CHANGES IN TESTICULAR ACTIVITIES INDUCED BY *PLASMODIUM BERGHEI* MALARIA PARASITE IN EXPERIMENTAL MICE

BY

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MARCH, 2017

CERTIFICATION

I declare that this research work was independently carried out by me **Oshiegbu Williams** in the Department of Biochemistry, Faculty of Science Delta State University Abraka for the award of M.Sc. Degree in Biochemistry and has not been carried out by any one for the award of any Diploma or Degree.

_____ _____

Oshiegbu Williams **Date**
(Researcher)

APPROVAL PAGE

This is to certify that this research work was carried out by **Oshiegbu Williams with** matriculation number **PG/13/14/223006** in the Department of Biochemistry, Faculty of Science Delta State University for the award of Master of Science (M.Sc.) Degree in Biochemistry.

_____ _____

Dr. I Onyesom **Date**
(***Supervisor***)

_____ _____

Prof. N.J.T Tonukari **Date**
(*Head of Department*)

DEDICATION

This work is dedicated to God who granted me Success through out the Study.

ACKNOWLEDGEMENTS

I am grateful to God, the Almighty for His grace, love, and mercies over the successful completion of this project.

Moreover, my sincere appreciation goes to my supervisor, Dr. I. Onyesom whose contributions have made this project a success. I equally wish to appreciate the efforts of Dr. F.I. Achuba for his fatherly role towards this work. Many thanks to other lecturers especially Prof. N.J Tonukari (Head of Department), Dr. E.Aganbi, Professor G.O.C. Onyeze, Professor B.O. George, Professor S.O. Asagba and other lecturers in the department for all of their kind ssistance.

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ABSTRACT

Changes in testicular activities induced by *plasmodium berghei* malaria parasite in experimental mice were investigated. The study comprised of twenty (10) Swiss albino male mice which were divided into two groups of 5 mice each. The first group served as the control while the second group served as the experimental group. The control group was innoculated with placebo-phosphates buffered saline (PBS) while the experimental group was infected with *Plasmodium berghei* in PBS. Animals were sacrificed at the end of seven days and their serum, and testes were collected for biochemical analysis using standard procedures. The results revealed that *P. berghei* malarial parasite infection significantly compromised ($p<0.05$) antioxidant defense capacity in testis SOD (0.07± 0. 03 mm0l/mL/min), CAT (11. 00 ±0.96 µmol/mL/min), GP_x (2 .26± 0.74^b µmol/mL/min), Acid phosphatase (0.60±0.16 IU/L), GSH (1.38±0.24 µM), Testosterone (0.05±0.26 ng/mL), MDA (61.84±14.55µM) relative to control SOD (1.03±0.16 mmol/Ml/min)· CAT (12.35±1.68 µmol/Ml/min) GP_x(10±0.84 µmol/ML/min), Acid phosphatase(0.54±0.22 IU/L), GSH (12.62±1.64µM),Testosterone (0.15±0.19(ng/mL), MDA (44.10±1.31µM) respectively. Semen antioxidant defense capacity also revealed significant reduction ($p<0.05$) in the experimental group; SOD(1.44±0.13mmol/mL/min) CAT(6.63±0.65µmol/Ml/min),GP_x(9.23±0.72µmol/mL/min), Acid phosphatase (0.48±0.72IU/L),GSH(18.50±1.67µM),Testosterone(0.02±0.13ng/mL),MDA(64.21±9.88µM) when compared with control values; SOD (1.07±0.15 mmol/Ml/min), CAT (12.30±2.10µmol/mL/min),GPx 4.97±0.83(µmol/mL/min),Acid phosphatase (0.32±0.02IU/L), GSH(13.94±1.57µM), Testosterone(0.10±0.36ng/mL), MDA(34.12±6.48 µM) respectively. The study further revealed alteration in testistecular ultra structure of the experimental group relative to control while semen quality showed varying alteration in sperm volume, fructose and glucose concentration as well as alteration in the micro-morphological appearance of sperms in mice infected with *P. berghei.* It was concluded that *P. berghei malarial* parasite infection in experimental mice induces oxidative damage to the testis, and this affected the ability of the organ to produce quality sperm cells and the hormone-testosterone.

CHAPTER ONE

INTRODUCTION

1.1 Background to Study

Malaria is a life threatening disease caused by the protozoan parasite, *plasmodium*. Malaria presents a global health problem with about two million cases and more than half a million deaths reported, most of which occurred in the sub-Saharan Africa (WHO, 2014). It is a major health challenge in developing countries, responsible for 1-3 million deaths per annum with over ten thousand maternal deaths (Schantz-Dunn and Nour, 2009). In Nigeria, malaria cases account for quarter of all cases in Africa, with about 30-50% morbidity and 25% mortality in infants (WHO, 2008, Idowu *et al.,* 2010). Malaria infection is usually associated with the generation of large amounts of reactive oxygen species (ROS) by both the host and parasite, he nce, inducing oxidative stress in the host system most of the time. Prolonged infection with malaria could, therefore, result in further systemic complications and death in some cases if not treated (Postma, *et al*., 1996).

Malaria parasite infection has reportedly been associated with male infertility (Viroj Wiwanikit, 2011; Yinusa, *et al*., 2006; Singer *et al,* 1987). Oxidative stress has been commonly seen in almost half of all infertile men (Agarwal, 1992). Reactive oxygen species have been reported to cause infertility in male subjects by either damaging the sperm membrane, thus, reducing the sperm's motility and ability to fuse with oocyte or by directly damaging sperm DNA (Tremellen, 2008; Zini *et* al., 2010). Several studies have suggested oxidative stress to be the main underlying pathology that connects varicocele with male infertility (Nellela *et al*., 2004; Smith *et al.,* 2006). Spermatozoa were the first type of cells reported to show susceptibility to reactive oxygen species (Macleod, 1943). This susceptibility is basically due to the high concentration of polyunsaturated fatty acids (PUFAS) in their cell membranes which could enhance lipid peroxidation. Lipid peroxidation has been reported to cause axonemal damage, decreased sperm viability and increased mid-piece morphological defect which contributes to decreased sperm motility (Bansal and Bilaspuri, 2010; Agarwal *et al.,* 2014; Aitken *et al.,* 2010).

Transmitted *Plasmodium berghei* infects the liver and after a short period of development and multiplication, these parasites leave the liver and invade red blood cells. The parasites in the blood stream cause pathological effect like anaemia and damage to essential organs of host (Zini and Sigman, 2009), possibly via free radicals and associated oxidative stress as indicated by lipid peroxidation products like malondialdehyde.

