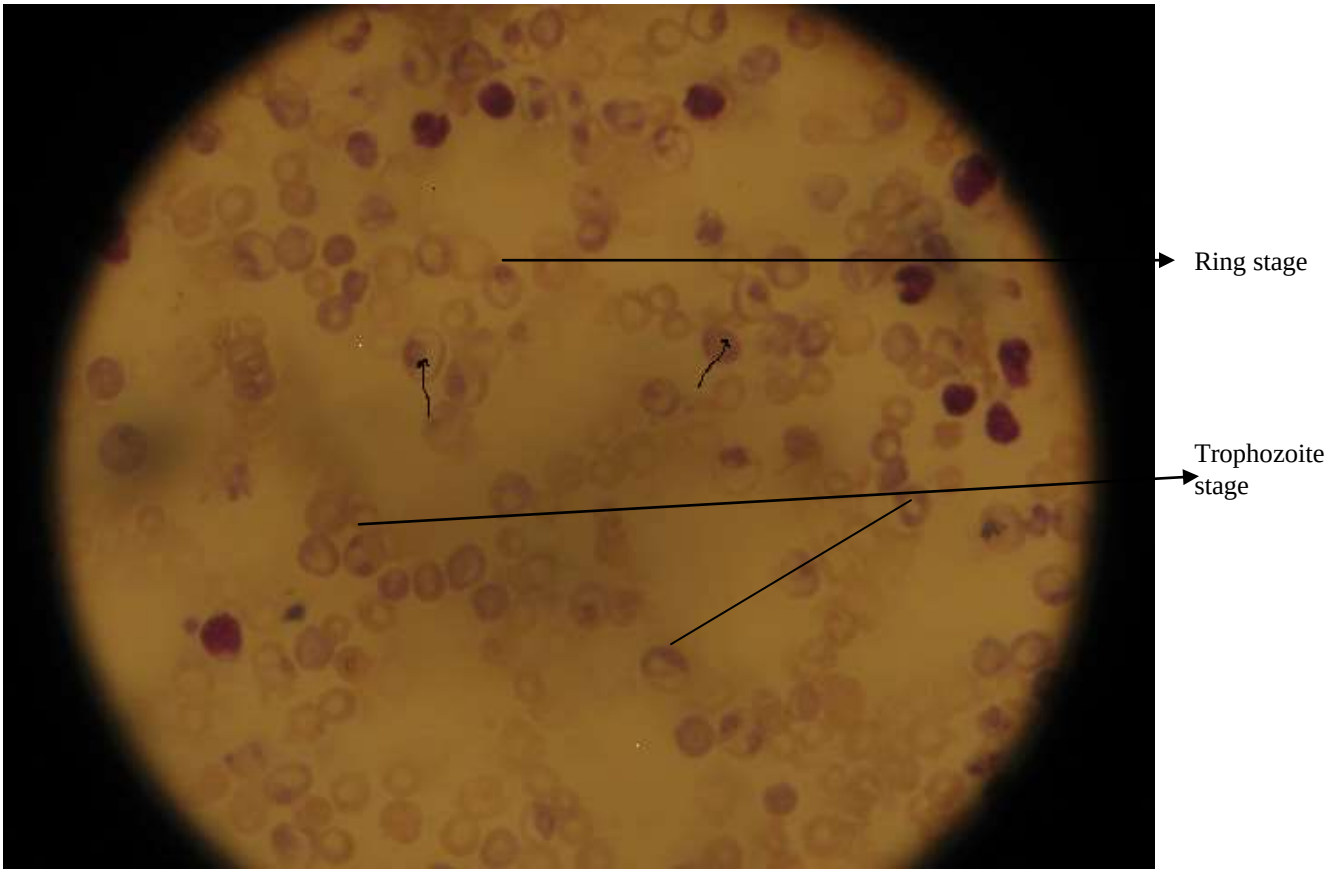

Figure 7: Thin Blood Film Micrograph Showing Established *P. berghei* Infection (72hrs post inoculation) in Passaged Mice (X 100 magnification)

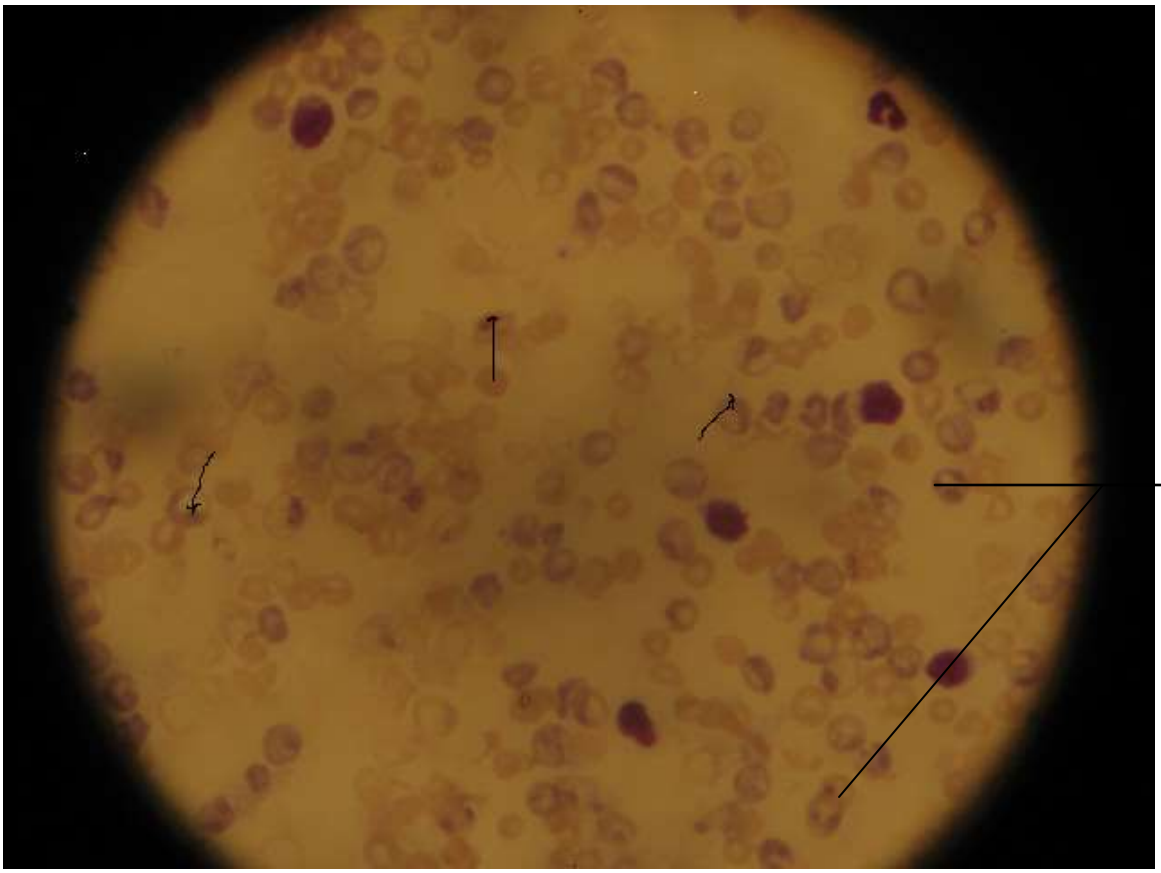

Figure 8: Thin Blood Film Micrograph Showing Progressive Parasitisation of RBC in Control Group (X 100 magnification)

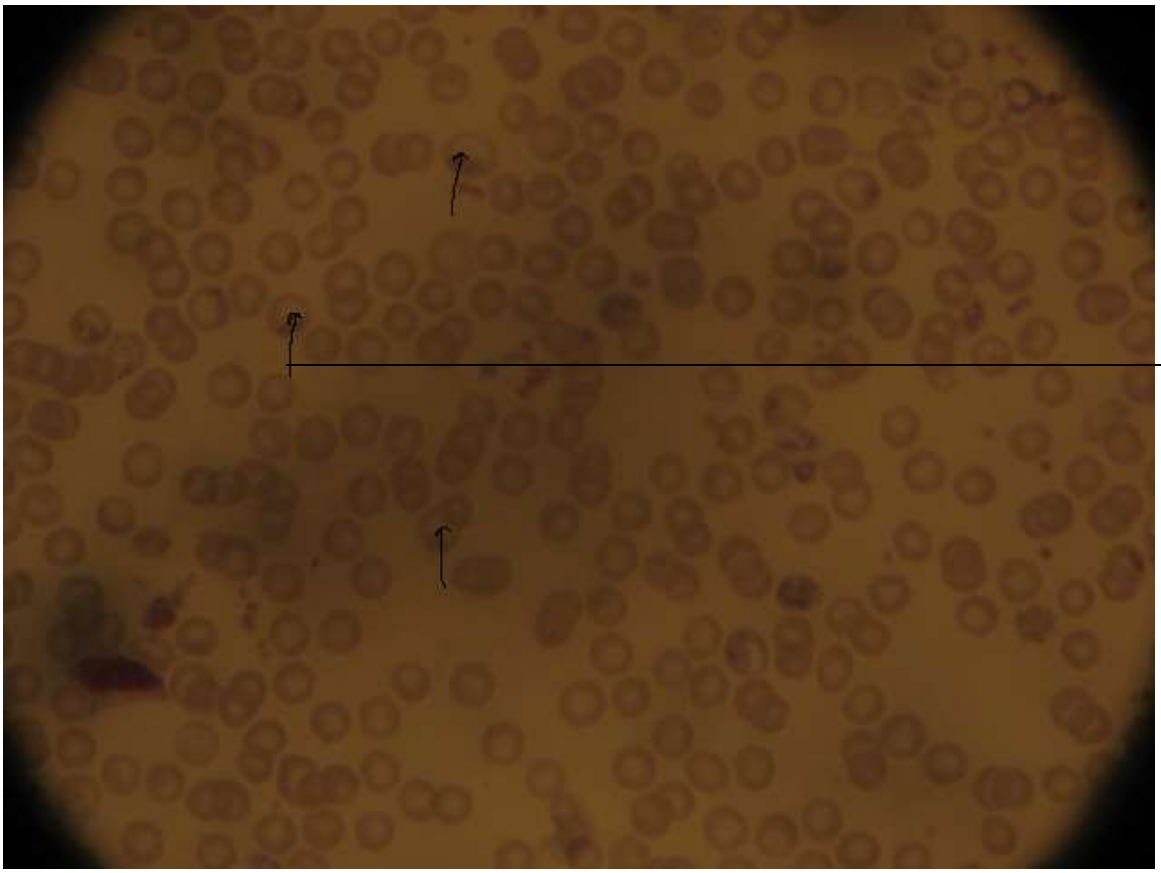

Figure 9: Thin Blood Film Micrograph Showing Reduced Erythrocyte Infection with *P. berghei* in Vit A Treated Group (X 100 magnification)

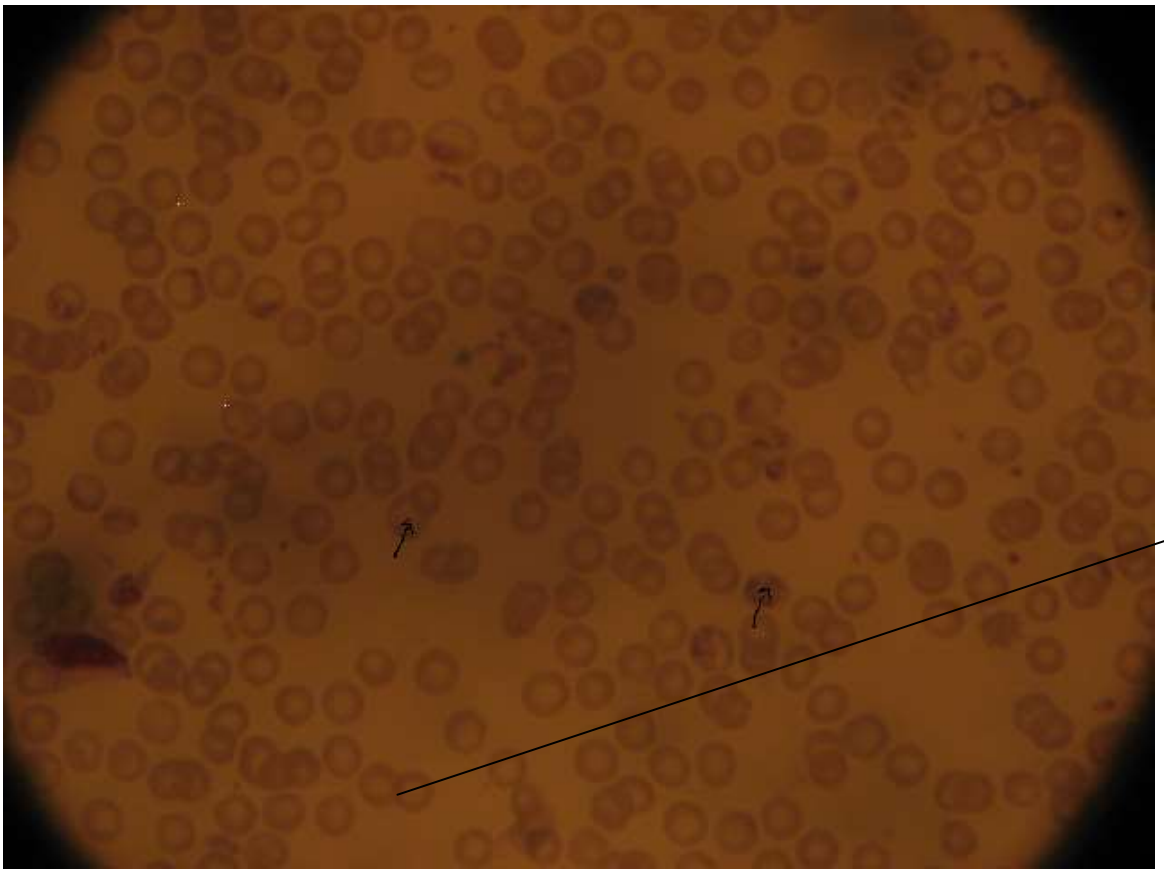

Figure 10: Thin Blood Film Micrograph Showing Reduced Erythrocyte Infection with *P. berghei* in Selenium Treated Group (X 100 magnification)

Table 6: % Chemosuppression in *P. berghei* Parasitized mice after 4 days of Treatment

Group	Dose (mg/kg)	Av % Parasitaemia	% Suppression
Control (not inoculated/no treatment)	-	-	-
Negative Control (Distilled H_2 0)	0.2ml	24.14±2.59	-
Tween-80	0.2ml	21.82±3.42	9.61
PositiveControl (Chloroquine)	25	0.00±0.00	100
Artesunate	4	0.00±0.00	100
Vitamin A	60	*8.90±2.78	63.13
Vitamin E	100	*11.61±2.78	51.90
Selenium	1	*5.08±1.85	78.95
Zinc	100	*9.89±1.11	59.03
ONE WAY ANOVA		**F = 17.88 P <0.05**	

Values are expressed as X±SEM. df = 4, *Mean difference is significant at p<0.05.

Table 7: % Hemolysis in Different Groups after 4 Days Curative Test in *P. berghei* Parasitized Mice

Conc. Nacl g/dl	% Hemolysis								
	Control(not inoculated)	**Dist. H_20**	**Tween-80**	**CQ**	**Arte**	**Vit A**	**Vit E**	**Selenium**	**Zinc**
0.0	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00	100±0.00
0.1	17.94±0.34	41.2±2.69	40.62±1.07	42.7±0.84	41.74±0.20	28.02±0.42	25.38±1.42	22.70±0.93	23.48±0.74
0.2	14.94±0.38	30.90±0.38	39.48±0.49	39.76±0.47	33.16±1.72	23.42±0.42	22.38±0.31	27.50±0.58	24.92±0.90
0.3	27.44±0.58	38.24±0.30	39.20±0.89	39.98±1.26	49.86±1.02	33.10±2.18	30.54±0.98	28.60±0.56	24.64±0.91
0.4	31.40±0.99	34.82±1.05	56.28±2.57	45.88±1.22	38.16±0.48	26.30±0.95	42.12±2.08	28.90±0.49	27.04±0.43
0.5	33.54±1.69	33.98±1.23	55.30±2.29	46.70±0.43	47.10±0.60	22.52±0.36	25.04±4.52	26.06±0.22	24.36±0.92
0.6	25.82±1.49	34.62±0.55	41.52±0.18	38.86±0.33	30.44±0.23	24.02±1.88	18.70±0.81	26.72±0.94	25.66±1.09
0.7	24.08±0.96	31.46±0.40	35.22±0.28	30.90±0.25	20.56±0.36	27.16±0.52	7.60±0.51	19.92±1.54	23.34±0.37
0.8	6.78±0.80	17.10±1.08	13.82±0.57	20.50±0.33	13.94±0.0.36	21.96±0.28	14.98±9.76	12.68±1.45	17.86±0.82
0.9	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

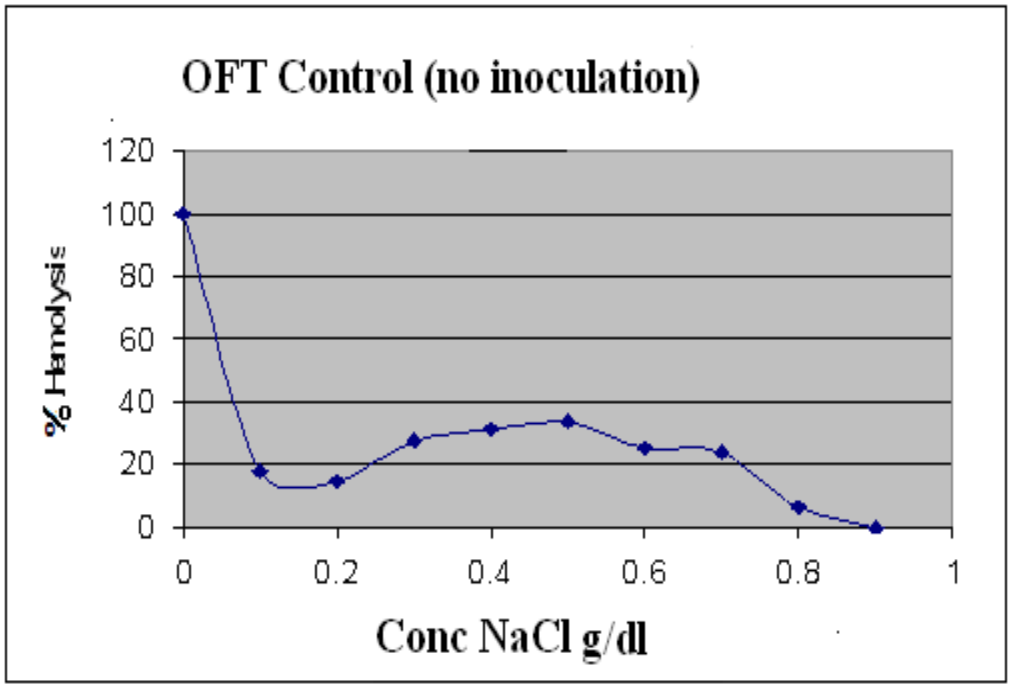

Figure 11: Osmotic Fragility Curve in Control Group

MCFI=0.065

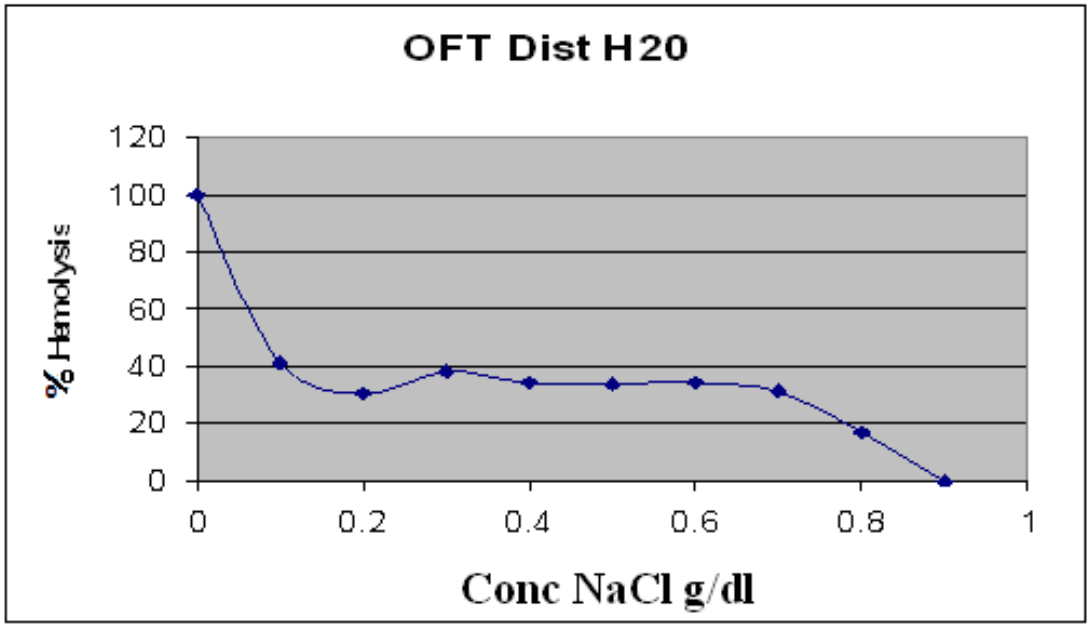

Figure 12: Osmotic Fragility Curve in Dist. H_2O Group

MCFI=0.239

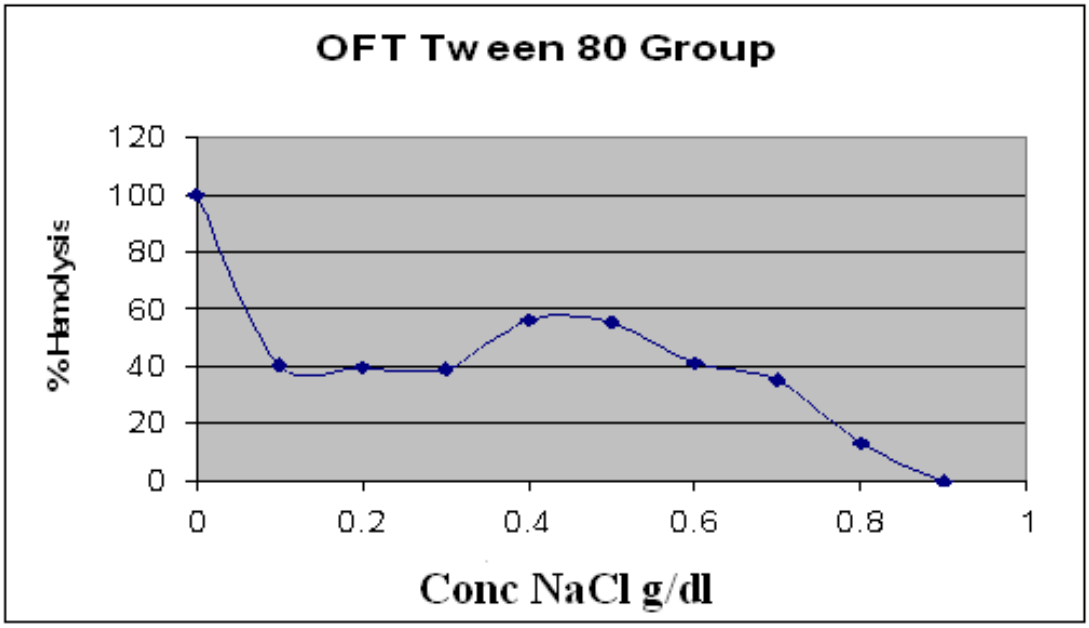

Figure 13: Osmotic Fragility Curve in Tween 80 Group

MCFI=0.343

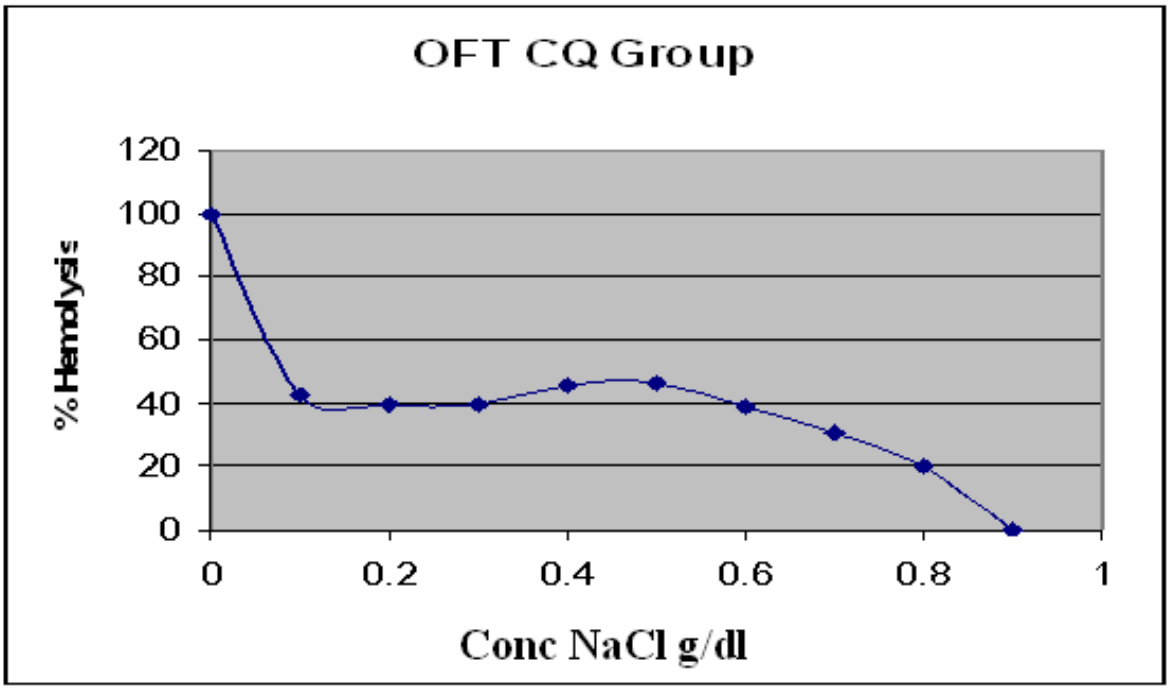

Figure 14: Osmotic Fragility Curve in Chloroquine Group

MCFI=0.308

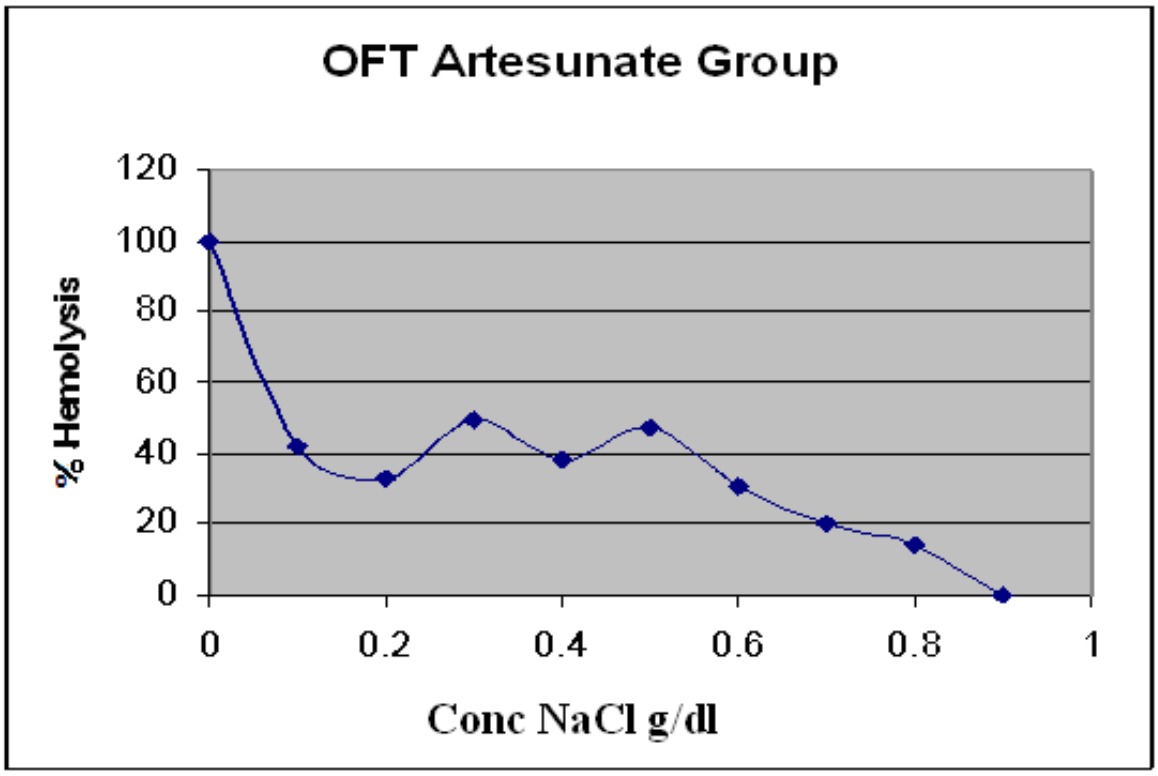

Figure 15: Osmotic Fragility Curve in Artesunate Group

MCFI=0.279

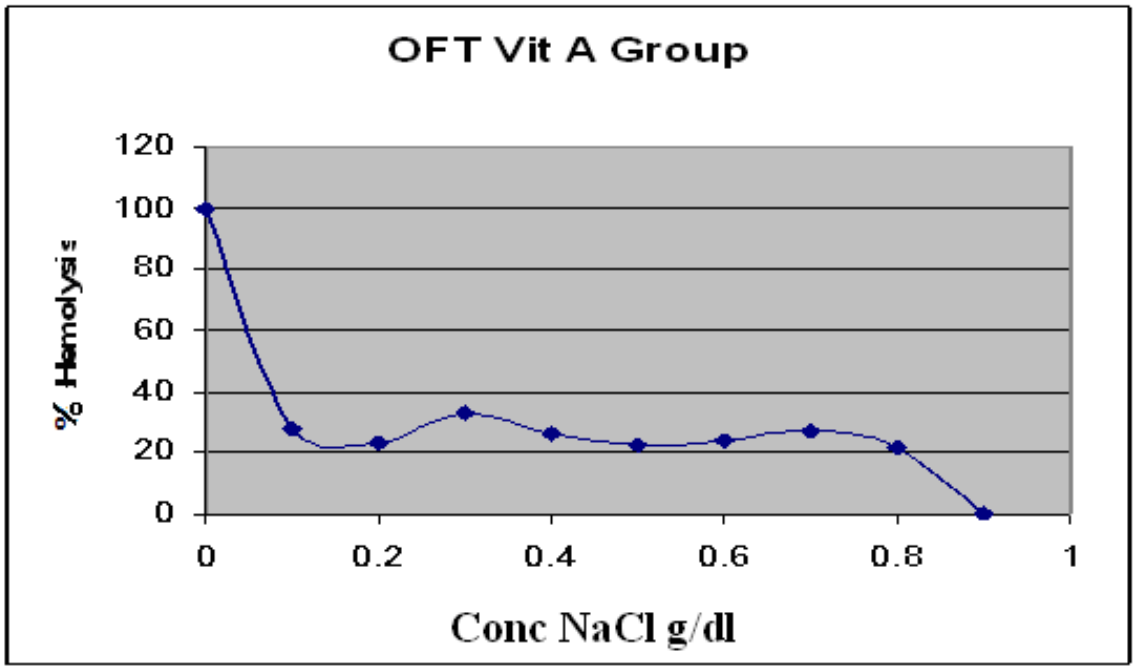

Figure 16: Osmotic Fragility Curve in Vitamin A Group

MCFI=0.115

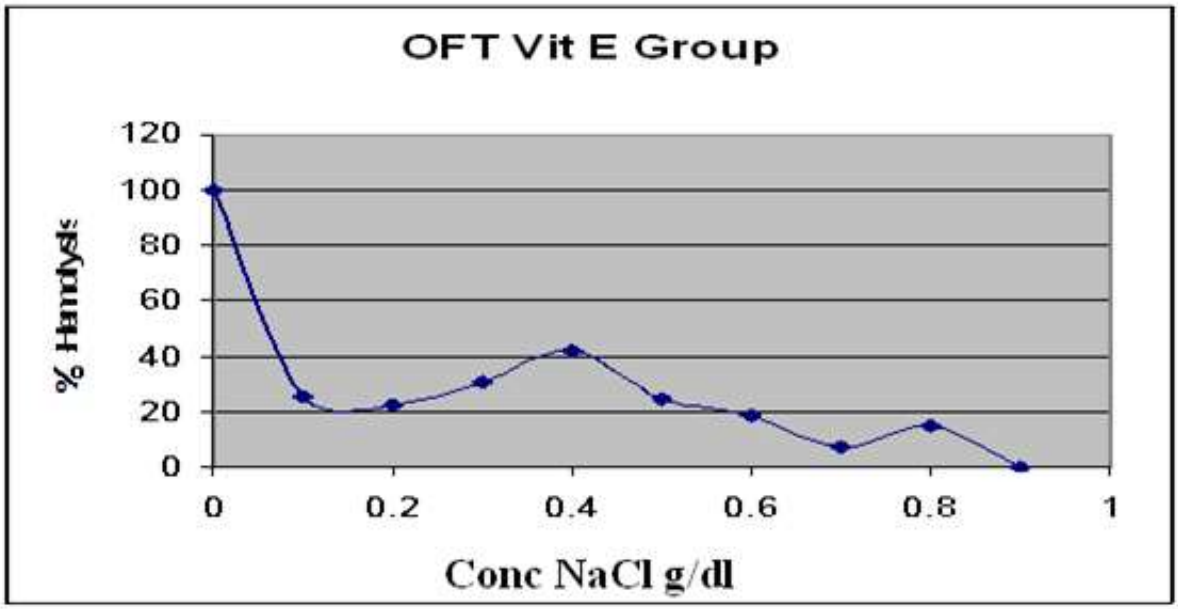

Figure 17: Osmotic Fragility Curve in Vitamin E Group

MCFI=0.129

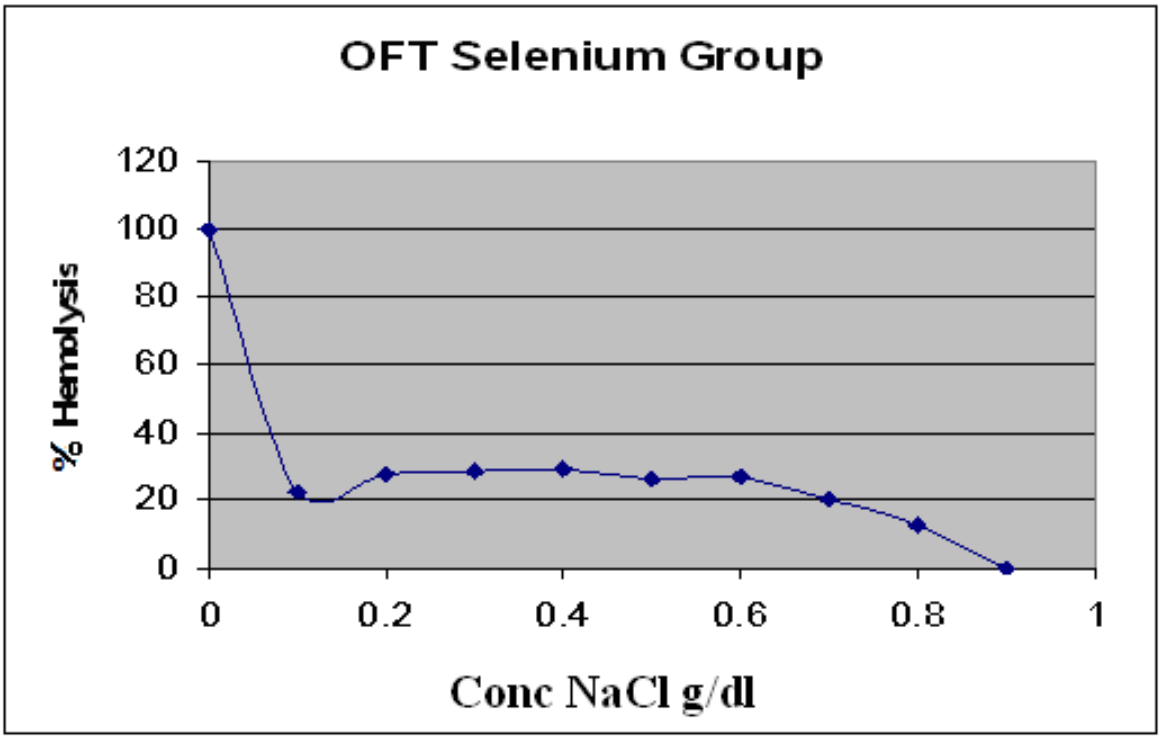

Figure 18: Osmotic Fragility Curve in Selenium Group

MCFI=0.114

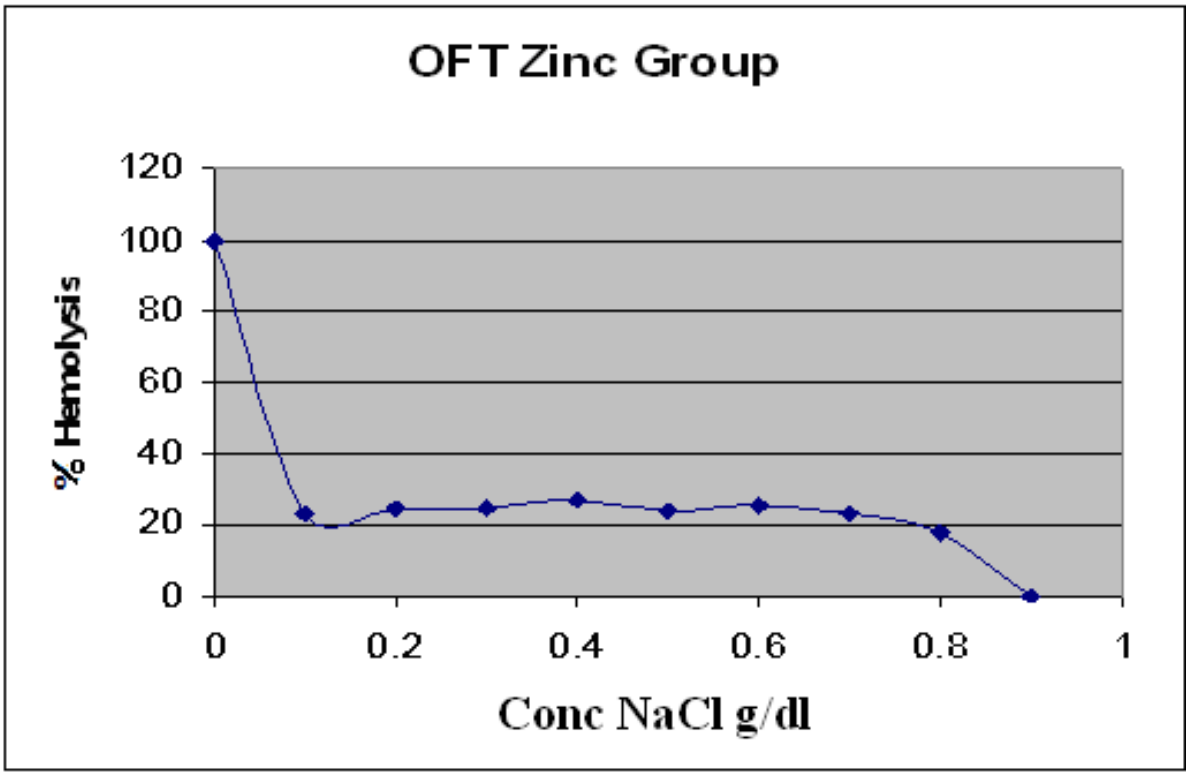

Figure 19: Osmotic Fragility Curve in Zinc Group

MCFI=0.08

As shown in Table 8, there is a significant difference in the mean corpuscular fragility index (MCFI) between groups ($F = 2275.65$; $P < 0.05$). This determines the % erythrocyte membrane stability or distability of the treatment groups. However, there was no statistically significant difference ($p > 0.05$) in MCFI values of vitamin A and selenium treated groups. Erythrocyte membrane distability (Figure 20) was most marked in the tween 80 group (426.15%), followed closely by the chloroquine (373.85%) treated group and artesunate group (329.23%) and least in the zinc treated group (32.31%)

Increased lipid peroxidation is associated with significant elevation of markers of oxidative stress. As shown in Table 9, malondialdehyde (MDA) levels were significantly higher in the distilled water, tween 80, chloroquine and artesunate treated groups when compared to apparently healthy uninfected control ($p < 0.05$). There was a significant difference in antioxidant activity between groups with the exception of superoxide dismutase ($F=0.81$; $P > 0.05$). However, catalase activity was significantly higher (Table 9; Figure 21) in the chloroquine, artesunate, vitamin A, E, selenium and zinc treated groups while glutathione peroxidase activity was significantly higher in the vitamin A, E, selenium and zinc treated groups respectively when compared to apparently healthy uninfected control ($p < 0.05$).