The human body has developed several antioxidant strategies to protect itself from ROS damage. This allows for normal oxidative metabolism to occur without damaging the cells, while still allowing for normal – mediated cellular reponses such as destruction of infectious pathogens and intracellular signaling (Tremellen, 2008). Oxidative stress occurs when the production of ROS overwhelms the antioxidant defense mechanisms leading to cellular damage. Seminal plasma (liquid supernatant after sedimentation of semen) and sperm themselves are well endowed with an array of protective antioxidants (Zini *et* al., 2010). Non-enzymatic antioxidants present within the semen include ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione, amino acids, albumin, carnithine, urates and prostasomes. These agents principally act by directly neutralizing free radical activity chemically. Enzymatic antioxidants also present within the semen are super oxide dismutase (SOD) and catalase, which inactivate the super oxide anion and peroxide radicals, respectively, by converting them into water ad oxygen. Antioxidants in the seminal plasma are obviously helpful for preventing sperm oxidative attack following ejaculation. Malaria infection decreases the levels of antioxidant enzymes and other antioxidants such as catalase, glutathione peroxidase, superoxide dismutase, GSH, ascorbate and plasma tocopherol (Clark *et al.*, 1989). In order to further enhance our understanding, this study investigates the changes in testicular activities induced by *plasmodium berghei* malarial parasite infection in mice.

1.2 Statement of Problem

Activities of the malaria parasite in host red blood cells have been observed to increase free radicals formation (Srivastava *et al.,* 1992).formation of free radicals could overwhelm antioxidant defense capacity in the testis and cause oxidative damage to sperm cells. This seemingly compromise in testicular defense capacity is not fully known and documented. There is need to therefore, investigate changes in testicular activities induced by *Plasmodium berghei* in mice.

1.3 General Objective

To assess changes in testicular activities induced by *plasmodium berghei* malarial parasite in experimented mice

1.3.1 Specific Objectives

1. To infect some group of mice with *plasmodium berghei*
2. To determine changes in testicular activities of mice infected with *plasmodium berghei* by assessing antioxidant defense capacity of testes and semen, and analyzing sperm cell quality, viability and morphology.

1.4 Significance of Study

This study reveals the extent of change in testicular activities in malarial infected mice and has, therefore, contributed to the accumulating knowledge and information regarding the impact of malarial infection on testicular antioxidant defense status, semen quality and sperm viability.

1.5 Justification

This study evaluates malarial-induced changes in testicular activities by examining antioxidant defense capacity of testes and semen, in addition to analyzing sperm quality and viability.

CHAPTER TWO

LITERATURE REVIEW

2.1. Malaria

Malaria is a mosquito borne infectious disease of human and other animals caused by parasitic protozoans (a group of single-celled microorganisms) belonging to the genus *Plasmodium* (WHO, 2014). Malaria was once common in most of Europe and North America, where it is no longer endemic, though imported cases due occur (Hay *et a*l., 2010). Malaria causes symptoms that typically include fever, fatigue, vomiting and headache. In severe cases, it can cause yellow skin, seizures, coma or death (Caraballo, 2014). The disease is transmitted by the biting of mosquitoes and the symptoms usually begin 10-l5days after been bitten. If not appropriately treated, people may have recurrence of the disease months later (WHO, 2014). In those who have recently survived an infection, re-infection typically causes milder symptoms. The partial resistance disappears over months to years if the person has no continuing exposure to malaria (Caraballo, 2014). The disease is transmitted most commonly by an infected female *Anopheles* mosquito. The mosquito bite introduces the parasites from the mosquito's saliva into a person's blood (WHO, 2014). The parasite travels to the liver where they mature and reproduce.

The risk of this disease can be reduced by preventing mosquito bites by using mosquito nets and insect repellants or with mosquito control measures such as spraying insecticides and draining stagnant water (Caraballo, 2014). Several medications are available to prevent malaria in travelers to areas where the disease is common. Occasion doses of the medication sulfacloxine/pyrimethamine are recommended in infants and after the first trimester of pregnancy in areas with high rate of malaria. Despite a need, no effective vaccine exists, although efforts to develop one are ongoing (WHO, 2014). The recommended treatment for malaria is a combination of antimalaria medications that includes an artemisinin (WHO, 2014; Caraballo, 2014). The second medication may be either mefloquine, lumefantrine or sulfadoxine/pyrimethmine (WHO, 2010). Quinine along with deoxycycline may be used if an artemisinin is not available (WHO, 2014). It is recommended that in areas where the disease is common, malaria is confirmed be for treatment is started due to concerns of increasing drug resistance. Resistance among the parasites has developed to several anti-

malaria medications; for example, chloroquine-resistance to *P. Falciparum* has spread to most malaria areas and resistance to artimisinin has become a problem in some parts of Southeast Asia (WHO, 2014).

The disease is widespread in the tropical and subtropical regions that exist in a broad bound around the equator (Caraballo, 2014). This includes most of sub-Saharan Africa, Asia and Latin America. Malaria is commonly associated with poverty and has a major negative effect on economic development (Gollin *and Zimmarman*, 2007; Worral, *et al*., 2005). In Africa, it is estimated to result in losses of US$1.2billion a year due to increased health care costs, lost of ability to work and effects on tourism (Greenwood *et al.*, 2005). The world health organization reports there were l98million cases of malaria worldwide in 2013 (WHO, 2014; GBD, 2013, 2014). This resulted in an estimated 584,000 to 855,000 deaths, the majority (90%) of which occurred in Africa (WHO, 2014).

2.1.1. Causes of Malaria

Malaria parasite belongs to the genus *plasmodium* (Phylum Apicomplexa). In humans, *malaria is caused by P. falciparum, P. ovale, P. vivax and P. lenowlesi (*Muller *et al.*, 2007; Collins, 2012). Among those infected, *P. falciparum* is the most common species identified followed by *P. vivax (*Caraballo, 2014). Each species has a distinctive appearance under the microscope and each one produces a somewhat different pattern of symptoms.

The species infecting humans are;

- *P. falciparum* is found in tropical and subtropical areas. It is the most malignant form of malaria. *P. falciparum* is able to infect red blood cells of all ages, resulting in high levels of parasitemia (greater than 5% RBCs infected). It is estimated that every year approximately 1million people are killed by *P. falciparum*, especially in Africa where this specie predominates.
- *P. Falciparum* can cause severe malaria because it multiplies rapidly in the blood which causes severe blood loss (anaemia). The infected parasites can clog small blood vessels. When this occurs in the brain, cerebral malaria results in a complication that can be fatal (Collins, *et al.*, 2007). Manifestation of *P. falciparum* include; hypoglycaemia, lactic acidosis, severe anaemia and multi organ dysfunction due to hypoxia.
- *P. Vivax* is found mostly in Asia, Latin America and in some parts of Africa and because of the population densities especially in Asia. It is probably the most prevalent human

malaria parasite. *P. vivax* (as well as *P. Ovale*) have dormant liver stages (hypnozoties) that can activate and invade the blood (relapse) several months or years after the infecting mosquito bite (Baird, 2013).