Table 8: Mean Corpuscular Fragility Index and % Membrane Distability after 4 Day Curative Test in *P. berghei* Parasitized Mice (n=5mice each)

Group	Dose mg/kg	% Parasitemia	MCFI (g/dl)	% Membrane Distability
Control (not inoculated/no treatment)	-	-	0.065±.003	0.00
Negative Control (Distilled H_2 0)	0.2ml	24.14±2.59	0.239±0.03	267.69
Tween-80	0.2ml	21.82±3.42	0.343±0.002	426.15
Positive Control (Chloroquine)	25	0.00±0.00	0.308±0.001	373.85
Artesunate	4	0.00±0.00	0.279±0.002	329.23
Vitamin A	60	8.90±2.78	[a]0.115±0.002	76.92
Vitamin E	100	11.61±2.78	0.129±0.002	98.46
Selenium	1	5.08±1.85	[a] 0.114±0.002	75.38
Zinc	100	9.89±1.11	0.086±0.003	32.31
ONE WAY ANOVA			**F = 2275.65** **P <0.05**	

Values are expressed as X±SEM. df = 4, Mean difference is significant at p<0.05. [a] No significant difference in MCFI value between vitamin A and selenium treated groups.

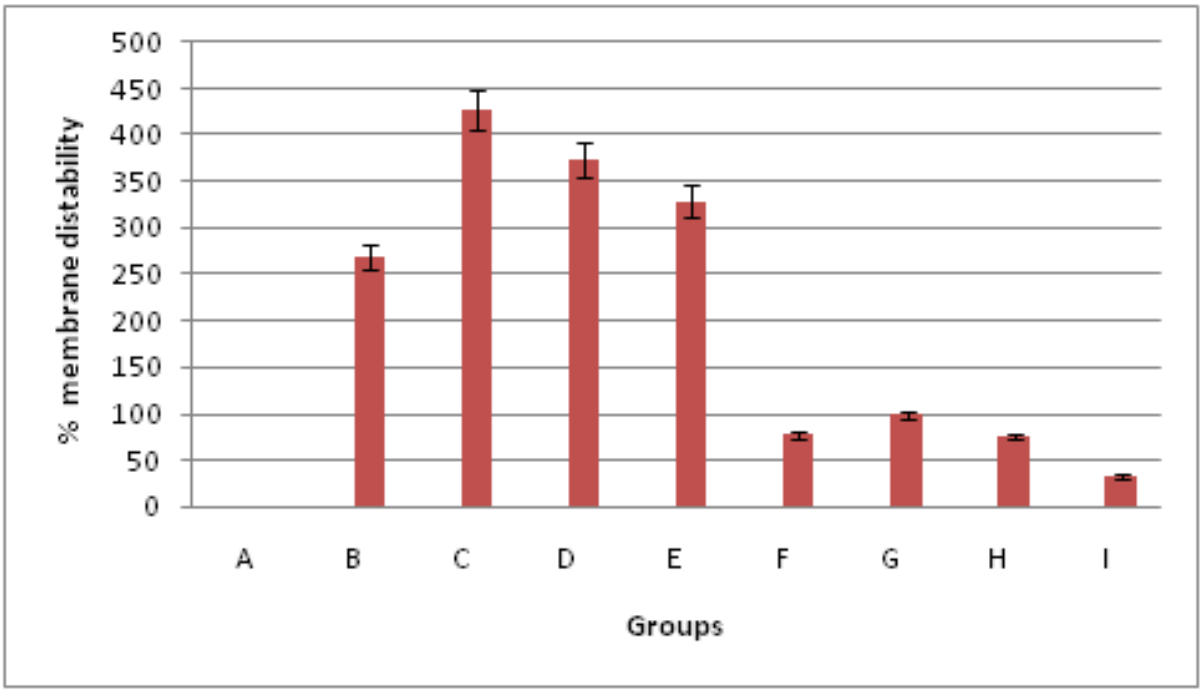

Figure 20: % Membrane Distability after 4 day Curative Test.

Key: A=Control (no inoculation, no treatment), B=Dist. H_2O, C=Tween-80, D=Chloroquine, E=Artesunate, F=Vit A, G=Vit E, H=Selenium, I=Zinc

Table 9: Lipid Peroxidation and Antioxidant Markers in *P. berghei* Parasitized Mice after 4 day Curative Treatment (n=5 mice per group)

Group	Dose mg/kg	% Parasitemia	MDA mg/ml	SOD min/mg protein	CAT µmol/min/mg protein	GPX µmol/ml	Total Protein g/L
Control (not inoculated)	-	-	2.020±0.020	0.062±0.001	1.085±0.028	4.580±0.045	47.59±0.60
Negative Control (Distilled H_2 0)	0.2ml	24.14±2.59	*6.020±0.296	0.043±0.120	1.396±0.033	4.213±0.056	44.56±0.76
Tween-80	0.2ml	21.82±3.42	*5.794±0.449	0.054±0.004	1.416±0.144	4.472±0.141	44.00±0.48
Positive Control (Chloroquine)	25	0.00±0.00	*4.340±0.068!	0.057±0.003	*1.932±0.017!	4.646±0.029	52.64±1.00
Artesunate	4	0.00±0.00	*8.200±0.491!	0.064±0.005	*1.880±0.050!	4.418±0.172	58.00±1.55
Vitamin A	60	8.90±2.78	2.060±0.024!	0.084±0.002	*2.374±0.170!	*5.444±0.100!	46.90±0.62
Vitamin E	100	11.61±2.78	2.080±0.020!	0.075±0.004	*2.745±0.055!	*5.420±0.091!	49.34±0.60
Selenium	1	5.08±1.85	2.080±0.020!	0.092±0.004	*2.754±0.056!	*6.484±0.189!	44.20±0.92
Zinc	100	9.89±1.11	2.040±0.025!	0.078±0.004	*2.467±0.106!	*5.842±0.037!	43.55±0.84
ONE WAY ANOVA		**F=19.76 P<0.05**	**F=91.65 P<0.05**	**F=0.81 P>0.05**	**F=47.81 P<0.05**	**F=48.69 P<0.05**	**F=30.54 P<0.05**

Values are expressed as X±SEM. *Mean difference is significant at p<0.05 when compared to control (not inoculated). ! Mean difference is significant at p<0.05 when compared to negative control. Key: MDA=Malondialdehyde, SOD=Superoxide dismutase, CAT=Catalase, GPX=Gluthatione peroxidase.

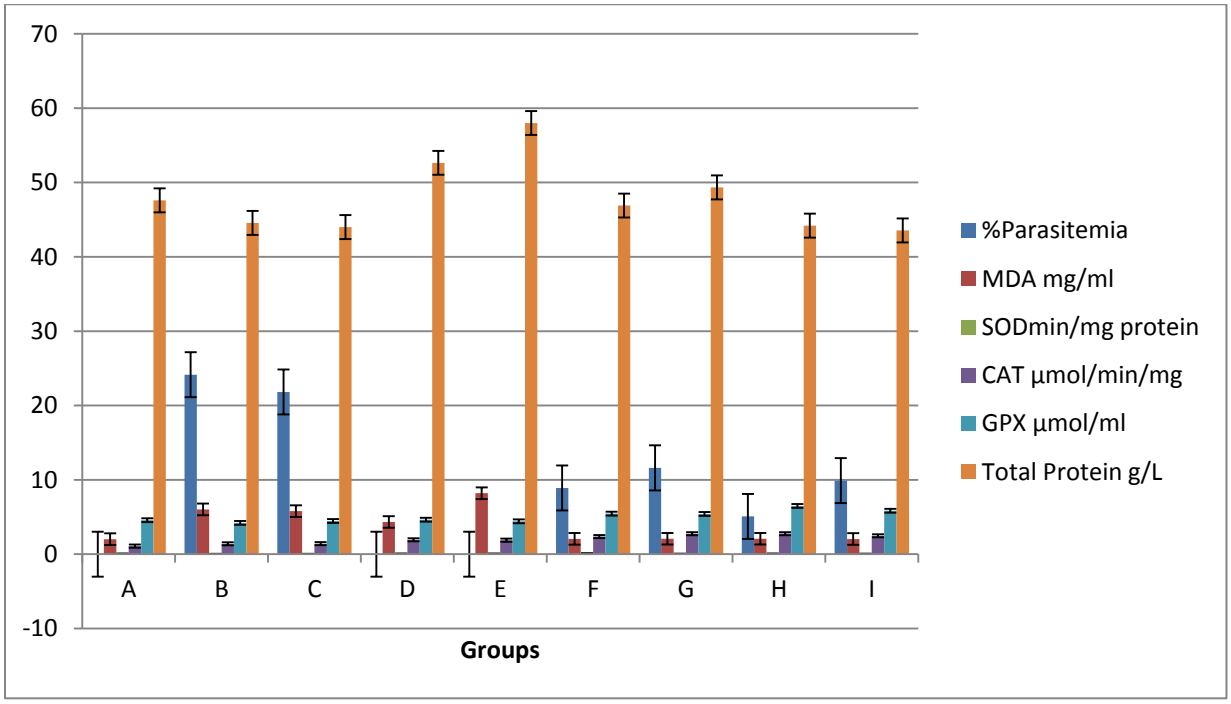

Figure 21: Oxidative Stress and Antioxidant Activity after 4 day Curative Treatment in *Plasmodium berghei* Parasitized Mice. Key: A=Control (no inoculation, no treatment), B=Dist. H_2O, C=Tween-80, D=Chloroquine, E=Artesunate, F=Vit A, G=Vit E, H=Selenium, I=Zinc. MDA=Malondialdehyde, SOD=Superoxide dismutase, CAT=Catalase, GPX=Gluthatione peroxidase.

As shown in Table 10, there is a statistically significant difference in total white blood cell count (WBC) in the artesunate, vitamin A, E, selenium and zinc treated groups when compared with apparently healthy non-inoculated control ($p<0.05$). This was also observed in the vitamin A, E, selenium and zinc treated groups when compared to negative control group treated with distilled water ($p<0.05$). Similarly, there was a significant difference in platelet count, differential lymphocyte, monocyte and eosinophil count between groups.

As shown in Table 11, the red blood cell count was significantly reduced in the chloroquine treated group and significantly increased in the artesunate, vitamin A, E, selenium and zinc treated groups when compared to apparently healthy uninfected control ($p<0.05$). Additionally, a significant reduction in hemoglobin concentration was observed in the negative control group while a significant increase in hemoglobin concentration was noted in the vitamin A, E, selenium and zinc groups respectively ($p<0.05$). Interestingly, there was a significant reduction in MCV and MCH values in all the treatment groups when compared with apparently healthy uninfected control. Furthermore, no significant change was observed in the MCHC values (Table 11) between groups and control except for a significant increase in MCHC in the zinc treated group when compared to apparently healthy uninfected control (34.21 ± 0.29 g/dl versus 31.88 ± 0.63 g/dl; $p<0.05$).

Table 10: WBC and Platelet Count in *P. berghei* Parasitized Mice after 4 days of Treatment (n= 5 mice per group)

Group	Dose mg/kg	WBC/mm^3 x10^3	PLT/mm^3 x10^5	LYM (%)	NEUT (%)	MO (%)	EO (%)	BASO(%)
Control (not inoculated)	-	4.72±132.15	1.21±3006.66	35.60±1.21	63.20±0.66	1.00±0.32	0.00±0.00	0.00±0.00
Negative Control	0.2ml	5.18±168.52	*9.16±7752.42	51.80±1.20	43.80±1.16	2.00±0.45	2.00±0.55	0.00±0.00
(Distilled H_2 0)	0.2ml	4.98±137.42	*3.32±10734.99$^!$	48.00±0.71	50.00±0.71	1.00±0.63	1.00±0.32	0.00±0.00
Tween-80	25	4.84±92.74	*4.46±5544.37$^!$	39.00±0.71	61.00±0.71	0.00±0.00	0.00±0.00	0.00±0.00
PositiveControl (Chloroquine)	4	*5.59±211.34	1.07±2302.17$^!$	55.00±0.89	45.00±0 .632	0.00±0.00	0.00±0.00	0.00±0.00
Artesunate	60	*7.13±139.18$^!$	*8.36±17162.75$^!$	60.00±0.71	40.00±0.71	0.00±0.00	0.00±0.00	0.00±0.00
Vitamin A	100	*7.10±100.48$^!$	*6.23±7756.29$^!$	62.00±0.84	38.00±1.22	0.00±0.00	0.00±0.00	0.00±0.00
Vitamin E	1	*7.680±345.54$^!$	*9.23±10610.84	68.00±0.84	32.00±0.84	0.00±0.00	0.00±0.00	0.00±0.00
Selenium	100	*6.97±67.68$^!$	*2.73±5124.45$^!$	58.00±1.67	42.00±0.84	0.00±0.00	0.00±0.00	0.00±0.00
Zinc								
ONE WAY ANOVA		**F=46.10 P<0.05**	**F= 1355.43 P<0.05**	**F=108.81 P<0.05**	**F=146.11 P<0.05**	**F=6.79 P<0.05**	**F=11.25 P<0.05**	

Values are expressed as X±SEM. *Mean difference is significant at p<0.05 when compared to control (not inoculated). $^!$ Mean difference is significant at p<0.05 when compared to negative control.

Key: WBC=White Blood Cell Count, PLT=Platelet count, LYM=Differential lymphocyte count, NEUT=Differential neutrophil count, MO=Differential monocyte count, EO=Differential eosinophil count, BASO=Differential basophil count.

Table 11: Hematological Profile in *P. berghei* Parasitized Mice after 4 day Curative Test

Group	Dose mg/kg	RBC (/mm^3) x10^6	Hb (g/dl)	PCV (%	MCV (fL)	MCH (pg)	MCHC (g/dl)
Control (not inoculated)	-	5.52±1.56x10^5	12.52±0.20	38.21±0.29	82.66±0.76	28.96±0.94	31.88±0.63
Negative Control (Distilled H_2 0)	0.2ml	5.13±1.01 x10^5	*10.90±0.36	33.68±0.62	*54.30±0.45	*17.03±0.12	31.85±0.25
Tween-80	0.2ml	5.06±1.03 x10$^{5!}$	11.39±0.18	34.62±0.84	*50.82±0.43$^!$	*17.49±0.27	32.51±0.50
PositiveControl (Chloroquine)	25	*3.68±5.54 x10$^{4!}$	12.86±0.22	35.08±0.47$^!$	*47.29±0.57$^!$	*16.83±0.17	33.53±0.30
Artesunate	4	*8.23±1.51x10$^{5!}$	13.62±0.25	42.22±0.64$^!$	*51.48±0.58$^!$	*16.96±0.18	32.40±0.56
Vitamin A	60	*8.58±8.66 x10$^{4!}$	*15.37±0.39	42.29±0.38$^!$	*52.17±0.68	*16.47±0.23	32.92±0.46
Vitamin E	100	*9.41±1.43 x10$^{5!}$	*15.70±0.26	49.02±0.36$^!$	*51.16±0.48$^!$	*16.22±0.16	31.85±0.35
Selenium	1	*8.48±2.72 x10$^{5!}$	*15.24±0.421	45.48±0.62$^!$	*51.42±0.37$^!$	*17.20±0.24	31.42±0.68
Zinc	100	*6.79±1.6772 x10$^{5!}$	12.09±0.21	37.75±0.26	*48.18±0.46$^!$	*27.31±0.17$^!$	*34.21±0.29$^!$
ONE WAY ANOVA		**F=179.68 P<0.05**	**F=38.17 P<0.05**	**F=98.36 P<0.05**	**F=388.23 P<0.05**	**F=368.96 P<0.05**	**F=3.72 P<0.05**

Values are expressed as X±SEM. *Mean difference is significant at p<0.05 when compared with control (not inoculated). ! Mean difference is significant at p<0.05 when compared with negative control.

Key: RBC=Red Blood Cell Count, Hb=Hemoglobin concentration, PCV=Packed Cell Volume, MCV=Mean Corpuscular Volume, MCH=Mean Corpuscular Hemoglobin, MCHC=Mean Corpuscular Hemoglobin Concentration.

4.2 CLINICAL STUDIES

Of the 150 participants recruited for this study, only 116 (77.33%) were successfully and completely followed up over a period of 4 weeks. The mean peak age of the participants in the study was 2.31±0.11 years. Male: Female ratio being 1:1 (75 males and 75 females respectively). Mean weight of the study population at presentation was 12.97±0.34 kg while the mean Temperature, Packed Cell Volume (PCV), Hemoglobin concentration and Total White Blood Cell Count (WBC) were $38.03\pm0.37^{0}C$, 33.21±0.37%, 11.01±0.13g/dL and $6{,}507.27\pm217.00/mm^{3}$ respectively.

The mean baseline parasite density in the study population was 17,677.67±17.68/µL. However, there was no significant difference ($P>0.05$) in base line parameters between groups (Table 12). As shown in Table 13, 57 (38%) of the participant's Parents/Guardians were traders, while 46 (30.7%) were civil servants.

All the participants 150 (100%) in the study presented with fever on day 0 of recruitment. Apart from fever which was a base line feature of the participants, loss of appetite 120 (80%) and weakness 47 (31.3%) appeared to be the most common symptoms at presentation (Figure 22). Other symptoms elicited from the study participants were abdominal discomfort 21 (14%), frequent passage of loose stools 30 (20%) and rigor 23 (15.3%).