- *P. Ovale* is found mostly in Africa (especially West Africa) and the islands of the Western Pacific. It is biologically and morphologically very similar to *P. vivax*. However, different from *P. vivax*, it can infect individuals who are negative for the Duffy blood group which is the case for many resistance of sub-saharan Africa. This explains the greater prevalence of *P. ovale* (rather than *P. vivax*) in most Africa.
- *P. Malariae*, is the human malaria parasite specie that has a quartan cycle (four day cycle). When *P. malariae* is not treated, it causes a long lasting chronic infection that in some cases can last a life time. In some chronically infected patients, *P. malariae* can cause serious complications such as the nephritic syndrome (Baird, 2013).
- *P. Knowlesi*, is found throughout southwest Asia as a natural pathogen of long- tailed and pig-tailed macaques. It has recently been sown to be a significant cause of zoonotic malaria in that region, particularly in Malaysia. *P. knowlesi* has a 24hours replication cycle and so can rapidly progress from an uncomplicated infection to a severe infection; fatal cases have been reported (Cyrus *et al.*, 2009).

2.1.2. Signs and Symptoms of Malaria

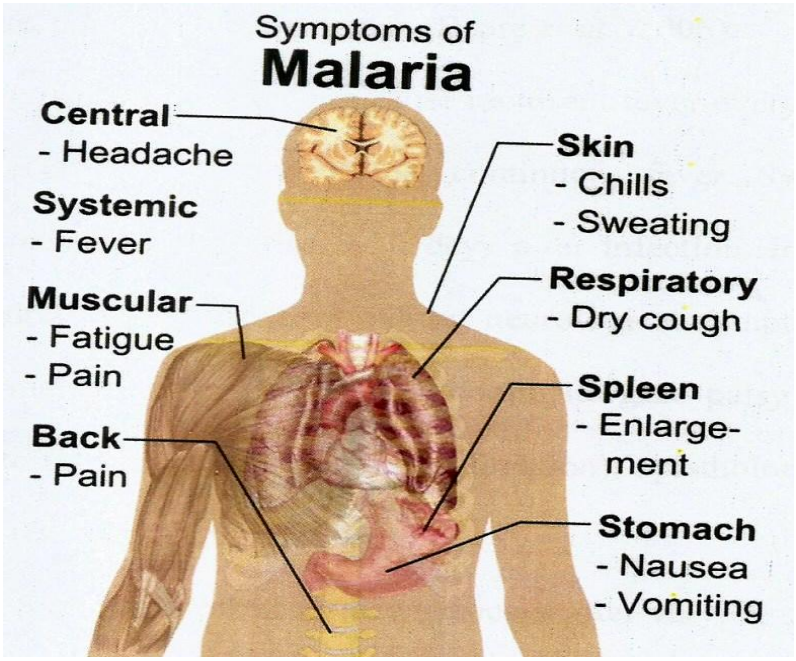


Fig. 1.0: Main symptoms of malaria
Source: Fairhurt and Wellens, (2009).

The signs and symptoms of malaria typically begin 8-25days Following infection; however, symptoms may occur later in those who have taken anti malaria medications as prevention. The clinical Symptoms include; headache, fever, shivering, joint pain, vomiting, haemolytic anaemia, jaundice, hemoglobin in urine, retinal damage, fatigue, cough and convulsions (Bartoloni *and Zammaihi,* 2012).

P. falciparum infection can cause recurrent fever every 36-48hours or a less pronounced and almost continuous fever. Symptoms of *P. falciparum* malaria arise 9-30 days after infection. Individuals with cerebral malaria frequently exhibit neurological symptoms, including abnormal posturing, nystagmus, conjugate gaze palsy (failure of the eyes to turn together in the same direction), opistholonus, seizures or coma (Bartoloni *and Zammaihi,* 2012).

Symptoms of malaria can reoccur after varying symptoms- free periods. Depending upon the cause, recurrence can be classified as recrudescence, relapse or reinfection. Recrudescence is when symptoms return after a symptoms free period. It is caused by parasites surviving in the blood as a result of inadequate or ineffective treatment. Relapse is

when symptoms reappear after the parasites have been eliminated from the blood but persist as dormant hypnozoites in liver cells. Relapse commonly occurs between 8-24weeks and is commonly seen with *P. vivax* and *P.* ovale infections (Nadjim and Behrens, 2012). Re-infection means the parasite that caused the past infection was eliminated from the body but a new parasite was introduced. Re-infection cannot readily be distinguished from recrudescence, although recurrence of infection within two weeks of treatments for the initial infection is typically attributed to treatment failure. People may develop some immunity when exposed to frequent infections (Schiagenhauf 2008).

2.1.3 Life Cycle of Malaria in Human

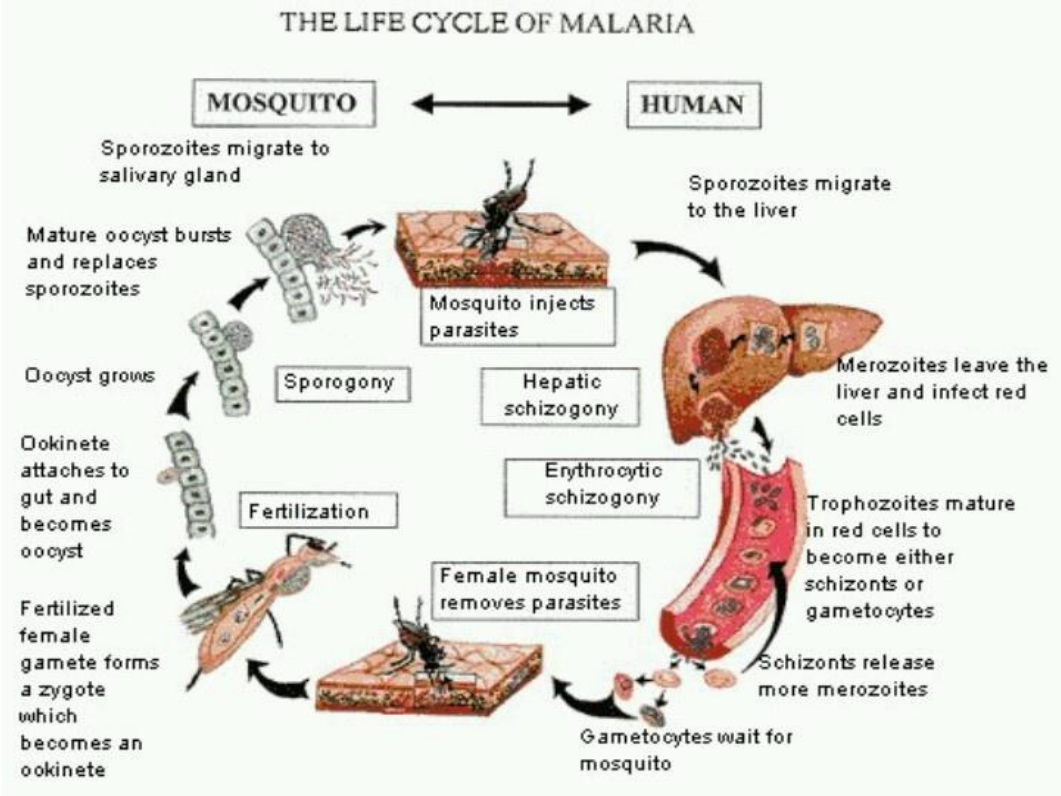


Fig 1.1: The Life Cycle of Malaria Parasite

Source: Schiagenhauf (2008)

In the life cycle of malaria in humans, sporozoites are injected into humans dermis through the bite of infected *Anopheles* mosquito. After incubation, sporozoites migrate to liver cells to re-establish the first intracellular replicative stage. Merozoites generated from this exoerythrocytic phase when invade erythrocytes (RBCs) and it is during this erythrocytic

stage that severe conditions of malaria occur. The cycle is completed when sexual stages (gametocytes) are ingested by a mosquito. Transgenic *Plasmodium berghei* sporozoites expressing green fluorescent protein were used by Amo *et al.*, (2002) to trace parasites migratory behaviour in mammalian skin and to observe sporozoite activity in draining nodes. Sporozoites deposited in the skin migrate rapidly through the region of the bite. Some eventually penetrate capillaries or lymph vessels. Those entering the lymph vessels will penetrate lymph vascular endothelial cells in lymph nodes to establish a lymph node form, which appears not to continue the life cycle but maybe significant in priming an immune response (Amo *et al*., 2002).

2.1.4. Classification of Malaria

Malaria is classified into either "severe" or "uncomplicated" by the world health organization (Nadjim and Behrens, 2012). It is deemed severe when any of the following criteria are present, otherwise, it is considered uncomplicated (WHO, 2010).

- Inability to feed
- Two or more convulsions
- Low blood pressure (less than 70mmHg in adults and 50mmHg in children)
- Pulmonary oedema
- Acidosis or lactate levels of greater than 5mmol/L
- Blood glucose less than 2.2mmol/L (40mg/dl)
- Kidney failure or hemoglobin in urine
- Bleeding problems or hemoglobin less than 50g/L or 50g/dl
- Circulation shock
- Breathing problems
- Decreased consciousness
- Significant weakness such that the person is unable to walk.
- A parasite level in the blood of greater than 100,000 per microlitre in
- low density transmission areas, or 250,000 per
- Microlitre in high intensity transmission areas.

2.1.5. Prevention of Malaria

Fig.1.2: Mosquito Net in Use
Source: Raghavendra *et al*., (2011)

Prevention of malaria maybe more cost- effective than treatment of the disease in the long run, but the initial costs required are out of reach of many of the world's poorest people. There is a wide difference in the costs of control (i.e maintenance of low endemicity) and elimination programs between countries. Methods used to prevent malaria include medications, mosquito elimination and the prevention of bites. There are no vaccines for malaria and the presence of malaria in an area requires a combination of high human population density, high *Anopheles* mosquito population density and high rates of transmission from humans to mosquitoes and from mosquitoes to humans. If any of these is lowered sufficiently, the parasite will eventually disappear from that area, as happened in North America, Europe, and parts of the Middle East. However, unless the parasite is eliminated from the whole world, it could become re-established if conditions revert to a combination that favours the parasite's reproduction. Furthermore, the cost per person of eliminating *Anopheles* mosquitoes rises with decreasing population density, making it economically unfeasible in some areas (WHO, 1958).

There are a number of other methods to reduce mosquito bites and slow the spread of malaria. Efforts to decrease mosquito larva by decreasing the availability of open water in

which they develop or by adding substances to decrease their development is effective in some locations. (Sabot *et al.*, 2010).

2.1.6. Treatment of Malaria

Malaria is treated with anti-malarial medications, the ones used depends on the type and severity of the disease. While, medications against fever are commonly used, their effects on outcomes are not clear (Meremikwu, 2012) uncomplicated malaria maybe treated with antimalarials medications. The most effective treatment for P. falciparum infection is the use of artemisinins in combination with other antimalarials (known as artemisinin-combination therapy or ACT), which decreases resistance to any single drug component (Kokworo, 2009).

2.1.7. Drug Resistance to Malaria

Drug resistance possesses a growing problem in 21st century malaria treatment (Sinha *et al.*, 2014). Resistance is now common against all classes of antimalarial drugs save the artemisinins. The cost of artemisinins limits their use in the developing world (White, 2008). Malaria strains found one the Cambodia-Thailand border are resistant to combination therapies that include artemisinins and may therefore be untreatable (Wongsrichanalai andMeshnick, 2008). Exposure of the parasite population to artemisinin monotherapies in sub therapeutic doses for over 30years and the availaibility of substandard artemisinins likely drove the selection of the resistant phenotype. (Dondorp *et al.*, 2010). Resistance to artemisinin has been detected in Cambodia, Myanmar,Thailand and Vietnam, (WHO, 2013) and there has been emerging resistance in Lagos (Briggs, 2014; Ashley *et al*., 2014).

2.1.8. Epidemiology of Malaria

Epidemiology can be described as the study of disease patterns. The epidemiology of malaria is described as endemic (stable) when a consistent pattern of transmission is found in humans over a number of years. Malaria epidemiology in areas stable transmission is typified by an age dependent pattern of non-sterilization immunity in human host. At other extreme epidemic (unstable) malaria occurs when there is a large increase in the prevalence of cases at any period in time. In epidemic malaria, parasites are generally less genetically diverse.

Globally, a spectrum of epidemiology of malaria showing varying transmission intensities has been found (Snow *et al.,* 2005). The major impact of the disease is almost entirely on the developing countries, with the heaviest burden in Africa. The WHO 2015

estimates that there were 214million cases of malaria resulting 438,000 deaths (range 236,000-235,000) (WHO, 2015). Others have estimated the number of cases at between 350 and 550million for *falciparum* malaria (Olupot and Maritland, 2013) and deaths in 2010 at 1.24million (Murray *et al*., 2012) up from l.0million deaths in 1990. The majority of cases (65%) occur in children under 5years old (Murray *et al*, 2012). About l25million pregnant women are at risk of infection each year; in sub-Saharan Africa, maternal malaria is associated with up to 200,000 estimated infant deaths yearly (Hartman *et al*., 2009). About 900 people died from the disease in Europe between 1993 and 2003 (Kajfasz, 2010). There are about 10,000 malaria cases per year in Western Europe and 1,300-1,500 in the United States (Taylor *et al.,* 2012). Both the global incidence of disease and resulting mortality has declined in recent years. According to the WHO, deaths attributable to malaria in 2010 were reduced by over a third from a 2000 estimate of 985,000, largely due to the whispered use of insecticides- treated nets and artemisinin-based combination therapies (Howtt, *et al.*, 2012).