Table 12: Base Line Characteristic of the Study Population (Mean ± SEM)

Groups N= 10	Age(Years)	Weight (kg)	Temperature(0C)	Parasite Density(/µL)
Amodia + Arte	1.95±0.42	13.20±1.58	38.06±0.07	8694.00±1668.20
Artemet +Lume	2.16±0.44	12.80±1.17	37.96±0.09	22459.00±5214.01
Arte + Vit A	2.55±0.35	14.40±0.87	38.10±0.09	21445.00±8628.96
Arte + Vit E	1.70±042	10.10±1.57	38.06±0.12	15390.00±6040.90
Arte + Zinc	2.30±0.53	12.00±1.02	38.01±0.06	20196.10±7336.57
Arte + Sele	2.29±0.47	13.50±1.57	38.01±0.05	36260.00±9067.43
Amodia + Vit A	2.48±0.53	13.98±1.78	38.04±0.08	15608.00±4872.51
Amodia + Vit E	2.17±0.47	13.20±1.55	38.06±0.14	20156.00±6007.27
Amodia + Zinc	2.61±0.41	13.80±1.08	37.95±0.05	16242.00±5421.19
Amodia + Sel	2.44±0.48	13.40±1.31	38.15±0.14	14070.00±4643.83
Arte + Vit A + Vit E	2.15±0.47	12.40±1.67	37.98±0.07	10202.00±2959.02
Arte + Vit A+ Zinc	2.44±0.31	12.60±1.01	38.15±0.09	16712.00±3463.18
Arte + Vit A + Sel	2.56±0.25	13.60±1.36	38.08±0.11	24619.00±4940.08
Arte + Vit E + Zinc	2.44±0.51	13.90±1.18	37.91±0.05	9060.00±2103.47
Arte + Vit E + Sel	2.33±0.57	11.60±1.15	37.93±0.06	14052.00±4901.90
ONE WAY ANOVA	**F = 0.30** **P > 0.05**	**F = 0.67** **P > 0.05**	**F = 0.73** **P > 0.05**	**F = 1.46** **P > 0.05**

Values are expressed as Mean±SEM. df = 149, P < 0.05 is considered significant.

Table 13: Socio-Demographic Characteristic of Participants' Parents/Guardian

SEX	**FREQUENCY**	**PERCENTAGE (%)**
Male	75	50.0
Female	75	50.0
TOTAL	150	100
OCCUPATION		
C/Servant	46	30.7
Clergy	2	1.3
Driver	2	1.3
Farmer	17	11.3
H/Wife	15	10.0
Hair Dresser	3	2.0
Seamstress	7	4.7
Student	1	0.7
Trader	57	38.0
TOTAL	**150**	**100**

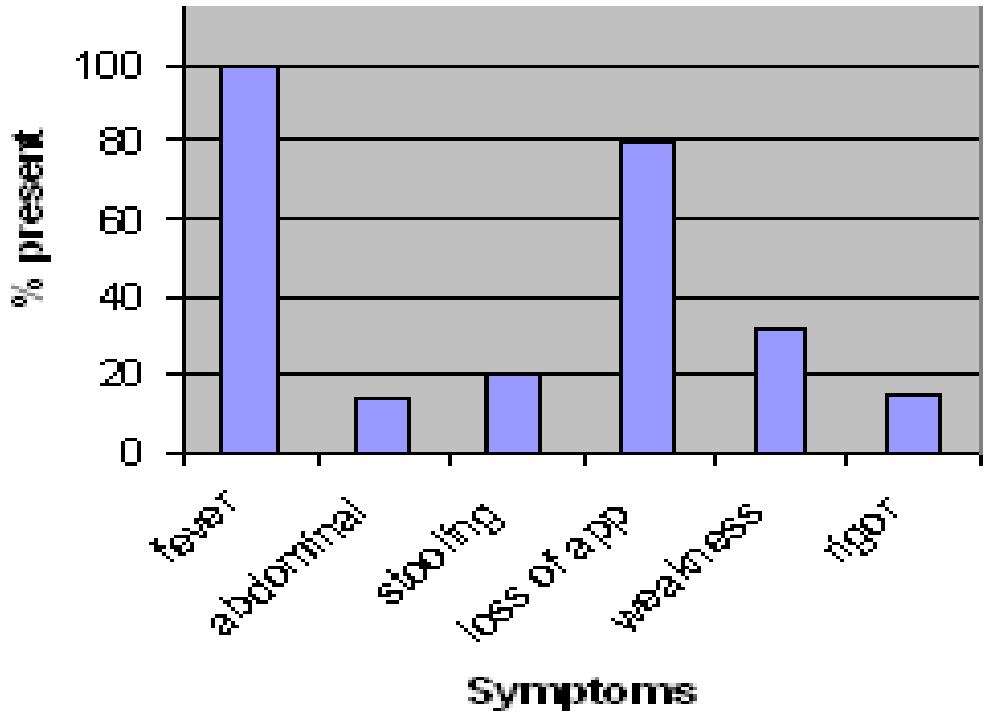

Figure 22: Frequency of Malaria Related Symptoms in Participants

There was no significant mean difference ($p > 0.05$) in the base line hemoglobin concentration, PCV and Total WBC count between the different study groups. However, a significant difference existed in the base line differential monocyte count between groups in the study population ($F = 2.27$, $P < 0.05$) as shown in Table 14.

As revealed in Table 15, the comparative analysis between the total WBC count on day 0 and day 28 post treatment revealed a significant increase in all the micronutrient treated groups on day 28 ($p<0.05$; $r = 0.76$; $P<0.01$). A comparatively significant increase ($p<0.05$) was also noted in the differential count of the micronutrient treated groups between day 0 and day 28 post treatment (Table 16).

As shown in Table 17, there was a consistent reduction of mean parasite density from day 0 to day 2 of treatment. The mean difference between groups was not significant ($p > 0.05$). However, the mean difference in the reduction of parasite density between groups on day 0/day 1, day 0/day 2 and day 1/day 2 respectively was statistically significant ($p < 0.05$).

Table 14: Base Line Hematological Profile of Participants (Mean ± SEM)

Groups N= 10	Hb (g/dL)	PCV (%)	WBC (/mm3)	Neutrophil (%)	Lymphocyte (%)	Monocyte (%)	Basophils (%)	Eosinophils (%)
Amodia + Arte	10.84±0.60	33.30±1.41	8130.00±1127.64	48.50±2.29	48.80±2.12	2.20±0.81	0.10±0.10	1.00±0.54
Artemet +Lume	11.85±0.33	35.60±1.05	5450.00±529.41	46.60±1.67	52.00±1.78	0.90±0.23	0.10±0.10	0.40±0.40
Arte +Vit A	11.09±0.50	33.10±1.52	6810.00±954.92	49.60±3.02	48.10±3.23	1.30±0.30	0.40±0.22	0.60±0.34
Arte + Vit E	10.52±0.36	31.80±1.04	7468.00±1008.80	52.40±2.24	44.70±2.31	1.70±0.75	0.20±0.13	0.90±0.53
Arte + Zinc	11.61±0.43	35.00±1.26	5429.00±482.03	50.50±1.96	48.70±1.87	0.80±0.20	0.10±0.10	0.10±0.10
Arte + Se	11.56±0.43	34.70±1.38	7840.00±1482.88	54.40±1.83	44.50±1.85	0.80±0.25	0.20±0.13	0.10±0.10
Amodia + Vit A	10.28±0.58	31.60±1.38	6510±768.32	53.20±1.99	46.00±2.01	0.40±0.22	0.20±0.20	0.20±0.20
Amodia + Vit E	10.86±0.52	32.80±1.51	5870.00±410.43	55.20±2.58	43.70±2.56	0.60±0.22	0.20±0.13	0.30±0.21
Amodia + Zinc	10.72±0.43	32.00±1.34	7272.00±845.37	47.50±1.92	51.30±1.90	0.60±0.22	0.40±0.22	0.10±0.10
Amodia + Se	11.08±0.55	33.80±1.60	5740.00±412.09	50.80±1.95	47.50±2.41	0.30±0.15	0.20±0.13	0.20±0.20
Arte+ Vit A + Vit E	11.19±0.38	33.70±1.17	6360±1246.08	51.40±2.73	47.90±2.64	0.50±0.17	0.10±0.10	0.20±0.13
Arte + Vit A+ Zinc	11.06±0.61	33.50±2.15	6240±1831.33	53.80±1.43	45.50±1.22	0.70±0.42	0.00±0.00	0.00±0.00
Arte + Vit A + Se	11.20±0.69	32.70±1.61	6260±288.75	50.30±1.54	49.10±1.62	0.40±0.16	0.00±0.00	0.20±0.13
Arte + Vit E + Zinc	10.66±0.48	32.00±1.48	6430±728.17	46.90±1.14	52.50±1.10	0.20±0.13	0.30±0.15	0.10±0.10
Arte+ Vit E+ Se	10.68±0.6	32.50±1.65	5800±728.77	48.50±2.77	49.60±2.59	1.20±0.36	0.20±0.13	0.50±0.27
ONE WAY ANOVA	**F = 0.71 P > 0.05**	**F = 0.68 P > 0.05**	**F = 1.01 P> 0.05**	**F = 1.66 P > 0.05**	**F = 1.61 P > 0.05**	**F = 2.27 P < 0.05**	**F = 0.75 P > 0.05**	**F = 1.22 P > 0.05**

Values are expressed as Mean±SEM. df = 149, P < 0.05 is considered significant

Table 15: Comparative Profile of Total WBC (/mm^3) Count on Day 0 of Treatment and Day 28 of Follow Up

Groups	**WBC_0 (/mm^3)**	**WBC_{28} (/mm^3)**
Apparently Healthy Control (N=10)	6400±1017.64	6985±1212.88
Amodia + Arte (N0=10,N28=8)	8130.00±1127.64	7110.00±443.83
Artemet + Lume (N0=10,N28=9)	5450.00±529.41	6410.00±507.38
Arte + vit A (N0=10,N28=6)	6810.00±954.92	9236.00±660.60
Arte + vit E(N0=10,N28= 7)	7468.00±1008.80	9609.00±746.76
Arte + Zinc (N0=10,N28=8)	5429.00±482.03	8648.00±338.12
Arte + Se (N0=10,N28=8)	7840.00±1482.88	10051.00±1025.41
Amodia + VitA (N0=10,N28=8)	6510±768.32	8463.00±616.93
Amodia + VitE (N0=10,N28=8)	5870.00±410.43	8759.00±451.68
Amodia + Zinc (N0=10,N28=7)	7272.00±845.37	8925.00±708.34
Amodia + Se (N0=10,N28=9)	5740.00±412.09	8570.90±449.04
Arte+VitA+VitE (N0=10,N28=7)	6360±1246.08	9488.00±819.86
Arte+VitA+Zic (N0=10,N28=6)	6240±1831.33	9498.00±490.32
Arte+VitA+Se (N0=10,N28=8)	6260±288.75	9002.00±513.22
Arte+VitE+Zinc (N0=10,N28=7)	6430±728.17	9290.00±459.58
Arte+VitE+Se (N0=10,N28=10)	5800±728.77	9176.00±413.20
ONE WAY ANOVA	**F = 1.01** **P > 0.05**	**F = 2.47** **P < 0.05**
PAIRED T-TEST **WBC_0 /WBC_{28}**	**P < 0.05**	
PEARSON'S CORRELATION	**0.76, P < 0.01**	

WBC_0 = white blood cell count on day 0 of Treatment; WBC_{28} = white blood cell count on day 28 of follow up, P < 0.05 is significant; P < 0.01 is strongly significant.

Table 16: Comparative Profile of WBC Differential Count on Day 0 of Treatment and Day 28 of Follow Up

Groups	N_0 %	L_0 %	M_0%	B_0 %	E_0 %	N_{28} %	L_{28} %	M_{28} %	B_{28} %	E_{28} %
Amodia + Arte (N0=10,N28=8)	48.50±2.29	48.80±2.12	2.20±0.81	0.10±0.10	1.00±0.54	53.80±1.11	45.90±1.22	0.20±0.13	0.10±0.10	0.00±0.00
Artemet +Lume (N0=10,N28=9)	46.60±1.67	52.00±1.78	0.90±0.23	0.10±0.10	0.40±0.40	54.40±1.10	44.80±0.84	0.30±0.15	0.10±0.10	0.30±0.15
Arte +Vit A (N0=10,N28=6)	49.60±3.02	48.10±3.23	1.30±0.30	0.40±0.22	0.60±0.34	38.70±1.23	60.80±1.27	0.40±0.22	0.10±0.10	0.00±0.00
Arte + Vit E (N0=10,N28= 7)	52.40±2.24	44.70±2.31	1.70±0.75	0.20±0.13	0.90±0.53	39.90±0.92	59.40±0.79	0.50±0.22	0.10±0.10	0.10±0.10
Arte+Zinc (N0=10,N28=8)	50.50±1.96	48.70±1.87	0.80±0.20	0.10±0.10	0.10±0.10	42.80±1.23	57.00±1.18	0.20±0.13	0.00±0.00	0.00±0.00
Arte+Se (N0=10,N28=8)	54.40±1.83	44.50±1.85	0.80±0.25	0.20±0.13	0.10±0.10	41.60±1.51	57.70±1.50	0.30±0.15	0.30±0.15	0.20±0.13
Amodia+VitA (N0=10,N28=8)	53.20±1.99	46.00±2.01	0.40±0.22	0.20±0.20	0.20±0.20	40.20±1.46	59.80±1.46	0.00±0.00	0.00±0.00	0.00±0.00
Amodia+VitE (N0=10,N28=8)	55.20±2.58	43.70±2.56	0.60±0.22	0.20±0.13	0.30±0.21	37.30±1.87	61.90±1.68	0.50±0.22	0.10±0.10	0.20±0.13
Amodia+Zinc (N0=10,N28=7)	47.50±1.92	51.30±1.90	0.60±0.22	0.40±0.22	0.10±0.10	37.30±1.68	62.00±1.67	0.70±0.30	0.00±0.00	0.00±0.00
Amodia+Se (N0=10,N28=9)	50.80±1.95	47.50±2.41	0.30±0.15	0.20±0.13	0.20±0.20	41.50±1..56	58.50±1.56	0.00±0.00	0.00±0.00	0.00±0.00
Arte+VitA+VitE (N0=10,N28=7)	51.40±2.73	47.90±2.64	0.50±0.17	0.10±0.10	0.20±0.13	40.00±1..50	59.50±1.46	0.30±0.21	0.10±0.10	0.10±0.10
Arte+VitA+Zinc (N0=10,N28=6)	53.80±1.43	45.50±1.22	0.70±0.42	0.00±0.00	0.00±0.00	38.40±2.02	61.40±1.96	0.20±0.13	0.00±0.00	0.00±0.00
Arte+VitA+Se (N0=10,N28=8)	50.30±1.54	49.10±1.62	0.40±0.16	0.00±0.00	0.20±0.13	41.00±1..51	59.00±1.51	0.00±0.00	0.00±0.00	0.00±0.00
Arte+VitE+Zinc (N0=10,N28=7)	46.90±1.14	52.50±1.10	0.20±0.13	0.30±0.15	0.10±0.10	37.70±1.76	62.80±1.51	0.40±0.22	0.00±0.00	0.10±0.10
Arte+VitE+Se (N0=10,N28=10)	48.50±2.77	49.60±2.59	1.20±0.36	0.20±0.13	0.50±0.27	37.40±1..22	61.90±1.03	0.60±0.27	0.00±0.00	0.10±0.10
ONE WAY ANOVA	**F = 1.66 P>0.05**	**F=1.61 P>0.05**	**F=2.27 P>0.05**	**F=0.75 P>0.05**	**F=1.22 P>0.05**	**F=13.52 P<0.05**	**F=15.01 P<0.05**	**F=1.38 P>0.05**	**F=1.23 P>0.05**	**F=1.40 P>0.05**
PAIRED T-TEST	**P <0.05**	**P<0.05**	**P<0.05**	**P<0.05**	**P<0.05**					
PEARSON'S CORRELATION	**-0.07, P>0.05**	**-0.03, P>0.05**	**0.06, P>0.05**	**-0.04, P>0.05**	**0.01, P>0.05**					

P < 0.05 is significant. N=Neutrophil, L=Lymphocytes, M=Monocytes, B=Basophils, E=Eosinophils (0=day 0 of treatment, 28=day 28 of follow up)

Table 17: Mean Level of Parasitemia (/µL) in Different Groups from Day 0 to Day 2 (D0-D2) of Treatment

Groups	D0	D1	D2	**Paired t-test D0/D1**	**Paired t-test D0/D2**	**Paired t-test D1/D2**
Amodia + Arte (N0=10,N1=8,N2=8)	8694.00±1668.20	220.00±170.21	0.00±0.00	**P < 0.05**	**P < 0.05**	**P <0.05**
Artem+Lume (N0=10,N1=9,N2=9)	22459.00±5214.01	160.00±106.47	0.00±0.00	**P < 0.05**	**P < 0.05**	**P <0.05**
Arte + Vit A (N0=10,N1=6,N2=6)	21445.00±8628.96	396.67±224.52	153.33±153.33	**P < 0.05**	**P < 0.05**	**P <0.05**
Arte + Vit E (N0=10,N1=7,N2=7)	15390.00±6040.90	645.71±234.08	0.00±0.00	**P < 0.05**	**P < 0.05**	**P <0.05**
Arte + Zinc (N0=10,N1=8,N2=8)	20196.10±7336.57	255.00±176.30	121.25±121.25	**P < 0.05**	**P < 0.05**	**P <0.05**
Arte + Se (N0=10,N1=8,N2=8)	36260.00±9067.43	263.75±173.41	0.00±0.00	**P < 0.05**	**P < 0.05**	**P <0.05**
Amod+Vit A (N0=10,N1=8,N2=8)	15608.00±4872.51	263.75±173.41	0.00±0.00	**P < 0.05**	**P < 0.05**	**P <0.05**
Amod+Vit E (N0=10,N1=8,N2=8)	20156.00±6007.27	701.25±395.55	171.25±171.25	**P < 0.05**	**P < 0.05**	**P <0.05**
Amod+Zinc (N0=10,N1=7,N2=7)	16242.00±5421.19	750.00±284.41	0.00±0.00	**P < 0.05**	**P < 0.05**	**P <0.05**
Amod+Se (N0=10,N1=9,N2=9)	14070.00±4643.83	354.44±222.31	0.00±0.00	**P < 0.05**	**P < 0.05**	**P <0.05**
Arte+Vit A+Vit E(N0=10,N1=7,N2=7)	10202.00±2959.02	418.57±282.95	124.29±124.29	**P < 0.05**	**P < 0.05**	**P <0.05**
Arte+Vit A+Zinc(N0=10,N1=6,N2=6)	16712.00±3463.18	206.67±206.67	0.00±0.00	**P < 0.05**	**P < 0.05**	**P <0.05**
Arte+ Vit A+Se(N0=10,N1=8,N2=8)	24619.00±4940.08	475.25±182.27	0.00±0.00	**P < 0.05**	**P < 0.05**	**P <0.05**
Arte+Vit E+Zinc(N0=10,N1=7,N2=7)	9060.00±2103.47	124.29±124.29	0.00±0.00	**P < 0.05**	**P < 0.05**	**P <0.05**
Arte+ VitE+Se (N0=10,N1=10,N2=10)	14052.00±4901.90	313.00±163.84	0.00±0.00	**P < 0.05**	**P < 0.05**	**P <0.05**
ONE WAY ANOVA	**F = 1.46** **P > 0.05**	**F = 0.79** **P > 0.05**	**F = 0.85** **P > 0.05**			
BONFERRONI CORRECTION				**P > 0.05**	**P > 0.05**	**P > 0.05**

Values are expressed as Mean ± SEM, D0/D1 df = 115, D0/D2 df = 114, D1/D2 df = 114. P<0.05 is considered significant. Key: N0=number of participants on day 0, N1= number of participants on day 1, N2=number of participants on day 2.

As shown in Table 18, the mean temperature difference between groups on day 0/day 1 and day 0/day 2 was statistically significant ($p < 0.05$). On day 1 of treatment (Figure 23 a-c) there was a rapid decline in the mean axillary temperature (38.03 ± 0.37^0C) on day 0 in all the groups.

Mean temperature was maintained below 37.5^0C during the period of follow up. However, the amodiaquine + zinc combination group showed a more rapid decline in temperature when compared to the other groups. This was sustained throughout the period of monitoring.

There was a negative correlation between the parasite density, PCV and hemoglobin concentration in the study population ($r = -0.102$; $P > 0.01$; $r = -0.08$; $P > 0.01$ respectively). This was also the case between the parasite density and WBC count, which showed a negative correlation ($r = -0.004$; $P > 0.01$) (Table 19).