An estimate for 2009 reported that countries with the highest death rate per 100,000 of population were Ivory Coast (86.15), Angola (56.93) and Burkina Faso (50.66) (Provost, 2011). A 2010 estimate indicated the deadliest countries per population were Burkina Faso, Mozambique and Mali (Murray, 2012). The malaria Atlas Project aims to map global endemic levels of malaria, providing a means with which to determine the global spatial limits of the disease and to assess disease burden (Guerra *et al*., 2007; Hay *et al*., 2007). This effort led to the publication of a map of *P. falciparum* endemicity in 2010 (Gething *et al.,* 2011).

2.1.9. Transmission of Malaria

Malaria transmission usually occurs through the bite of an infected female Anopheles mosquito. It can also occur through contact with infected blood (transfusion malaria). The disease can also be transmitted from a mother to her foetus before or during delivery (congenital malaria).

2.2. Transmitting Malaria through Mosquito Bite

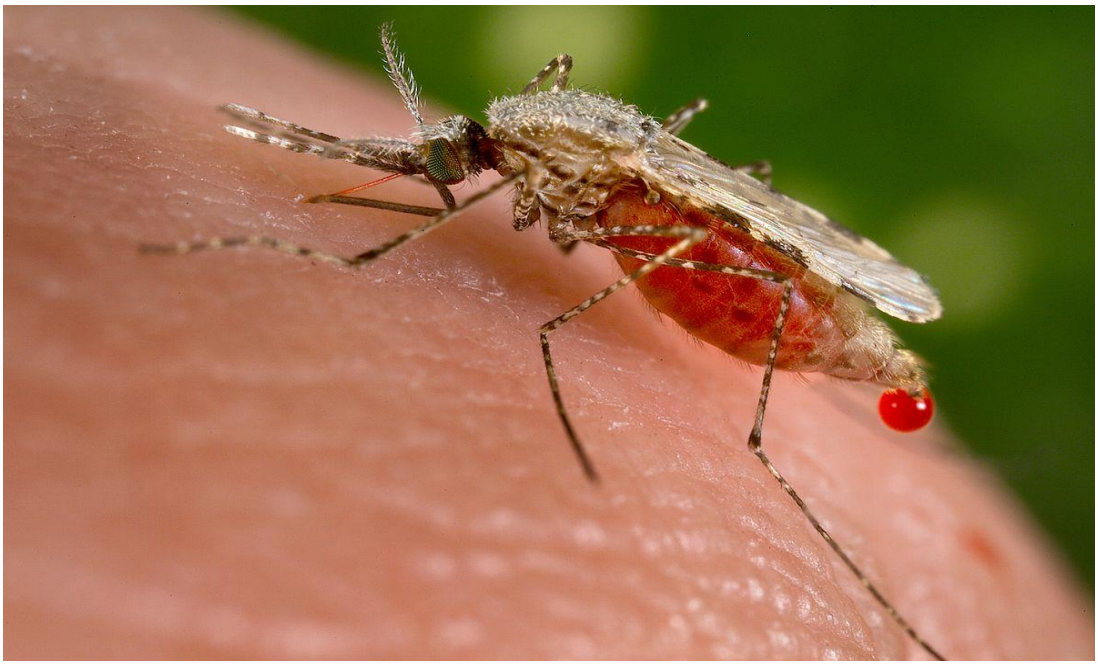


Fig. 1.3: An *Anopheles stephensi* mosquito shortly after obtaining blood from a human (the droplet of blood is expelled as a surplus).
Source: Sabot *et al.* (2010).

Malaria transmission most often occurs through the bite of an infected Anopheles mosquito. No other type of mosquitoes is known to transmit this disease. This type of mosquito becomes infected with one of the four plasmodium parasites that cause malaria in human through a previous blood meal from an infected person.

When an *Anopheles* mosquito bites an infected person, a small amount of blood infected with microscopic malaria parasite is taken. The parasite grows and matures in the mosquito's gut for a week or more, then travels to the mosquito's salivary glands. When the mosquito next takes a blood meal, these parasites mixed with the saliva are injected with the bite, and the transmission of malaria is complete. The parasite in the blood travels to the liver and enter liver cells to grow and multiply. After as few as seven days or as long as several years, the parasite leaves the liver cells and enters red blood cells, which normally carry oxygen in the blood to the tissues that need it.

Once the parasite is in the red blood cells it continues to grow and multiply. After they mature, the infected red blood cells rupture, freeing the parasites to attack and enter other red blood cells. Toxins are released when the red cells burst and these toxins cause the typical symptoms of malaria such as fever, chills, flu like symptoms, etc. If a mosquito bites this infected person and ingests certain types of malaria parasite, the malaria transmission cycle continues (Heidrick, 2011).

2.2.1 *Plasmodium Berghei*

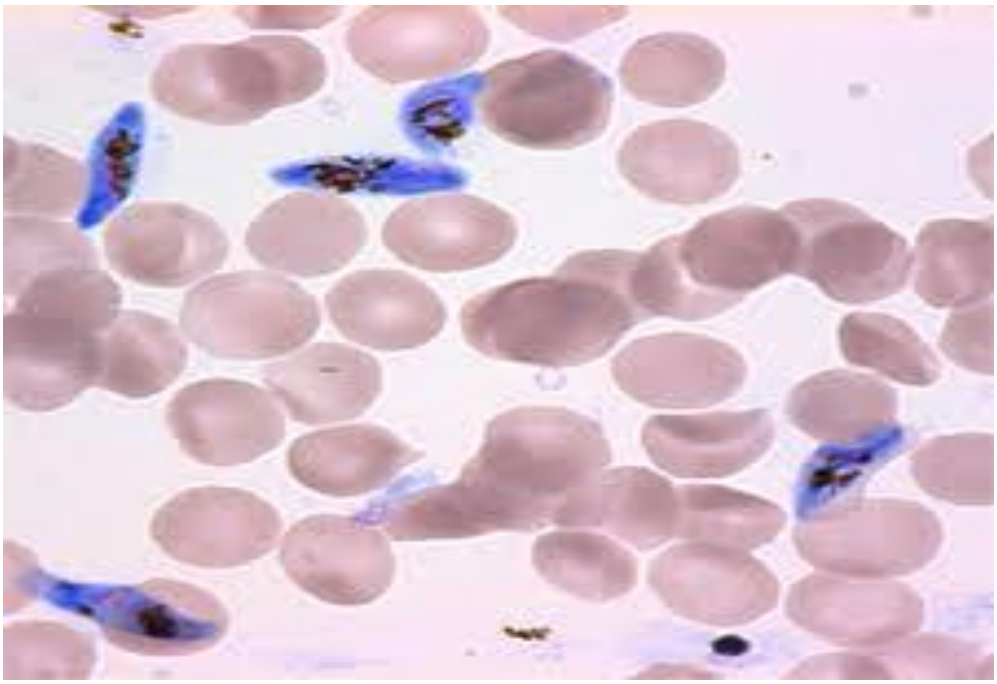


Fig. 1.4*: Plasmodium berghei forms in Red Blood Cell*

Source: Heidrick, (2011)

Plasmodium berghei is a unicellular parasite (protozoan) that infects mammals other than humans. It is one of the four *Plasmodium* species that have been described in African Murine rodents, the others been; *Plasmodium chabaudi, Plasmodium vinckei and Plasmodium yeolli. Plasmodium berghei* is found in the forest of Central Africa, where its natural cyclic host is the thicket rat (*Grammonys surdaster*) and the mosquito (*Anopheles dureni*)

Like all malaria parasites of mammals, including the four human malaria parasites, *P. berghei* is transmitted by *Anopheles* mosquitoes and it infects the liver after been injected into the blood stream 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 anemia and damage of essential organs of the host such as lungs, liver and spleen. *Plasmodium berghei* infections may also affect the brain and can be the cause of cerebral complications in laboratory mice. The symptoms are to a certain degree comparable to symptoms of cerebral malaria in patients infected with the human malaria parasite (*P. falciparum*) (Franke *et al.,* 2010).