Table 18: Mean Temperature Level (Mean ± SEM) in Different Groups from Day 0 to Day 2 (D0-D2) of Treatment

Groups	T0 (^{0}C)	T1 (^{0}C)	T2 (^{0}C)	**Paired t-test T0/T1**	**Paired t-test T0/T2**	**Paired t-test T1/T2**
Amodia+Arte (N0=10,N1=8,N2=8)	38.06±0.07	36.98±0.10	36.89±0.08	**P < 0.05**	**P <0.05**	**P >0.05**
Artemet+ Lume (N0=10,N1=9,N2=9)	37.96±0.09	36.81±0.07	36.96±0.07	**P < 0.05**	**P <0.05**	**P >0.05**
Arte+Vit A (N0=10,N1=6,N2=6)	38.10±0.09	37.02±0.10	37.05±0.12	**P < 0.05**	**P <0.05**	**P >0.05**
Arte+ Vit E (N0=10,N1=7,N2=7)	38.06±0.12	37.10±0.14	36.89±0.08	**P < 0.05**	**P <0.05**	**P >0.05**
Arte + Zinc (N0=10,N1=8,N2=8)	38.01±0.06	37.03±0.12	36.99±0.06	**P < 0.05**	**P <0.05**	**P >0.05**
Arte + Se (N0=10,N1=8,N2=8)	38.01±0.05	37.16±0.14	36.89±0.08	**P < 0.05**	**P <0.05**	**P >0.05**
Amodia + Vit A (N0=10,N1=8,N2=8)	38.04±0.08	37.05±0.15	36.99±0.06	**P < 0.05**	**P <0.05**	**P >0.05**
Amodia+Vit E (N0=10,N1=8,N2=8)	38.06±0.14	37.05±0.13	36.89±0.08	**P < 0.05**	**P <0.05**	**P >0.05**
Amodia + Zinc (N0=10,N1=7,N2=7)	37.95±0.05	31.84±5.31	36.95±0.08	**P < 0.05**	**P <0.05**	**P >0.05**
Amodia + Se (N0=10,N1=9,N2=9)	38.15±0.14	36.81±0.08	31.695±5.28	**P < 0.05**	**P <0.05**	**P >0.05**
Arte+Vit A+VitE(N0=10,N1=7,N2=7)	37.98±0.07	37.20±0.13	37.01±0.07	**P < 0.05**	**P <0.05**	**P >0.05**
Arte+Vit A+Zinc(N0=10,N1=6,N2=6)	38.15±0.09	36.87±0.12	37.03±0.17	**P < 0.05**	**P <0.05**	**P >0.05**
Arte+Vit A+Se (N0=10,N1=8,N2=8)	38.08±0.11	37.01±0.12	36.85±0.06	**P < 0.05**	**P <0.05**	**P >0.05**
Arte+Vit E+Zinc(N0=10,N1=7,N2=7)	37.91±0.05	37.08±0.10	36.94±0.05	**P < 0.05**	**P <0.05**	**P >0.05**
Arte+VitE+Se(N0=10,N1=10,N2=10)	37.93±0.06	36.87±0.87	36.97±0.05	**P < 0.05**	**P <0.05**	**P >0.05**
ONE WAY ANOVA	**F = 0.73** **P > 0.05**	**F = 1.07** **P > 0.05**	**F = 1.11** **P > 0.05**			
BONFERRONI CORRECTION				**P > 0.05**	**P > 0.05**	**P > 0.05**

Values are expressed as Mean±SEM. T0/TI df = 115, T0/T2 df = 115, T1/T2 df = 115. P < 0.05 is considered significant. Key: N0=number of participants on day 0, N1= number of participants on day 1, N2=number of participants on day 2. T0, T1, T2=Temperature on day 0, 1 and 2 respectively.

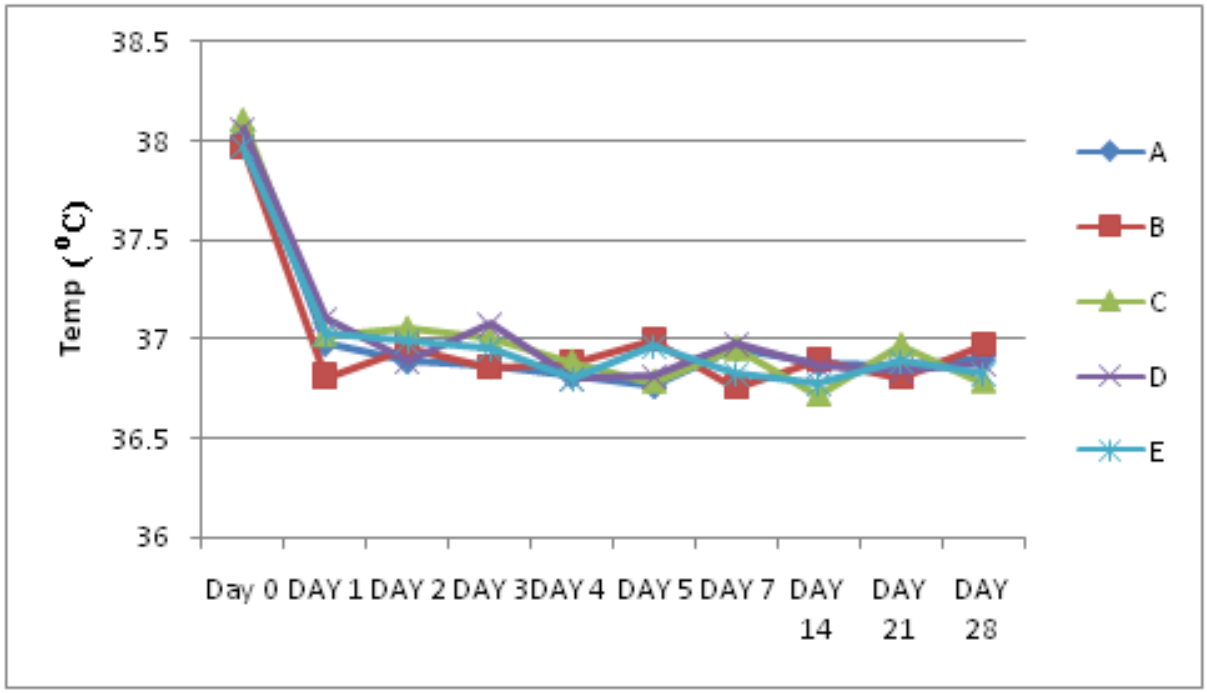

Figure 23a: Temperature Profile in the Treated groups from day 0 to day 28.

Key: A=artesunate+amodiaquine, B=artemether+lumefantrine, C=artesunate+vitA, D=artesunate+vitE, E= artesunate+zinc.

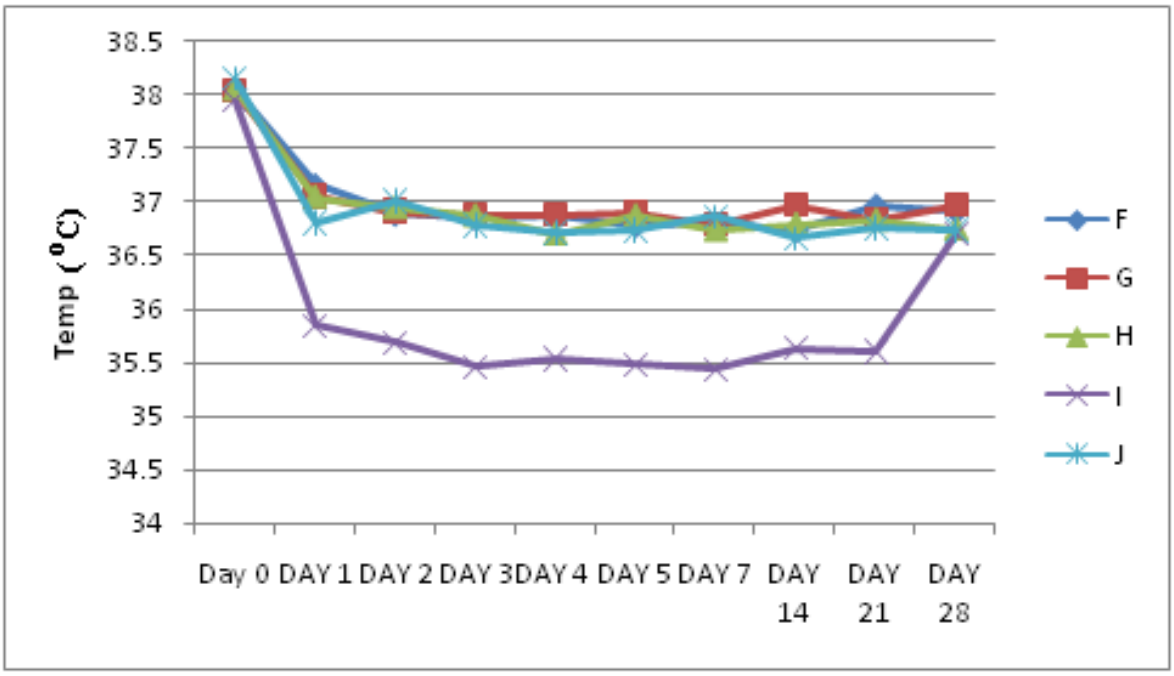

Figure 23b: Temperature Profile in the Treated groups from day 0 to day 28

Key: F = artesunate + selenium, G=amodiaquine+vitA, H= amodiaquine+vitE, I= amodiaquine+zinc, J=amodiaquine+selenium.

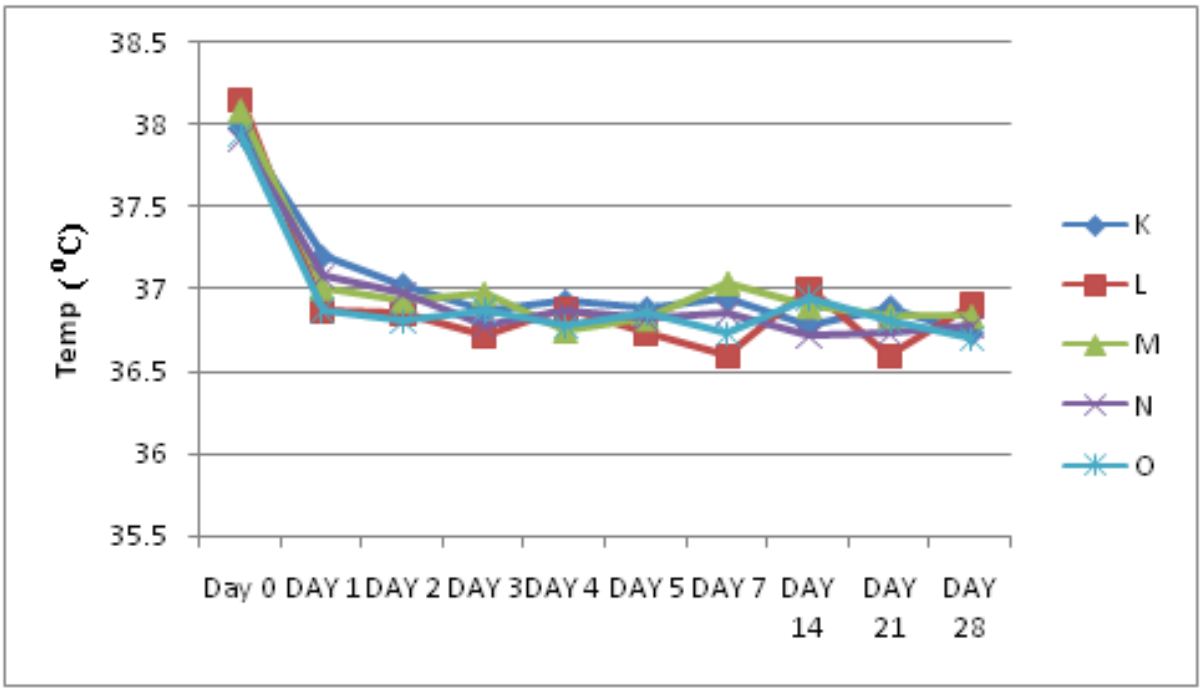

Figure 23c: Temperature Profile in the Treated groups from day 0 to day 28

Key: K=artesunate+vitA+vitE, L=artesunate+vitA+zinc, M=artesunate+vitA+selenium, N= artesunate+vit E + zinc, O =artesunate+vit E+selenium.

Table 19: Correlational Relationship between Parasite Density, Packed Cell Volume, Hemoglobin Concentration and White Blood Cell Count on Day 0

Parameters	**PCV (%)** **(r value)**	**Hb (g/dl)** **(r value)**	**WBC (/mm^3)** **(r value)**
Parasite Density **(day 0)**	-0.102*	-0.084*	-0.004*
Pearson's Correlation **(2tailed)**	P=0.019	P=0.025	P=0.016

P < 0.05 = significant

The mean parasite and fever clearance time in different treatment groups are shown in Table 20. A more rapid parasite clearance time (PCT) was recorded in the Artesunate + vitamin A + zinc treated group (26.00±4.82 hours) when compared with the active comparator groups (Amodiaquine + Artesunate; 27.00±3.00 hours and Artemether + Lumefantrine; 29.33±3.53 hours). However, this difference was not statistically significant ($F = 0.93$, $P > 0.05$) between the different treatment groups, since complete parasite clearance was observed within 48 hours of commencement of antimalarial therapy in all the groups.

Additionally, complete parasite clearance was maintained throughout the period of monitoring with no emergence of recrudescence in all the treatment groups in the succeeding 48 hours of consecutive microscopic monitoring. Similarly, a rapid fever clearance time was observed in the Artesunate + Zinc; 21.00±4.39 hours, Amodiaquine + Selenium; 22.67±1.33 hours, Artesunate + Vitamin A + Zinc group; 22.00±2.00 hours, Artesunate + Vitamin E + Zinc; 22.29±4.85 hours and Artesunate + Vitamin E + Selenium; 21.20±1.96 hours treated groups respectively when compared to the active comparator groups (Artesunate + Amodiaquine; 21.00±1.96 hours and Artemether + Lumefantrine; 21.33±1.76 hours respectively).

The mean difference in fever clearance time between groups was not statistically significant ($F = 1.02$, $P > 0.05$). However, a paired t test comparison between PCT and FCT in all the groups showed a statistically significant difference ($p < 0.05$). Additionally, there was a strongly positive correlation between the FCT and PCT ($r = 0.67$; $P < 0.01$). The 7 day cure rate per protocol population was consistently 100% in all the groups examined.

Table 20: Mean Parasite and Fever Clearance Time (Mean ± SEM) in Different Treatment Groups

Groups	PCT (HRS)	FCT (HRS)	7 DAY CURE RATE PER PROTOCOL POPULATION (%)	Paired t-test PCT/FCT
**Ref.Cntrl Artes+Amodia	27.54±2.00	33.6±12.00	98.70	**P < 0.05**
*Ref. CntrlArtemet +Lume	18.91 ± 11.5	31.17 ± 11.05	100.00	**P < 0.05**
Amodia + Arte, N,8	27.00±3.00	21.00±1.96	100.00	**P < 0.05**
Artemet +Lume, N,9	29.33±3.53	21.33±1.76	100.00	**P < 0.05**
Arte +Vit A, N,6	$^{+}$40.00±8.00	32.00±8.00	100.00	**P < 0.05**
Arte + Vit E, N7	37.71±4.85	30.86±4.43	100.00	**P < 0.05**
Arte + Zinc, N,8	$^{+}$33.00±6.31	21.00±4.39	100.00	**P < 0.05**
Arte + Se, N,8	30.00±3.93	27.00±4.94	100.00	**P < 0.05**
Amodia + Vit A,N,8,	36.00±6.40	28.50±4.50	100.00	**P < 0.05**
Amodia + Vit E, N,8	$^{+}$39.00±6.31	27.00±3.00	100.00	**P < 0.05**
Amodia + Zinc, N,7	*41.14±4.43	29.14±5.14	100.00	**P < 0.05**
Amodia + Se, N,9	32.00±4.00	22.67±1.33	100.00	**P < 0.05**
Arte +Vit A + Vit E, N,7	$^{+}$37.71±7.14	32.57±10.38	100.00	**P < 0.05**
Arte + Vit A+ Zinc,N,6	26.00±4.82	22.00±2.00	100.00	**P < 0.05**
Arte + Vit A + Se, N,8	*36.00±4.54	27.00±3.00	100.00	**P < 0.05**
Arte +Vit E + Zinc,N,7	27.43±3.43	22.29±4.85	100.00	**P < 0.05**
Arte + Vit E+ Se,N,10	27.20±3.67	21.20±1.96	100.00	**P < 0.05**
ONE WAY ANOVA	**F = 0.93** **P > 0.05**	**F = 1.02** **P >0.05**		
PEARSON'S CORRELATION	**r = 0.67** **P<0.01**			

Values are expressed as Mean±SEM. df = 115, P < 0.05 is considered significant. Key: N=number of participants on day 7. $^{+}$ Although parasite was present in some participant on day 2, parasite was completely cleared in some of the participants within 24 hours. *Parasite clearance occurred much ealier in some of the participants; this reduced the mean value to less than 48hours. * Reference control value for Artemether +Lumefantrine and **Artesunate +Amodiaquine in Africans (Michael and Srivicha, 2009; Abdul *et al.,* 2011).

This was also the 14 day and the 28 day cure rates. However, with the 'intention to treat analysis' cure rate was different. This difference was hinged on the fact that the evaluation made was based on the number of patients recruited per group, irrespective of the follow up or treatment status of the patients (Table 21).

The parasite clearance rate within 48 hours of treatment was 100% in groups (A,B,D,F,G,I,J,L,M,N,O). In addition, group C, E, H, and K comparatively showed a more reduced 48 hours clearance rate (99.29%, 99.40%, 99.23% and 98.78% respectively) (Figure 24). However, no recrudescence was observed during the 4 weeks of follow up.

The side effects of the antimalarials/micronutrients administered showed a satisfactory safety profile, with only a few patients presenting with moderate weakness (3.5%) and pruritus (1.72%) during the follow up period. This was attributed to the amodiaquine and lumefantrine component of the drug combinations since the adverse events were noticed predominantly in the amodiaquine and lumefantrine combination groups (Table 22).

Table 21: Cure Rates (%) in Different Groups Using the "Intention to Treat Population Analysis"

Groups	7 Day Cure Rate	14 Day Cure Rate	21 Day Cure Rate	28 Day Cure Rate
Amodia + Arte, N,8	80	80	80	80
Artemet +Lume, N,9	90	90	90	90
Arte +Vit A, N,6	60	60	60	60
Arte + Vit E, N7	70	70	70	70
Arte + Zinc, N,8	80	80	80	80
Arte + Se, N,8	80	80	80	80
Amodia + Vit A,N,8,	80	80	80	80
Amodia + Vit E, N,8	80	80	80	80
Amodia + Zinc, N,7	70	70	70	70
Amodia + Se, N,9	90	90	90	90
Arte +Vit A + Vit E, N,7	70	70	70	70
Arte + Vit A+ Zinc,N,6	60	60	60	60
Arte + Vit A + Se, N,8	80	80	80	80
Arte +Vit E + Zinc,N,7	70	70	70	70
Arte + Vit E+ Se,N,10	100	100	100	100

Key: N= number of participants (the number of participants was constant on day 7, 14, 21 and 28).