Plasmodium berghei can be genetically manipulated in the laboratory using standard genetic engineering technologies. Consequently this parasite is often used for the analysis of the function of malaria genes using the technology of genetic modification (Janse *et al.,* 2006; Janse, 2011; Khan *et al.*, 2013).

2.2.2. Scientific Classification of *Plasmodium berghei*

Dormain: Eukarya

Kingdom: Chronalveo Lata

Super Phylum: Alveolata

Phylum: Apicomplexa

Order: Chromatorida

Species: Berghei

Genus: Plasmodium

Family: Plasmodiidae

Suborder: Laveraniina

Subclass: Haemosphoridiasinia

These species were first described by Vinke and Lips (1948) in the Belgran Congo.

2.2.3. Importance of Plasmodium Berghei

Plasmodium berghei has been an important element in the attempts to learn how to manage and eradicate malaria. 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 *P. falciparum* (Kulkanium *et al.,* 2003). The use of this model malaria parasite has provided biologist and medical researchers with more insight into developing and researching for anti-malarial drugs for the effective treatment/vaccine production against malaria (Kulkanium *et al.,* 2003)

The use of *P. berghei* has also provided researchers with the biochemical effects which infection could have on the metabolism of some biomolecules in the body and the transformation that may occur when treatment are made with some research anti-malarial agents (drugs) (WHO, 2000). The process of infection of some organs (the pancreas and other vital organs of the body) by the malaria parasites. Researchers focused more on the last two points listed above. Malaria plasmodia are highly similar in their patterns of infection and life cycle, making the berghei stains which is not transmitted to humans, a safe choice for experimental and observation.

2.2.4 Diagnosis of Malaria

Due to the non specific nature of the presentation of symptoms, diagnosis of malaria in non-endemic areas requires a high degree of suspicion, which might be elicited by any of the following: recent travel history, enlarged spleen, fever, low number of platelets in the blood, and higher than-normal level of bilirubin in the blood combined with normal level of white blood cells. (Nadjim and Behrens, 2012).

- Malaria is usually confirmed by the microscopic examination of blood films or by antigen-based rapid diagnostic tests (RDT). Microscopy is the most commonly used method to detect the malaria parasite- about 165 Million blood films were examined for malaria in 2010. The sensitivity of blood films ranges from 75-90% in optimum conditions, to as low as 50%. Commercially available RDT's are often more accurate than blood films at predicting the presence of malaria parasites, but they are widely variable in diagnostic sensitivity and specifically depending on manufacturer, and are unable to tell how many parasites are present. In areas that cannot afford laboratory diagnostic tests, it has become common to use a history of fever as indication to treat for malaria-thus the common teaching "fever equals malaria unless proven otherwise" Although, Polymerase chain reaction-base tests have been developed, they are not widely

used in areas where malaria is common as of 2012, due to their complexity. (Nadjim and Behrens, 2012).

2.2.5 Complications

Several complications that may arise due to malaria include respiratory distress, usually up to 25% of adults and 40% of children with severe P. Falciparum malaria. This is rare in children with severe malaria but acute respiration distress syndrome occurs in 5-25% of adults and up to 29% of pregnant women (Taylor *et al.*, 2012).

- Co infection of HIV with malaria increases mortality, renal failure is a feature of black water fever, where hemoglobin from lysed red blood cells leaks into the urine (Batoloni and Zammardii, 2012).
- Infection with *P. falciparum* may result in cerebral malaria a form of severe malaria that involves encephalopathy. It is associated with retinal whitening, which may be a useful clinical sign in distinguishing malaria from other causes of fever, Splenomegaly, severe headache, hepatomegaly (enlarged liver), hypoglycemia with renial failure may occur. Complications include spontaneous bleeding and coagulopathy, may cause shock (algid malaria).
- Malaria in pregnant women is an important cause of still birth, infant mortality, abortion and low birth weight, (Hartman *et al*, 2010) particularly in *P falciparum* infection but also *P. vivax*

2.2.6 Recurrent Malaria

Recurrence can be classified as recrudescence, relapse and reinfection. recrudenscence is when symptoms returns after a symptoms free-period. it is cause by parasites surviving in the blood as a result of inadequate or infective treatment. relapse is when symptoms reappear after the parasite have been eliminated from blood but persist as dominant hypnozoites in liver cells. it occurs mainly within 8-24 weeks and common to *p. vivax* and *p. ovale* infections. in the case of reinfection, the parasite that caused past infection is eliminated from the body but a new parasite is introduced.

- in most cases, it is difficult to distinguish re-infection from recrudescence, although recurrence of infection within two weeks of treatment for the initial infection is typically

attributed to treatment for the initial infection is typically attributed to treatment failure (WHO, 2010).

2.2.7 Malaria Eradication Efforts

Notable efforts are being made to eradicate malaria parasite worldwide. one of the attempts was in 2006, when the organization, malaria no more set a public goal of eliminating malaria from africa by the year 2015 and the organization plans to dissolve if the goal is accomplished. (Strom, 2011). A lot of malaria vaccines are in clinical trials which are intended to provide protection for children in academic areas and lower the rate of transmission of the disease. As of 2012, the global fund to fight aids, tuberculosis and malaria has distributed 230 million insecticide treated nets with attempt to stop mosquito borne transmission of malaria. The US based clinton foundation has worked to manage demand and stabilize prices in the artemisinin market. (Schoof, 2008) malaria atlas projects, focus on analyzing climate and weather information required to accurately predict the rate of malaria based on the availability of habitat of malaria-carrying parasite. the malaria policy advisory committee (mpac) of the world health organization (who) was formed in 2012 "to provide strategic advice and techniques input to who on all aspects of malaria control and elimination". World Health Organization on malaria report. 13 February, 2014. in November, 2013, who and the malaria vaccine funders group set a goal to develop vaccine designed to interrupt malaria transmission with the malaria has been successfully eliminated or greatly reduced in certain areas. Vector control programmes in conjunction with the monitoring and treatment of infected humans, eliminated malaria from United States and southern Europe. draining of wetland breeding grounds for agriculture and other changes in water management practices, greater use of glass windows and screen in dwellings contributed (Mead, 2010).