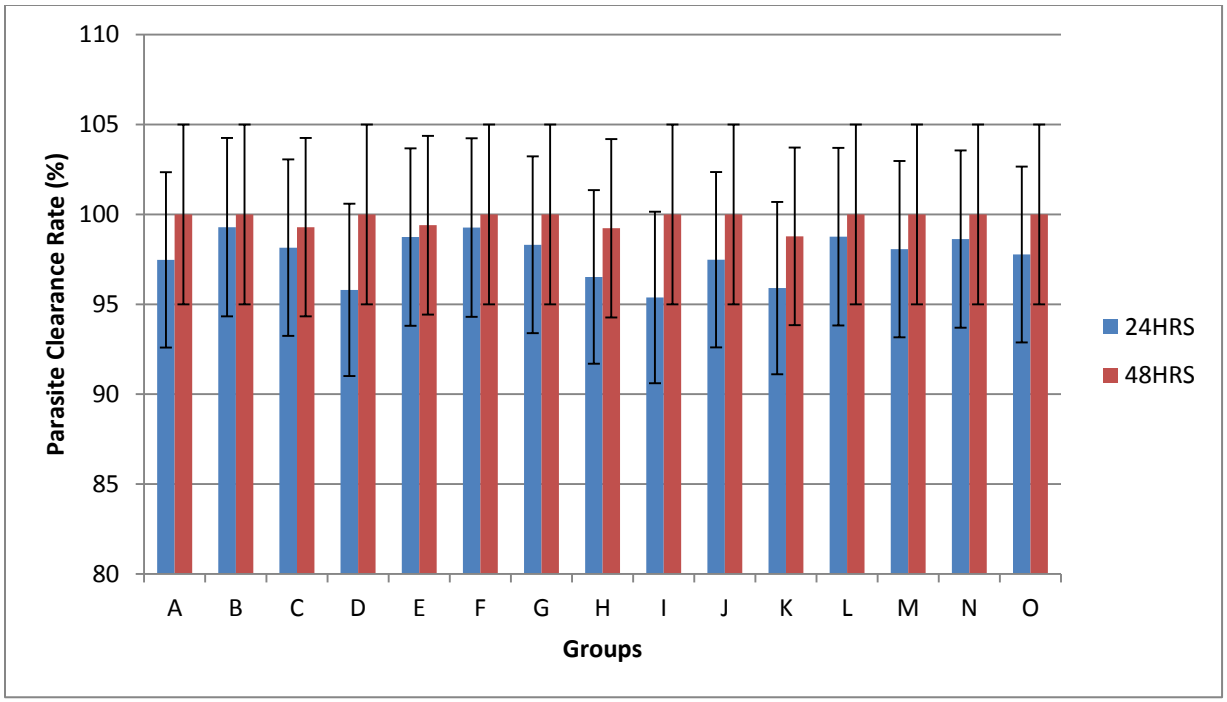

Figure 24: Parasite Clearance rate in Study Participant.

Key: A= artesunate+amodiaquine, B= artemether+lumefantrine, C= artesunate+vit A, D=artesunate+vitE, E= artesunate+zinc, F = artesunate + selenium, G=amodiaquine+vitA, H= amodiaquine+vitE, I= amodiaquine+zinc, J= amodiaquine+selenium, K=artesunate+vitA+vitE, L=artesunate+vitA+zinc, M =artesunate+vitA + selenium, N = artesunate+vit E + zinc, O =artesunate+vit E+selenium.

Table 22: Side Effect Profile of Participants

Groups	GIT	CVS	CNS	MSK	SKIN (pruritus)	ENT	UGS	OCULAR	RS	Weakness
Amodia + Arte, N,8	-	-	-	-	+(N, 1)	-	-	-	-	+(N, 2)
Artemet+Lume, N,9	-	-	-	-	-	-	-	-	-	+(N, 1)
Arte +Vit A, N,6	-	-	-	-	-	-	-	-	-	-
Arte + Vit E, N7	-	-	-	-	-	-	-	-	-	-
Arte + Zinc, N,8	-	-	-	-	-	-	-	-	-	-
Arte + Se, N,8	-	-	-	-	-	-	-	-	-	-
Amodia+ Vit A,N,8,	-	-	-	-	+(N, 1)	-	-	-	-	-
Amodia +Vit E, N,8	-	-	-	-	-	-	-	-	-	-
Amodia + Zinc, N,7	-	-	-	-	-	-	-	-	-	+(N, 1)
Amodia + Se, N,9	-	-	-	-	-	-	-	-	-	-
Arte+VitA+VitE,N,7	-	-	-	-	-	-	-	-	-	-
Arte+VitA+Zinc,N,6	-	-	-	-	-	-	-	-	-	-
Arte +VitA+ Se, N,8	-	-	-	-	-	-	-	-	-	-
Arte+VitE+Zinc,N,7	-	-	-	-	-	-	-	-	-	-
Arte+VitE+ Se,N,10	-	-	-	-	-	-	-	-	-	-

Key:

+ = Side Effect Present

- = Side Effect Absent

GIT=Gastrointestinal System, CVS=Cardiovascular System, CNS=Central Nervous System, MSK=Musculoskeletal system, ENT=Ear, Nose & Throat, UGS=Urogenital System, RS=Respiratory System

As shown in Figure 25, base line serum retinol, tocopherol, zinc and selenium levels was significantly reduced ($p < 0.05$) in the participants on day 0 of treatment when compared to apparently healthy control. However, there was no significant difference in ascorbic acid level. There was a marked elevation of serum retinol, tocopherol, zinc and selenium levels on the 28th day of follow up in all the treated groups (Figure 26). However, retinol, tocopherol, zinc and selenium levels were most markedly elevated in the artesunate + vitamin A + zinc (82.30±4.89µg/dl), artesunate + vitamin E + zinc (74.50±6.00µmol/L), artesunate + vitamin E + zinc (159.50±6.11µg/dl) and artesunate + vitamin A + selenium (8.65±1.31µg/dl) treated groups respectively (Table 23).

As shown in Table 23, multiple comparison with bonferroni correction revealed a strongly significant reduction ($P<0.01$) in serum retinol on day 0 of treatment in groups A, B, C, D and F while the reduction in serum selenium was strongly significant ($P<0.01$) in all the treatment groups on day 0 when compared to apparently healthy control. Comparatively, on day 28 post-treatment the increase in serum retinol was strongly significant ($P<0.01$) in groups C, E, G, L and M while that of tocopherol and zinc was only strongly significant in group D, H, L, N, O and group L, N respectively when compared to control. There was a significant negative correlation between serum retinol ($r = -0.131$; $P<0.05$), serum tocopherol ($r = -0.178$; $P<0.05$), zinc ($r = -0.171$; $P<0.05$) and parasite density on day 0 of treatment. Additionally, a strongly significant negative correlation also exist between selenium ($r = -0.211$; $P<0.01$) and parasite density on day 0 of treatment (Table 24).

Table 23: Comparative Profile of Serum Antioxidant Micronutrients in Children with Uncomplicated Malaria on Day 0 and Day 28 of Treatment

Groups	sRetin0 µg/dl	sToco0 µmol/L	sAscorb0 mg/dl	sZinc0 µg/dl	sSel0 µg/dl	sRetin28 µg/dl	sToco28 µmol/L	sAscorb28 mg/dl	sZinc28 µg/dl	sSel28 µg/dl
Ctl, $n_0/_{28}10$	27.60±1.67	23.00±3.09	0.94±0.20	92.00±10.09	3.76±0.68	25.90±2.54	21.55±2.13	0.97±0.10	91.45±7.56	3.89±0.53
$An_0 10, n_{28,} 8$	[a]14.30±3.97	[a]11.20±1.40	1.28±0.23	[a]30.60±7.04	[a] 0.69±0.41	47.60±3.76	32.20±2.91	1.17±0.17	118.80±8.79	4.36±1.03
$Bn_0 10, n_{28,} 9$	[a]17.60±8.46	[a]10.60±1.17	0.67±0.30	64.20±8.32	[a] 0.22±0.08	50.90±4.85	34.20±5.13	1.42±0.29	129.10±6.86	4.27±0.88
$Cn_0 10, n_{28,} 6$	[a]17.10±3.78	[b]13.70±1.37	1.11±0.21	70.00±10.04	[a] 0.13±0.04	[a]73.30±4.14	42.80±3.34	0.99±0.18	117.20±10.33	3.49±0.75
$Dn_0 10, n_{28,} 7$	[a]12.90±5.13	[a]11.90±1.33	1.05±0.18	71.80±7.91	[a] 0.13±0.03	43.80±4.34	[a]48.40±.3.03	1.39±0.22	122.10±9.10	4.27±1.05
E $n_0 10, n_{28,} 8$	19.10±4.41	[a]12.40±1.29	0.79±0.25	88.30±5.31	[a] 0.11±0.03	[a]55.20±7.10	36.90±4.41	1.06±0.15	[b]136.00±7.75	3.96±0.95
F $n_0 10, n_{28,} 8$	[b]18.90±4.53	[b]13.00±1.20	0.70±0.19	85.30±5.36	[a] 0.11±0.04	47.30±5.46	39.10±4.95	1.23±0.22	122.10±9.75	7.66±1.44
$Gn_0 10, n_{28,} 8$	[b]18.20±4.32	[b]13.40±2.31	0.28±0.06	76.40±8.37	[a] 0.19±0.06	[a]61.00±6.35	40.00±4.63	1.47±0.26	106.60±9.36	3.19±0.61
$Hn_0 10, n_{28,} 8$	[b]18.70±3.80	16.30±1.37	0.68±0.23	72.40±7.40	[a] 0.21±0.08	47.50±6.96	[a]62.40±5.65	0.98±0.12	111.20±4.77	4.04±0.73
I $n_0 10, n_{28,} 7$	[b]18.00±4.06	16.30±1.16	0.14±0.03	64.30±13.15	[a] 0.14±0.04	[b]58.80±5.42	40.70±5.70	1.71±0.33	[b]136.2±9.91	3.95±1.02
J $n_0 10, n_{28,} 9$	[a]16.67±3.35	16.60±1.18	0.63±0.14	93.70±5.27	[a] 0.18±0.06	[b]54.20±5.97	43.10±4.18	1.20±0.34	125.50±8.70	7.91±1.32
$Kn_0 10, n_{28,} 7$	[a]16.00±4.07	[a]14.40±1.03	0.50±0.18	60.70±8.04	[a] 0.21±0.07	[b]52.40±4.79	45.20±4.97	1.04±0.10	125.00±5.17	4.17±0.79
L $n_0 10, n_{28,} 6$	[a]15.30±4.55	15.40±1.33	0.41±0.11	83.30±2.27	[a] 0.15±0.04	[a]82.30±4.89	[a]48.60±4.65	1.71±0.38	[a]156.10±4.82	4.31±0.74
$Mn_0 10, n_{28} 8$	[a]16.70±1.95	17.60±0.91	0.37±0.11	76.40±6.40	[a] 0.15±0.03	[a]78.20±3.74	39.90±3.75	1.26±0.23	123.20±4.23	8.65±1.31
$Nn_0 10, n_{28}$	[b]18.80±3.55	18.20±0.70	0.75±0.16	[b]47.50±4.71	[a] 0.15±0.04	42.50±4.14	[a]74.50±6.00	1.79±0.40	[a]159.50±6.11	4.05±0.92
$On_0 10 n_{28} 10$	[a]15.30±3.86	[b]14.60±1.23	0.64±0.16	65.10±8.50	[a] 0.25±0.08	40.60±4.35	[a]73.10±4.54	1.31±0.30	113.50±7.61	8.13±1.22
ONE WAY ANOVA	F=5.14 P<0.01	F=4.40 P<0.01	F=2.80 P<0.05	F=4.49 P<0.01	F=19.42 P<0.01	F=13.86 P<0.01	F=14.86 <0.01	F=2.71 P<0.05	F=21.22 P<0.01	F=5.20 P<0.01

Values are expressed as X±SEM. Bonferroni correction = [a]P <0.01 is strongly significant when compared with apparently healthy control; [b] P <0.05 is significant when compared to apparently healthy control.

Key: Ctl=apparently healthy conrol; n_0 =number of participants on day 0; n_{28} = number of participants on day28; A=artesunate+amodiaquine, B=artemether+lumefantrine, C=artesunate+vitA, D=artesunate+vitE, E= artesunate+zinc, F = artesunate + selenium, G=amodiaquine+vitA, H= amodiaquine+vitE, I= amodiaquine+zinc, J= amodiaquine+selenium, K=artesunate+vitA+vitE, L=artesunate+vitA+zinc, M =artesunate+vitA + selenium, N = artesunate+vit E + zinc, O =artesunate+vit E+selenium.

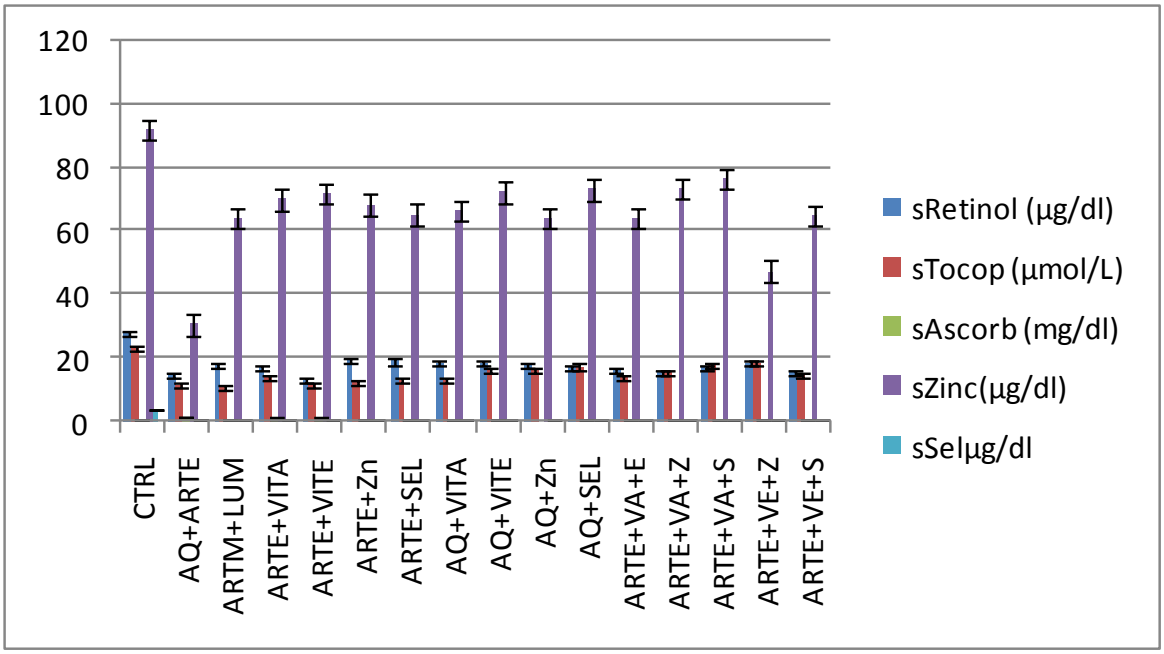

Figure 25: Serum Level of some Antioxidant Micronutrients in Children on Day 0 of Treatment

CTRL= Apparently healthy control, sRetinol=Serum level on day 0, sTocop=SerumTocopherol level on day 0, sAscorb= Serum Ascorbic acid level on day 0, sZinc= Serum level on day 0, sSel= Serum Selenium level on day 0.

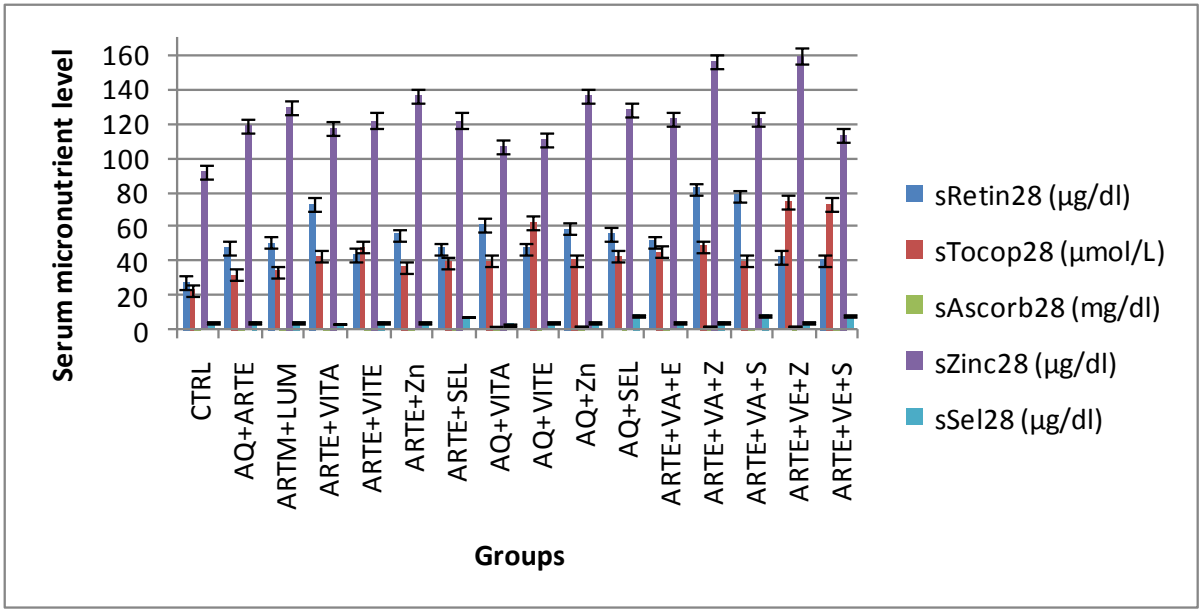

Figure 26: Serum Level of some Antioxidant Micronutrients in Children on Day 28 Post-treatment

Key: CTRL= Apparently healthy control, sRetin 28(retinol), sTocop 28 (Tocopherol), sAscorb28 (Ascorbic acid), sZinc28, sSel (Selenium) =serum levels on day 28.

Table 24: Correlational Relationship between Parasite Density, Serum Retinol, Tocopherol, Ascorbic acid, Zinc and Selenium on Day 0

Parameters	**sRetinol (µg/dl) (r value)**	**sTocopherol (µmol/L) (r value)**	**sAscorbic acid (mg/dl) (r value)**	**sZinc (µg/dl) (r value)**	**sSelenium (µg/dl) (r value)**
Parasite Density (day 0)	-0.131*	-0.178*	-0.091	-0.171*	-0.211**
Pearson's Correlation (2tailed)	P=0.043	P=0.025	P=0.255	P=0.033	P=0.007

***P < 0.05 = significant; **P < 0.01 = strongly significant**

CHAPTER FIVE

5.0 DISCUSSION

Malaria commonly afflicts populations that are both impoverished and malnourished, and a large proportion of the burden falls upon children. Vitamin A is an essential micronutrient required for maintaining immune function. It plays an important role in the regulation of cell mediated immunity and humoral antibody responses (Shankar 1995; Shankar 2000; Villamor and Fawzi, 2005; Villamor *et al.*, 2005).

Results from the *in vivo* study revealed that vitamin A has antimalarial activity. It caused 43.1% chemosuppression during the 4-day suppressive test. This finding is in agreement with the finding of Oreagba and Ashorobi, (2006) which demonstrated a similar antimalarial activity against *Plasmodium berghei berghei* in rodents and stated that the antimalarial activity of retinol became stronger with chronic administration. However, no complete parasite clearance was recorded after the 4 day curative treatment which also corroborates the findings of Oreagba and Ashorobi, (2006). The antimalarial activity of vitamin A as demonstrated in this study is supported by the study of Hamzah *et al.*, (2004) which revealed that retinol inhibits the growth of cultured *Plasmodium falciparum*.

A study conducted in Calabar by Akpotuzor *et al.,* (2007), revealed that there was a significant reduction in the level of total antioxidants and vitamin A which correlated strongly (negatively) with the severity of falciparum malaria; this signifies that antioxidant vitamins may have potential benefit in malaria therapeutics.

Vitamin A supplementation in pre-school children is known to decrease the risk of morbidity and mortality from diarrhea, measles, HIV infections and malaria. These

effects are likely the action of vitamin A on immunity. The immunomodulatory role of vitamin A has been described in clinical trials and can be correlated with outcome of supplementation (Villamor and Fawzi, 2005). Vitamin A is an important nutrient required for maintaining immune function, playing an important role in humoral antibody responses (Shankar, 2000; Stephensen, 2000; Villamor *et al.,* 2005).

The present study revealed that vitamin C has little or no antimalarial activity. Results from this study revealed that parasitemic levels after a 4 day suppression test were not significantly different from the control (4.70±0.33% versus 4.78±0.65%). Results from other studies in mice have been varied. One study by Godfrey, (1957) revealed that large doses of vitamin C abrogated the protective antimalarial effect of cod liver oil in mice. Levander and Ager, (1993), concluded that vitamin C supplementation did not modify the course of parasitemia in normal mice. This finding supports the present study. However, the study by Oreagba *et al.,* (2008), revealed that extremely high doses of ascorbic acid and grape juice possessed antimalarial activity in *Plasmodium berghei* infected mice.

Vitamin E administration also demonstrated moderate antimalarial activity as evidenced by its chemosuppressive activity in the 4 day suppressive and the 4 day curative test (36.82% and 34.56% respectively). This finding was however in agreement with two previous studies. In one of such studies, the morbidity and outcome of avian malaria infection with *Plasmodium spartani* was more severe in ducklings fed with vitamin E and selenium deficient diets than in ducklings fed with vitamin E and selenium adequate diets (Yarrigton *et al.,* 1973). In another study conducted in Kampala Uganda by Amy *et al.,* (2001), parasite clearance in children with uncomplicated *falciparum* malaria was associated with elevated levels of antioxidants (vitamin A, B-carotene, Lycopene and

vitamin E). This study suggests that children with acute malaria have depressed plasma concentration of antioxidants and that an increased concentration of antioxidants including vitamin E was associated with more rapid clearance of malaria parasite. Other studies however, have a contravening result. Animal studies by Taylor *et al.,* (1997) revealed that mice fed with vitamin E deficient diet containing omega-3-fatty acids survive infection with lethal *Plasmodium yoelii* infection. This study was supported by other studies linking vitamin E deficiency with malaria suppression (Levander *et al.*, 1989; Orville *et al.,* 1995; Levander and Ager, 1993). However, Maria *et al.,* (2010) suggested that the malaria suppression linked to vitamin E deficiency was primarily due to the disruption in alpha tocopherol transfer protein, which acts as an important regulator of vitamin E concentration in the circulation. Futhermore, a combination with the antimalarial drug chloroquine resulted in an even more dramatic reduction in parasitemia and increased survival rate (Arita *et al.,* 1995; Jishage *et al.,* 2001; Maria *et al.,* 2010). However, these findings are inconclusive. From the study conducted, selenium was found to exhibit a marked chemosuppressive activity against *Plasmodium berghei* infected mice in the 4 day suppressive test and curative test (82.01% and 76.16% respectively). Apart from the study done by Yarrigton *et al.,* (1973), no other *in vivo* study has been done till date to corroborate these findings. The work by Yarrigton *et al.,* (1973) was inconclusive as it suggested that ducks fed with vitamin E and selenium deficient diet had a more severe manifestation of avian malaria compared with ducks fed with vitamin E and selenium supplemented diet. However, he noted that vitamin E and selenium supplemented diet did not influence the survival time in avian malaria. In a separate experiment he stated that selenium deficient duck were more susceptible to avian malaria

(Yarrigton *et al.,* 1973). In this same study the author noted that vitamin E and selenium deficiency in swine was associated with a high incidence of microbial infection and that studies in mice fed a diet deficient in factor 3, vitamin E and cystine had diminished natural resistance to *Schistosoma mansoni* infection. The immune system relies upon the generation of reactive oxygen species for microbicidal activity. Release of reactive oxygen species generates inflammation and destroys microbicidal invaders. However, chronic production and release of other reactive oxygen species can lead to oxidative damage in the host. Studies by Spallhoiz *et al.,* (1990) showed that injection of this element into rodents enhance host antibody and complement responses showing immunoprotective role of selenium. Selenium augments host antibody and complement responses to both natural and experimental immunogen such as tetanus toxoid, typhoid toxin, sheep red blood cells and immunoglobulins (Spallhoiz *et al.,* 1990). Deleterious effect on immunity was also demonstrated in selenium deficient host. These include defective neutrophil function, reduced natural killer cell activity, inactivation of NADPH-dependent generation of superoxide by granulocytes, decreased antibody response to sheep RBC etc. Evidence that the immune system is being directly affected by selenium is supported by studies showing that selenium injection or supplementation resulted in enhanced immunity against malaria, increased antibody production by B cell members, increased T-cell dependent antibody production and increase in lymphocyte glutathione peroxidase activity. These findings were corroborated by the present study which revealed that selenium has potent antimalarial activity in *Plasmodium berghei* infected mice evidenced by marked chemosuppression and increased schizonticidal activity in 4 day suppressive and 4 day curative tests respectively. However, the stimulatory effect on

immunity was not recorded by all investigators and in some cases high level of selenium supplements actually decreased immunity (Spallhoiz *et al.,* 1990). The clinical trial presented in this work appears to be the first clinical trial to demonstrate the beneficial effect of selenium as an adjuvant in the management of acute uncomplicated *falciparum* malaria in preschool children. Findings from the present study revealed that selenium, when used as an adjuvant to artesunate and amodiaquine as well as in the presence of other micronutrients demonstrates a remarkably beneficial antimalarial activity. This is supported by; a more rapid parasite clearance demonstrated in the *in vivo* study when used as an adjunct to artesunate in the 4 day curative synergistic test using chloroquine resistant ANKA strain of *Plasmodium berghei*, a rapid parasite and fever clearance time in all the groups supplemented with selenium and a 100 % 7 day and 28 day cure rate in all the groups supplemented with selenium.

The findings of this study revealed that zinc supplements have similar chemosuppressive activity with vitamin A (43.51% versus 43.10%) after a 4 day suppressive test. This antimalarial activity is supported by the study of Shankar *et al.*, (1995) who indicated that moderate zinc deficiency resulted in an increased mortality from normally non lethal rodent malaria from *Plasmodium yoelii* 17 X-NL strains. Other studies reveal that zinc is essential for a variety of lymphocyte function implicated in resistance to malaria including production of Immunoglobulin G, IF-γ, TNF-α and enhances the microbicidal activities of macrophages (Shankar and Prasad, 1998). However, animal studies on the synergistic antimalarial effects of different antioxidant micronutrient combinations are quite scanty. Results from the present study revealed enhanced antimalarial activity following different micronutrient combination.

Amongst the micronutrient combination groups, vitamin A + E and vitamin A + selenium had a more prominent schizonticidal activity in the 4 day curative test. This was closely followed by the vitamin A + Zinc combination group. This suggests synergism in antimalarial action following antioxidant micronutrient combinations. In addition, mouse survival time was significantly prolonged in the micronutrient combination groups when compared with control ($p<0.05$). Results from other *in vivo* studies are quite scanty, however, in a recently published clinical trial, using combined vitamin A and zinc supplementation in young children with uncomplicated *falciparum* malaria in Burkina Faso, a major reduction in malaria morbidity was observed in the supplemented group (34%) compared with placebo (3.5%) $p<0.001$. Time to first malaria episode was higher in the supplemented group. The supplemented group also had 22% fewer fever episodes when compared to the placebo group (Augustin *et al.,* 2008). Three zinc supplementation trials have been done so far. Studies in The Gambia and Papua New Guinea showed reduction of about one third (38%) reduction in the rate of visit to a health facility for a clinical syndrome consistent with malaria and confirmed by parasitological examination of the blood (Shankar *et al.,* 2000). The third trial was done in Burkina Faso by Müller *et al.,* (2001). The study found no effect of zinc supplementation on the rate of malaria episode as ascertained from house hold visits. These results are however, conflicting and inconclusive. In a study by Duggan *et al.,* (2005) plasma zinc concentration were found to be depressed during the acute phase response in children with *falciparum* malaria. According to a report from a randomized control trial by Zinc Against Plasmodium Study Group in 2002, children between the ages of 6 months – 5 years with fever and asexual parasitemia where placed on zinc supplements (20 mg/day for infants, 40 mg/day for

older children) for 4 days. Results obtained showed no significant effect of zinc on the median time to reduction of fever, no significant reduction in parasitemia and no significant change in Hemoglobin concentration in the 3 day period of treatment and 4 weeks period of follow up. Plasma zinc levels were found to be consistently low in all the children at base line. Hence the author concluded that zinc does not appear to have a beneficial effect in the treatment of acute uncomplicated malaria in preschool children. Children less than 5 years old are at increased risk of protein energy malnutrition (PEM), as well as deficiencies in micronutrients including zinc (Gibson *et al.,* 1991). Zinc deficiency in humans leads to growth retardation , thymic atrophy, lymphopenia, impaired T and B cell function, impaired chemotactic activity of neutrophils and a reduction in thymic activity, interferon-γ concentration and the number of CD4+ lymphocytes (Shankar and Prasad, 1998). These alterations in the cellular and humoral functions may increase host susceptibility to *Plasmodium falciparum* (Shankar and Prasad 1998; Good *et al.,* 1988; Good *et al.,* 1998). Zinc supplementation in developing countries has resulted in improvement in delayed cutaneous hypersensitivity (Sempertegui *et al.*, 1996) and an increase in CD4+ lymphocytes (Sazawal *et al.,* 1998). Zinc supplementation have been found to reduce the incidence of diarrhea and pneumonia (Zinc Investigators Collaborative Group, 1999), to be beneficial when used as adjunctive therapy for acute diarrhea (Zinc Investigators Collaborative Group, 2000; Dutta *et al.*, 2000). This is in consonance with results from this study which showed a remarkable recession of diarrhea in patients who received zinc supplementation in addition to other antimalarials. The community based zinc supplementation trial in the Gambia showed a reduced health centre attendance for malaria in children who received

zinc (Bates *et al.,* 1993). In Papua New Guinea, zinc supplementation in pre-school children reduced malaria attributable hospital attendance (Shankar *et al.,* 2000). This is in agreement with the findings of this study which revealed a more rapid parasite clearance time (PCT) in the Artesunate + vitamin A + zinc treated group (26.00±4.82 hours) when compared to the active comparator groups (Amodiaquine + Artesunate; 27.00±3.00 hours and Artemether + Lumefantrine; 29.33±3.53 hours). In the *in vivo* study, parasite clearance was more rapid in the artesunate + zinc group (2.60±0.40 days) when compared with the artesunate group (3.00±0.32 days). Comparatively the parasite clearance time in the artesunate + chloroquine group (2.60±0.24 days) was not significantly different from the artesunate + zinc group (2.60±0.40 days).

Zinc and vitamin A interact in several ways; zinc is a component of retinol binding protein, a protein necessary for the transporting of vitamin A in the blood. It is also important in activating the enzyme that converts retinol to retinal (Arif *et al.,* 1987). This is supported by the finding in the *in vivo* study which showed that zinc and vitamin A combination resulted in a remarkable schizonticidal activity (91.82%) in the 4 day curative synergistic study when compared to other combination groups. This demonstrates the synergism between vitamin A and zinc.

Till date, only one clinical trial has been done to evaluate the effect of zinc as an adjunct in the treatment of acute, uncomplicated *falciparum* malaria in under five's. In this study the children received zinc supplements at 20mg/day for infants and 40mg/day for older children for 4 days in addition to chloroquine (Zinc Against Plasmodium Group, 2002). The results were quite disappointing as the result showed no beneficial effect when used as an adjunct to chloroquine in the management of uncomplicated *falciparum* malaria in

pre-school children. Although, this finding contravanes the finding of this clinical study which revealed that zinc when used as adjuvant to artesunate and amodiaquine demonstrated beneficial antimalarial activity in acute uncomplicated malaria in under five or pre-school children. This is evidenced by; the occurrence of a more rapid parasite clearance time in the artesunate + vitamin A + zinc combination group when compared to the active comparator groups, the occurrence of a rapid fever clearance in all the zinc supplemented groups when compared with the active comparator group and a 7 day cure rate of 100% in all the zinc supplemented groups.

Other results from this study revealed that the antioxidant micronutrients (vitamin A, E, zinc and selenium) demonstrated marked repository activity against *Plasmodium berghei* NK 65 infected mice 72 hours post inoculation. The repository activity demonstrated by zinc and selenium is comparable to that of pyrimethamine (91.61% and 94.94% versus 95.32%). Studies by Oreagba and Ashorobi, (2006) are in agreement with the chemoprophylactic effect of vitamin A in *Plasmodium berghei* infected mice as revealed by this study.

Present study revealed that artesunate + selenium combination in the 4 day curative treatment of established *Plasmodium berghei* (chloroquine resistant ANKA strain) infection in mice, caused a more rapid clearance which was statistically significant ($p<0.05$) when compared to the chloroquine + artesunate combination (2.40±0.40 days versus 2.60±0.24 days). All the micronutrient combination group had a more rapid parasite clearance time when compared with the artesunate monotherapy group ($p<0.05$). These findings reveal the potential benefit of these micronutrients when used as adjuvants in artemisinin based therapy.

In a placebo controlled trial by Villamor *et al.,* (2007), using different vitamin combinations (vitamin B, C and E multivitamins, vitamin A and β- carotene) were found to significantly reduce the incidence of clinical malaria by 71% while vitamin A, B and C multivitamins caused a non significant 63% reduction in malaria incidence in children born to HIV positive mothers. The effect of vitamin A on clinical malaria could be mediated in part by increased phagocytosis of infected red cells and decreased pro-inflammatory activity (Serghides *et al.,* 2002).

In a study conducted in Owerri, Nigeria by Ekeanyanwu *et al.,* (2009), serum levels of antioxidant vitamins estimated in 43 children (0-5 years) with *Plasmodium falciparum* infection showed that serum vitamin A, C and E levels were significantly reduced in malaria infected children when compared with control. The relationship between malaria parasitemia and serum concentration of vitamin E were strongly and positively correlated ($r = 0.42$), but vitamin A and C levels though reduced, were negatively correlated with parasite density. In a similar Nigerian study in the University of Calabar Teaching Hospital by Akpotuzor *et al.,* (2007), there was a significant reduction in total antioxidant level (vitamin A, C and β-carotene) in malaria infected children when compared with control. In addition, malaria parasitemia correlated strongly and negatively with total antioxidant level ($r = -0.432$, $p < 0.01$) but weakly with vitamin A level. This finding corroborates the result of the present study which showed a marked decrease in serum antioxidants (retinol, tocopherol, zinc and selenium) on day 0 of treatment in children with uncomplicated *falciparum* malaria. Additionally, there was a significant negative correlation between serum retinol ($r = -0.131$; $P<0.05$), serum tocopherol ($r = -0.178$; $P<0.05$), zinc ($r = -0.171$; $P<0.05$) and parasite density on day 0 of treatment.

Other studies have shown the potential benefit of selenium in the treatment of other infectious disease like Chagas disease. In a study by Souza *et al.,* (2010), selenium was shown to be of potential benefit as an adjuvant in the prevention of right ventricular chamber dilation and reversal of *Trypanosoma crutzi* induced acute and chronic cardiomyopathy in mice.

In a study designed to evaluate the potential benefit of combination therapy consisting of an immunomodulator, picroliv and an antimalarial such as chloroquine against drug resistant *Plasmodium yoelii* infection in mice, it was observed that picroliv evoked a T-cell proliferating response as well as antibody producing response with an associated enhancement of the efficacy of chloroquine against murine malaria (Puri *et al.*, 1992; Bone, 1995). Selenium has similar immunomodulatory properties with picroliv; hence it is expected to evoke a similar enhancement of antimalarial activity when combined with antimalarials. This may be one of the potential mechanisms of action of selenium as an adjuvant in malaria chemotherapy.

Dietary zinc deficiency is wide spread in developing countries and is often aggravated by intermittent acute and chronic infections (Cuevas and Koyanagi, 2005). Recent studies have demonstrated that zinc supplementation of apparently well nourished children can significantly reduce morbidity and mortality and shorten the time to recovery from common infectious diseases (Cuevas and Koyanagi, 2005). In another study conducted in Ado-Ekiti, Nigeria, malaria infection was associated with significantly reduced serum micronutrient and antioxidant levels in pregnant women aged between 20-43 years (Asaolu and Igbaakin, 2009). In the present study, serum retinol, tocopherol, zinc and selenium levels were significantly reduced ($p < 0.05$) in children with uncomplicated

malaria on day 0 of treatment when compared to apparently healthy control. Additionally, the marked elevation of serum retinol, tocopherol, zinc and selenium levels on the 28th day of follow up in all the treated groups supports the report from earlier findings.

Mechanism of Action

Findings from the present study demonstrated that antioxidant micronutrients have antimalarial activity both in *Plasmodium berghei* parasitized mice and in human subjects (early childhood) with uncomplicated *falciparum* malaria. These findings are supported by the recent study of Ries *et al.,* (2010) who found increased production of molecules indicative of high oxidative stress in the brains of mice with cerebral malaria. The authors also found that treating mice with a combination of chloroquine and two antioxidant agents, at the first signs of cerebral malaria prevented both inflammatory and vascular damage in the tissues of the brain, as well as the development of persistent cognitive damage. The addition of antioxidants did not diminish the efficacy of chloroquine in eliminating *Plasmodia* from the blood. Similarly combination therapy with antioxidants and a newer antimalarial called artesunate was also effective in treating cerebral malaria and preventing subsequent cognitive impairment in mice. Based on the above findings, Ries *et al.,* (2010) suggested that antioxidant drugs should be studied as additive therapy to antimalarial drugs in clinical trials. Interestingly, the clinical trials conducted in this present study revealed that use of antioxidant micronutrient as adjuvant to antimalarials have comparative efficacy with standard artemisinin based combinations in the therapy of uncomplicated *Plasmodium falciparum* malaria. The proposed mechanism for this is via the modulation of cell mediated immune response and erythrocyte membrane protection due to increased antioxidant activity. This proposed mechanism is further corroborated by

the work of Stocker *et al.,* (1985) which revealed that erythrocyte membranes are better protected by antioxidants than parasite membrane. According to Kraus *et al*., (1997), vitamins C and E supplementation reduced erythrocyte osmotic fragility and oxidative damage in rats. Similarly, exercise stress, heat stress and other forms of oxidative stress have been associated with increased erythrocyte osmotic fragility, concurrently with elevated levels of thiobarbituric acid reacting substances (TBARS) and malondialdehyde (MDA) which are products of lipid peroxidation in the erythrocyte membrane (Kelle *et al*., 1999; Ozturk and Gumuslu, 2004). This finding corroborates the results obtained in this present study which revealed a significantly higher level of MDA in the distilled water and vehicle treated groups when compared to uninfected control. This suggests increased oxidative stress in malaria infection in the groups treated with distilled water and vehicle. In the present study, the MDA levels were significantly higher in the artesunate and chloroquine treated groups when compared with the antioxidant micronutrient treated groups. This presupposes that oxidative stress in malaria infection may be further increased by free radicals generated from artesunate and chloroquine metabolism. Findings from earlier studies revealed a reduction in the levels of intra-erythrocytic copper, zinc, superoxide dismutase (SOD) and glutathione peroxidase with an associated elevation of catalase and oxidized glutathione levels in oxidative stress (Kelle *et al*., 1999; Ozturk and Gumuslu, 2004; Aguilo *et al*., 2005). A slightly similar pattern was observed in the present study. There was no significant change in the superoxide dismutase level between the antimalarial and antioxidant treated groups, this was also not significantly different from the control groups. However, catalase and glutathione peroxidase levels were significantly elevated in the antioxidant treated groups

when compared with the control and antimalarial groups. Damage to the erythrocyte membrane proteins and lipids due to oxidative stress leads to hemolysis as a result of destruction of the spectrin bands which is the base of the erythrocyte cytoskeleton (Reid and Mohandas, 2004). Also damaged by oxidative stress according to Reid and Mohandas, (2004) are band 3, glycophorin C and RhAG; the membrane proteins that link the lipid bilayer to the spectrin cytoskeleton. These linkages play a significant role in regulating cohesion between the lipid bilayer and the cytoskeleton; the loss of which results in lipid loss, decreased membrane surface area and loss of deformability of erythrocytes. Hence, the erythrocyte membrane appears to be protected by the antioxidant micronutrients (vitamin A, E, Zinc and Selenium) as evidenced by a significantly lower MCFI in the antioxidant treated groups when compared to the infected control groups. Additionally, the % membrane distablity was also significantly lower in the antioxidant groups when compared to infected control. Consequent upon these findings, it is pertinent to state that with membrane stabilization, hemolysis is impaired, merozoite release is reduced and progressive parasitisation of uninfected RBC is also significantly reduced. It has been revealed that the trace element zinc plays an important role in the structure and function of biological membranes (Bettger and O'Dell, 1993). Dietary zinc deficiency in rats is associated with increased hemolysis of erythrocytes in hypotonic saline (O'Dell *et al.*, 1987; Paterson and Bettger, 1985; Roth and Kirchgessner, 1994) and in the presence of various detergents, alcohols and toxins (Paterson and Bettger, 1985). *In vitro* addition of zinc to red blood cells is also protective against hemolysins (Avigad and Bernheimer, 1976; Takeda *et al.*, 1977). Alterations in the composition of the erythrocyte membrane have been detected in zinc-deficient rats (Avery and Bettger, 1988; Avery and Bettger,

1992; Driscoll and Bettger, 1991; Johanning and O'Dell, 1989; Paterson *et al.*, 1987). These findings corroborate the finding of the present study which revealed that zinc had the lowest MCFI value among the antioxidants used for treatment of malaria.