2.2.8 Medications for Malaria

there are a number of drugs that can help prevent malaria. chloroquine may be used where the parasite is still sensitive. Because most *plasmodium* is resistant to one or more medications, one of three medications mefloquine, doxycyline or the combination of atovaquone and proguanil hydrochloride is frequently needed. doxycycline and the atoquone and proquanil combination are the best tolerated; mefloquine is associated with death, suicide and neurological and psychiatric symptoms, (Jacqueroz and Croft, 2009). The use of preventive drugs where malaria bearing mosquitoes are present may encourage the

development of partial resistance. an exception to this is during pregnancy when taking medication to prevent malaria has been found to improve the weight at birth and decrease the risk of anemia, the mother, (Radeva-petrova *et al.*, 2014).

- Malaria parasite contains apicoplasts, organelles usually found in plant which play crucial role in various aspect of parasite metabolism such as fatty acid biosynthesis. not less than 400 proteins have been found to be produced by apicoplasts these are now being investigated as possible targets for novel anti-malaria drugs (Kalanon and McFadden, 2010).
- With the onset of drug-resistant plasmodium parasite new strategies, such as introduction of synthetic pyridoxal amino-acid adducts have been developed to combat the wide spread disease. they are taken up by the parasite and ultimately interfere with its ability to create several essential b vitamins. (Jacqueroz and Croft, 2009).

2.2.9 Prognosis of Malaria

Severe malaria can progress extremely rapidly and cause death within hours or days but when properly treated, the people can have complete recovery. in the most severe cases of the disease, fatality rates can reach 20%, even with intensive care and treatment (Nadjim and Behrens, 2012). Over the longer term, developmental impairments have been documented in children who have suffered episodes of severe malaria (Fernando *et al.*, 2010). Chronic infection without severe disease can occur in an immune deficiency syndrome associated with decrease responsiveness to salmonela bacteria and the epstein- barr virus.

During a period of rapid brain development (childhood) malaria causes anemia and direct brain damage resulting from cerebral malaria. (fernando *et al*; 2010). some survivors of cerebral malaria have an increased risk of neurological and cognitive deficits, behavioural disorders, and epilepsy (idro *et al;* 2010) malaria prophylaxis was shown to improve cognitive function and school performance in clinical trials when compared to placebo groups (Fernando *et al*., 2010).

2.3 Semen

Semen is a grayish white bodily fluid that is secreted by the gonads of male animals. It carries sperm or spermatozoa and fructose and other enzymes that help the sperm to

survive to facilitate successful fertilization. The whitish opalescence is due to the large amount of protein that it contains and it's slightly turbid appearance is due to the spermatozoa contained within it.

Sperm is released during the process of ejaculation and is processed in the seminal vesicle in the pelvis, which is where it is produced. Ejaculation is controlled by the central nervous system and occurs when there is friction on the genitalia and other forms of sexual stimulation. The stimuli lead to impulses that are sent up the spinal cord and into the brain. Ejaculation has two phases:

Phase 1: Emission in which the vas deferens (the tubes that store and transport sperm from the testis) contract to squeeze the sperm toward the base of the penis through the prostate gland and into the urethra. The seminal vesicles release their part of the semen that combines with the sperm. The ejaculation is unstoppable at this stage.

Phase 2: Ejaculation in which the muscles at the base of the penis and urethra contract. This leads to forcing the semen out of the penis (ejaculation and orgasm) and this phase also has a bladder neck contraction. The bladder neck contracts to prevent the back flow of the semen into the urinary tract. Dry orgasm can occur even without delivery of semen (ejaculation) from the penis. Erection declines normally following ejaculation.

2.3.1 Semen Composition

The semen travels through the ejaculatory ducts and mixes with fluids from the seminal vesicles, the prostrate, and the bulbourethral glands. The seminal vesicles produce a viscous, fructose-rich fluid forming around 65-70% of the semen base. The white color of the semen is due to secretion from the prostate glands containing enzymes, citric acid, lipids, and acid phosphatase. This forms around 25-30% of the semen base. At each ejaculation around 200- 500 million sperms are released by the testes. This forms about 2-5% of the semen composition. Apart from these, the bulbourethral glands produce a clear secretion. This helps in mobility of the sperm cells in the vagina and cervix. The glands' secretion contributes less than 1% to the overall semen composition. The semen comprises of: Fructose, Ascorbic acid, Zinc, Cholesterol, Protein, Calcium, Chlorine, etc.

2.3.2 Sperm Cells

Sperm is a male reproductive cell and is derived from the Greek word sperma (meaning "seed") A uniflagellar sperm cell that is motile is referred to as spermatozoan, whereas a non-motile sperm cell is a referred to as a spermatium. Sperm is secreted by the gonads (sexual glands) and other sexual organs like testicles, prostate gland and seminal vesicles of male or hermaphrotidic animals and can fertilize female ova. In humans, seminal fluid contains several components besides spermatozoa: proteolysis and other enzymes as well as fructose are the elements of seminal fluid which promotes the survival of spermatozoa and provide a medium through which they can move or swim. Semen is produced and originates from the seminal vesicles, which are located in the pelvis.

2.3.3 Internal and External Fertilization by Sperm Cells

Depending on the species, spermatozoa can fertilize ova externally or internally. In external fertilization, the spermatozoa fertilize the ova directly, outside of the female's sexual organs. Internal fertilization takes place in the female's sexual organs. It takes place after insemination of a female through copulation. In most vertebrates, including amphibians, reptiles, birds and monotreme mammals, copulation is achieved through the physical mating of the male and female. In marsupial and placental mammals copulation occurs through the vagina.

2.3.4 Sperm Motility

Sperm motility describes the ability of sperm to move properly through the female reproductive tract (internal fertilization) or through water (external fertilization) to reach the egg. Sperm motility can also be thought of as quality, which is a factor in successful conception; sperm that do not swim properly will not reach the egg in order to fertilize it. Sperm motility in mammals also facilitates the passage of the sperm through the cumulus oophorus (a layer of cells) and the zona pellucid (a layer of extracellular matrix), which surround the mammalian oocyte. In the wood mouse Apodemus sylvaticus, sperms aggregate in trains that are able to fertilize eggs because they are more capable of navigating the viscous environment of the female reproductive tract. The trains move in a sinusoidal motion.

Sperm motility is also affected by certain factors released by eggs. Sperm movement is activated by changes in intracellular ion concentration. The changes in ion concentration that provoke motility are different among species. In some mammals, sperm motility is activated by increase in pH, calcium ion and CAMP, yet it is suppressed by low pH in the epididymis. The tail of the sperm- the flagellum - confers motility upon the sperm, and has three principal components:

1. A central skeleton constructed of 11 microtubules collectively termed the axoneme and similar to the equivalent structure found in cilia.
2. A thin cell membrane covering the axoneme.
3. Mitochondria arranged spirally around the axoneme.