Oxidative modifications of the membrane increases fragility of red blood cells (Stern 1986; Wagner *et al.*, 1988). There is evidence for a physiological role of zinc as an antioxidant (Bettger, 1993; Bray and Bettger, 1990), greater oxidative damage in zinc deficiency could be responsible for impaired stability of erythrocytes. In a previous study by Kraus *et al.*, (1997), enrichment of the diet with antioxidants in combination (vitamin C, vitamin E and β-carotene) prevented the elevated osmotic fragility of erythrocytes in zinc-deficient rats. Indeed, this suggested an important role of oxidative damage in the impaired stability of erythrocytes in zinc deficiency. In the present study, increased antioxidant activity in the micronutrient treated groups as suggested by significant elevation in catalase and GPx is responsible for a significantly lower erythrocyte membrane distability in these groups. This is suggestive of erythrocyte membrane protection.

Vitamin E on the other hand is a natural constituent of biological membranes; it acts as an antioxidant by donating hydrogen atom at 6-hydroxyl group on the chromatin ring and by scavenging singlet oxygen and other reactive species (Lee *et al*., 2004; Powers and Jackson, 2008).

An association between reactive oxygen species (ROS) generation and erythrocyte loss has been observed in malarial infection, when such markers were measured in the erythrocyte (Das and Nanda, 1999). The malarial parasite is known to perturb the lipid composition of the host RBC (Sherman, 1979), with RBCs in *in vitro* culture showing an

increase in total lipid content and a decrease in percentage polyunsaturated fatty acid (PUFA) in the erythrocyte membrane (Hsiao *et al.,* 1991).

The effect of ROS on erythrocytes is probably a balance between the parasite and host response. The significant reduction in membrane percentage polyunsaturated fatty acid (PUFA) and α-tocopherol concentration in malarial subjects supports oxidative stress.

Support for increased ROS generation in malaria has been previously provided by indirect markers of lipid peroxidation, such as plasma malondialdehyde and thiobarbituric acid reactive substances (Thurnham *et al.,* 1990; Das *et al.,* 1993). More recently, Kremsner *et al.,* (2000) reported an increase in ROS generation in whole blood of African children with malarial anaemia compared with mild malaria subjects. These micronutrients are well known to have antioxidant effects but not much work has been done in relation to their interaction with *falciparum* malaria infection. However, one study carried out in children with acute malaria infection in Kampala, Uganda, showed that the plasma level of these vitamins were depressed at enrollment and increased by day 7 (Metzger *et al.,* 2001). The study showed that higher plasma lycopene concentrations at enrollment were associated with clearance of parasitemia between enrollment and day 3. Their study suggested that the children with acute malaria have depressed concentrations of antioxidants. Wo and Yang (1986), observed that during the ageing of erythrocyte membrane, the spectrin content, Na/K-ATPase activity as well as the lipid fluidity were obviously decreased. However, supplementation of a trace amount of a selenium compound (Na_2SeO3) in the medium prevented the dissociation of spectrin from membrane and delayed the changes of Na/K-ATPase activity and lipid fluidity. The

effectiveness is proportional to selenium concentration within the range of 0.1-1.0 ppm (Wo and Yang, 1986). A similar effect of supplementation of selenium on the intact erythrocytes during ageing has also been observed (Wo and Yang, 1986). This supports the potent membrane protective role of selenium as evidenced in the present study, which showed a significantly lower MCFI and % membrane distability when compared to other treated groups. The protective action of selenium on biomembranes is generally interpreted in terms of the activity of selenium-containing glutathione peroxidase (GPx). However, GPx mainly distributes in the cytoplasm of erythrocytes, therefore it seems that the protective action of supplemented selenium on the isolated erythrocyte membrane might not be related to the activity of GPx alone.

Findings from this present study has further reiterated the fact that antioxidant play a significant role in membrane protection. This is further corroborated by the findings of Wambi *et al.*, (2008), which observed that dietary antioxidant supplements increased survival when administered as a preventive measure prior to radiation exposure as well as when given as treatment after radiation exposure. The administration of dietary antioxidants prior to the radiation exposure was associated with significant protective effects against radiation-induced leukocyte depletion in peripheral blood and bone marrow, suggesting that antioxidants may improve the survival of irradiated animals by attenuating the deleterious effects of radiation on the host immune system. Earlier studies have previously demonstrated the preventive effect of antioxidants against radiation-induced oxidative stress *in vitro* and *in vivo,* which was measured by radiation-induced reductions of serum or plasma total antioxidant status in animals (Guan *et al.,* 2004; Guan *et al.,* 2006; Kennedy *et al.,* 2004; Wan *et al.,* 2005;Wan *et al.,* 2006). Other studies

assessed the hematopoietic radioprotective efficacy of an oral formulation consisting of selenomethionine, α-lipoic acid, N-acetylcysteine, vitamin C and vitamin E succinate *in vivo* using animal survival and immune cell counts as the end points. These studies demonstrated that orally administered antioxidants were effective in protecting the hematopoietic system from the deleterious effects of ionizing radiation as well as in increasing survival. Other data indicate a highly significant effect of dietary antioxidants in the prevention of neutropenia after low- and high-dose total body irradiation (TBI) (Wan *et al.,* 2005; Wan *et al.,* 2006). Dietary antioxidants were also effective in preventing peripheral lymphopenia associated with low-dose TBI but not high-dose TBI (Wanbi *et al.,* 2008; Wan *et al.,* 2005; Wan *et al.,* 2006).

Nutritional status plays an important role in humoral and cell-mediated immune function. Micronutrient deficiencies in HIV-infected women impair immune responses, increasing the risk of infection, HIV disease progression, and possibly vertical transmission. Systemic immune response to HIV infection in infants and children may also be impaired by maternal micronutrient deficiencies. Most evidence of the effects of micronutrients on immune function comes from studies that examined a single nutrient, often used pharmacologic doses, and did not involve HIV infection. Vitamin A has an important regulatory role in systemic immune function (Ross and Stephenson, 1996; Semba, 1998). Vitamin A deficiency impairs cytotoxic T lymphocyte activity (Sijtsma *et al.*, 1990) and neutrophil function in animals (Twining *et al.,* 1997). Vitamin A supplementation improves natural killer cell cytotoxicity in rats (Zhao and Ross, 1995) and increases the number of natural killer cells in HIV-infected children (Hussey *et al.,* 1996). Antibody responses to tetanus toxoid (Semba *et al.,* 1992) and measles vaccines (Coutsoudis *et al.,*

1992) are also enhanced by vitamin A supplementation. In epidemiologic studies, megadose vitamin A supplementation of children reduced the severity of infectious morbidity such as measles (Hussey and Klein, 1990), malaria (Shankar *et al.,* 1999), and diarrhea, and increased overall survival (Fawzi *et al.,* 1993).

Antioxidant vitamins are important enhancers of immune function. Vitamin E deficiency is associated with impairment of cell-mediated immune functions, such as the delayed type hypersensitivity (DTH) skin response, neutrophil phagocytosis, and lymphocyte proliferation in human and animal studies (Bendich, 1988). Vitamin E supplementation has immunostimulatory benefits in HIV-infected mice, including increased IL-2 production and natural killer cell cytotoxicity and a reduced production of inflammatory cytokines such as tumor necrosis factor α and IL-6 (Wang *et al.,* 1994; Wang *et al.,* 1995). Short-term, high-dose vitamin E supplementation in elderly subjects significantly increased lymphocyte proliferation from mitogen stimulation, IL-2 production, and the DTH response (Meydani *et al.,* 1996). Longer-term vitamin E supplementation at lower dosages also increased the DTH response and improved the antibody response to T cell–dependent vaccines (Meydani *et al.,* 1997). Vitamin C deficiency negatively affects cellular immune responses in animal studies (Bendich, 1988).

Zinc deficiency has widespread negative effects on immunity and increases the risk of infections because of zinc's central role in many aspects of immune function (Shankar and Prasad, 1998). Zinc is necessary for the normal function of neutrophils, natural killer cells, and macrophages and for the production and activity of T and B lymphocytes. Zinc supplementation trials in children showed significant reductions in diarrheal and respiratory infections and malaria (Black, 1998). Zinc is also crucial for the normal

function of cells which mediate nonspecific immunity, such as neutrophils and natural killer cell. B lymphocyte development and antibody production, particularly immunoglobulin G, is compromised by zinc deficiency. The macrophage, a pivotal cell in many immunologic functions, is adversely affected by zinc deficiency. These can dysregulate intracellular killing, cytokine production, and phagocytosis.

The effects of zinc on these key immunologic mediators is rooted in the myriad roles for zinc in basic cellular functions such as DNA replication, RNA transcription, cell division, and cell activation. Apoptosis or programmed cell death is potentiated by zinc deficiency. Zinc also functions as an antioxidant and can stabilize membranes.

Selenium is an essential structural component of the antioxidant enzyme glutathione peroxidase, and it has numerous important functions in the maintenance of humoral and cell-mediated immunity (Kiremidjian-Schumacher and Stotzky, 1987). Deficiency inhibits neutrophil function, the cytoxic activity of T lymphocytes and natural killer cells, lymphocyte proliferation in response to mitogens, the DTH response, antibody production, and resistance to pathogens (Kiremidjian-Schumacher and Stotzky, 1987). In a small study of selenium depletion and supplementation in patients with gut failure and receiving parenteral nutrition, 2–4 months of supplementation with a moderate dose of selenium improved lymphocyte responses to various mitogens and antigens (Peretz *et al.*, 1991).

In the present study, a significant increase in total WBC count was observed in the artesunate, chloroquine, vitamin A, vitamin E, zinc and selenium groups when compared with apparently healthy uninfected control. Although there was a non significant increase

in total WBC count in the negative control and vehicle group, this increase is a reflection of the elevation in WBC count associated with malaria infection as reported by Kamga *et al.,* (2010). The marked elevation of total WBC count in the antioxidant micronutrient treated group as observed in the present study is a reflection of the additional immunostimulating and immunomodulatory role of the micronutrients in malaria infection. Recent studies among children with *falciparum* malaria revealed that a fairly low lymphocyte and monocyte counts were independently associated with morbidity (Kamga *et al.*, 2010). However, in the present study no significant change was observed in lymphocyte and monocyte count in the micronutrient treated groups. This suggests that antioxidant micronutrients may actually be involved in the modulation of lymphocyte and monocyte activity. A platelet count of less than 150 x 10^3/mm^3 of blood was found in 13% of the subjects with *falciparum* malaria in the study of Kamga *et al.,* (2010). However, the finding in the present study revealed a markedly significant elevation of absolute platelet count in the micronutrient group. This suggests that antioxidant micronutrient may also be involved in the modulation of platelet activity in malaria.

5.1 SUMMARY OF FINDINGS

S/N	Objectives of Study	Summary of Findings
1.	To determine: Antiplasmodial activity using 4 day suppressive test and also the chemopreventive and chemotherapeutic role of selected antioxidant micronutrients.	There was a significant shizonticidal activity in the early phase of *Plasmodium berghei* infection ($p < 0.05$). However, this was more marked in selenium treated group (82.01%) and insignificant in vitamin C treated group. Selenium demonstrated a more marked repository activity (94.94%) which is comparable to pyrimethamine (95.32%). Reduction in mean parasitemic levels after 4 days curative treatment was also more marked in the selenium group 5.82± 1.48% when compared with other micronutrient groups and control (24.42 ± 3.84%).
2.	Synergistic effect of selected antioxidant combination and standard antimalarial agents when used in varying combinations in the treatment of *P. berghei* malaria infection.	Synergistic schizonticidal activity was more marked with the vitamin A + E combination therapy (94.52%) when compared with any other micronutrient combination after 4 days treatment of established infection. This was closely followed by the vitamin A + selenium combination (94.43%), the mean difference in parasitemic levels was significant between groups ($F = 2.59$; $P < 0.05$)

3.	The effect of selected antioxidant micronutrients when used in varying combinations as adjuvants in uncomplicated *falciparum* malaria as well as the effect of selected standard agents when used in combination with selected micronutrients in the therapy of uncomplicated *falciparum* malaria.	A more rapid parasite clearance time (PCT) was observed in the Artesunate + vitamin A + zinc treated group (26.00±4.82 hours) when compared with the active comparator groups (Amodiaquine + Artesunate; 27.00±3.00 hours and Artemether + Lumefantrine; 29.33±3.53 hours) combination therapy. Parasite Clearance was sustained through out the 28-day follow up period. There was a consistent reduction of mean parasite density from day 0 to day 2 of treatment. The mean difference in the reduction of parasite density between groups on day 0/day 1, day 0/day 2 and day 1/day 2 respectively was statistically significant ($p < 0.05$). Parasite clearance rate was 100% in all the treatment groups within 48 hours. The artesunate + selenium group showed a comparatively similar 24 hours parasite clearance rate when compared to artemether + lumefantrine combination group.
4.	Possible mechanisms of antimalarial action of selected antioxidant micronutrients	Erythrocyte membrane distability was more marked in the tween 80 group (426.15%), followed closely by the chloroquine (373.85%) treated group and artesunate group (329.23%) and least in the zinc treated group (32.31%). Catalase and glutathione peroxidase activity was significantly higher in the vitamin A, E, selenium and zinc treated groups respectively when compared to apparently healthy uninfected control ($p<0.05$). There is a statistically significant difference in total white blood cell count (WBC) in the vitamin A, E, selenium and zinc treated groups when compared with apparently healthy non-inoculated control ($p<0.05$). A comparative analysis between the total WBC count on day 0 and day 28 post treatment revealed a significant increase in all the micronutrient treated groups on day 28 ($p<0.05$; $r = 0.76$; $P<0.01$).

5.	Evaluation of serum antioxidants (vit A, C, E) and trace elements (zinc and selenium) in under five's with uncomplicated malaria.	The study showed a marked decrease in serum antioxidants (retinol, tocopherol, zinc and selenium) on day 0 of treatment. Additionally, there was a significant negative correlation between serum retinol ($r = -0.131$; $P<0.05$), serum tocopherol ($r = -0.178$; $P<0.05$), zinc ($r = -0.171$; $P<0.05$) and parasite density on day 0 of treatment.

5.2 CONCLUSION

This study on antioxidant micronutrients and malaria has revealed that antioxidant micronutrients have a remarkably potent antimalarial activity and are of benefit in the management of uncomplicated *falciparum* malaria. They can be used as adjuvants to standard antimalarial drugs like artesunate and amodiaquine for a low cost effective management of uncomplicated *falciparum* malaria in vulnerable population. Under five's in resource poor settings where malaria is endemic will benefit maximally from this measure since they represent the non-immune population at risk of malaria and micronutrient deficiency. This will help to expand the therapeutic options available for the treatment of uncomplicated *falciparum* malaria and reduce the potential emergence of resistance to existing drugs and the overall burden of malaria.

5.3 RECOMMENDATIONS

Based on the findings of this study, antioxidant micronutrients are of immense benefit in malaria therapeutics, hence the following recommendations are made;

- Antioxidant micronutrients such as zinc, selenium, vitamin A and E should be used as adjuvants to standard antimalarial drugs in the management of malaria in vulnerable population. This will provide; (a) a low cost therapeutic benefit in resource poor settings (b) reduce the emergence of resistance to existing drugs (c) expand the therapeutic options available in the treatment of malaria (d) reduce the incidence of malaria attack through immunomodulation.
- Antioxidant micronutrients should be used in the chemoprophylaxis of malaria since it offers a low cost and comparatively effective option in malaria chemoprophylaxis.
- Antioxidant micronutrients should be compounded with existing formulations during drug manufacturing by pharmaceutical companies to give rise to organometallic and organonutritional complexes which can be used in malaria therapeutics.

5.4 CONTRIBUTIONS TO KNOWLEDGE

The contributions to knowledge as elucidated by this study are as follows:

1. The study has established that artesunate + vitamin A + zinc combination has therapeutic benefit in the management of uncomplicated falcciparum malaria which is more effective than standard artemisinin based combination therapy.
2. The study has also established that the antioxidants (selenium, zinc, vitamin A and E) help to stabilize erythrocyte membrane and prevent progressive malaria infection in children
3. The study also established that there is no deleterious effect when these micronutrients are co-administered with artemisinin based combination antimalarials.

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APPENDICES

Appendix 1

Preparation of Drugs

Chloroquine

50 mg of powdered chloroquine sulphate were dissolved in 20 ml of distilled water. So that 1 ml will contain 2.5 mg of chloroquine sulphate. Dosage administered to the animals in the standard drug group (A) was 25 mg/kg. Hence the 0.2 ml of solution administered contained 0.5 mg of chloroquine sulphate.

Vitamin A

200,000 IU of vitamin A caplet, which is equivalent to 60 mg of vitamin A, was used to prepare the dose administered (60 mg/kg). The drug was dissolved in 0.2 ml of Tween 80 used as a vehicle and distilled water in a ratio of 0.2:0.2:9.6. To make up a total volume of 10 ml. The final volume of drug administered was 0.2 ml, which is equivalent to 0.495 mg of vitamin A.

Vitamin E

100 mg of vitamin E caplet was dissolved in 0.2 ml of Tween 80 and distilled water in a ratio of 0.2:0.2:9.6 making up a total volume of 10 ml. The dose administered to the animal was 100 mg/kg. Hence the final volume of drug administered to the animal was 0.2 ml, which is equivalent to 1.6 mg of vitamin E.

Selenium

1 mg of selenium was dissolved in 10 ml of distilled water in its powdered form. A dose of 1 mg/kg body weight was administered to the animals. The final volume of drug administered was 0.2 ml equivalent to 0.0145 mg of selenium.

Zinc

The dose of zinc administered was 100 mg/kg. 100 mg of zinc was dissolved in 10 ml of distilled water in its powdered form. 0.2 ml of the solution was administered which is equivalent to 1.91 mg of zinc.

Vitamin C

200 mg of the powdered form of vitamin C was dissolved in 10 ml of distilled water. Dosage administered to the animals was 200 mg/kg body weight. A final volume of 0.2 ml of solution was prepared which is equivalent to 1.8 mg of vitamin C.