Back and forth movement of the tail results from a rhythmical longitudinal sliding motion between the anterior and posterior tubules that makes up the axoneme. The energy for this process is supplied by ATP produced by mitochondria. The velocity of a sperm in fluid medium is usually 1-4 mm/min. This allows the sperm to move towards an ovum in order to fertilize it.

In mammals, spermatozoa mature functionally through a process which is known as capacitation. When spermatozoa reach the isthmic oviduct, their motility has been reported to be reduced as they attach to epithelium. Near the time of ovulation, hyperactivation occurs. During the process, the flagella move with high curvature and long wavelength (Mortimer *et al;* 1995).

Without technological intervention, a non-motile or abnormally motile sperm is not going to fertilize. Therefore, the fraction of a sperm population that is motile is widely used as a measure of semen quality. Insufficient sperm motility is a common cause of sub-fertility or infertility. Several measures are available to improve sperm quality. Motility is classified into straight moving, zig-zag moving, vibrating and non-motile.

2.3.5 Sperm Viability

Sperm viability is the percentage of sperm alive. Although normal seminal fluid contains huge numbers of sperm, not all of these sperm will be viable (alive). Sperm viability is an important measure of fertility. For normal fertility, about 60% or more of a male sperm need to be viable. Viable sperm are those which are healthy and without defects of the sort

that might prevent conception. These defects might include damage to DNA and its substrate, or the presence of anti-sperm antibodies, or other chemical issues which would prevent the sperm fertilizing an egg.

Sperm viability is important because many sperm will die in the acid environment of the vagina. If there are too few viable sperm to start with, it is unlikely that any will survive to reach the egg. So anything that reduces the number of a male viable sperm reduces his chances of fatherhood. To get an accurate picture of fertility the Percentage of viable sperm is normally looked at alongside the percentage of motile sperm and the total sperm count. Because it is the total number of viable and motile sperm that is important a very high sperm count can sometimes help mitigate low viability

2.4 Malondialdehyde

Malondialdehyde is an organic compound with the formular ch_2 $(cho)_2$. it is not typically observed in pure form. it results from lipid peroxidation of polyunsaturated fatty acid (Davey *et al.,* 2005). It is a prominent production in thromboxane a2 synthesis wherein cyclooxygenase 1 or cyclooxygenase 2 metabolizes arachidonic acid to prostate gland in oxygen by platelets and a wide array of the cell types and tissues. this product is further metabolized by thromboxane synthesis to thromboxane a2, 12-hydroxyheptadecatrienoic acid malonyldialdehyde (it is one of the most prevalent byproduct of lipid peroxidation during oxidative stress. the degree of lipid peroxidation can be estimated by the amount of malondialdehyde in tissues (Davey *et al.,* 2005).

Oxidative stress is one of the most important pathological consequences of malaria infections. recent studies suggest that the generation of reactive oxygen and nitrogen species (ros and rns) associated with oxidative stress plays a crucial role in the development of systemic complications caused by malaria (Klein, 2004). Oxidative stress occurs when the production of potentially destructive reactive oxygen species (ROS) exceeds the body's own natural antioxidant defenses resulting in cellular damage.

Malaria infection decreases the level of antioxidant enzymes and other antioxidants such as catalase, glutathione peroxidase, superoxide dismutase, GSH, Ascorbate and plasma to copherol (Clark *et al.,* 1989).

2.4.1 Biochemistry of Malondialdehyde

Reactive oxygen species degrade polyunsaturated lipids forming malondialdehyde (Pryor and Stanley, 1995). this compound is a reactive aldehyde and one of the many reactive species that cause toxic stress in cells and form covalent protein adducts referred to as advanced lipoxidation end-product (ale), in analogy to advanced glycation end-products (age) (Farmer and Davonic, 2007). The product of this aldehyde is used as a biomarker to measure the level of oxidative stress in an organism (Del *et al*., 2005).

2.5 Superoxide Dismutase

Superoxide dismutases are enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. Thus, they are important antioxidant defense in nearly all cells exposed to oxygen.

Reactions of SOD

The SOD-catalyzed dismutation of superoxide may be written ‘with the following half-reactions:

- $M^{(n+1)+}SOD + O^{-} \rightarrow M^{n+}\text{-}SOD + O_2$
- $M^{n+}\text{-}SOD + O^{-} + 2H^{+} \rightarrow M^{(n+1)+}\text{-}SOD + H_2O$.

Where M = Cu (n=1); Mn (n=2); Fe (n=2); Ni (n=2).

In this reaction the oxidation state of the metal cation oscillates between n and n+1.

2.5.1 Types of SOD

Irwin Fridovich and Joe McCord discovered the activity of superoxide dismutase. SODs were previously known as a group of metalloproteins with unknown function; for example, CuZn SOD was known as erythrocuprein and as the veterinary anti-inflammatory drug “Orgotein” (McCord and Fridovich, 1988). Likewise, (Brewer, 1967) identified a protein that later became known as superoxide dismutase as an indophenol oxidase by protein analysis of starch gels using the phenazinetetrazoliwn technique.

Several common forms of SOD exist: they are proteins cofactored with copper and zinc, or manganese, iron, or nickel. Thus, there are three major families of superoxide dismutase, depending on the metal cofactor: Cu/Zn (which binds both copper and zinc), Fe and Mn types (which bind either iron or manganese), and the Ni type, which binds nickel.

• Copper and zinc — most commonly used by eukaryotes. The cytosols of virtually all eukaryotic cells contain an SOD enzyme with copper and zinc (Cu-Zn-SOD). For example, Cu-Zn-SOD available commercially is normally purified from the bovine erythrocytes: The Cu-Zn enzyme is a homodiiner of molecular weight 32,500. The bovine Cu-Zn protein was the fir St SOD structure to be solved in 1975 (Richardson and Richardson, 1975). It is an 8-stranded "Greek key" beta-barrel, with the active site held between the barrel and two surface loops. The two subunits are tightly joined back-to-back, primarily by hydrophobic and some electrostatic interactions. The ligands of the copper and zinc are six histidine and one aspartate side-chains; one histidine is shared between the two metals (Tamer *et al.*, 1983).

• Iron or manganese — used by prokaryotes and protists, and in mitochondria

• **Iron:** E. coil and many other bacteria also contain a form of the enzyme with iron (Fe-SOD); some bacteria contain Fe-SOD, others Mn-SOD, and some contain both. (For the E. coil Fe-SOD). Fe-SOD can be found in the plastids of plants. The 3dimiensional structures of the homologous Mn and Fe superoxide dismutases have the same arrangement of aipha-helices, and their active sites contain the same type and arrangement of amino acid side-chains.

• **Manganese:** Chicken liver, mitoehondria, and many bacteria (such as E. coil), contain a form with manganese (Mn- SOD): for example, the Mn-SOD found in human mitochondria. The ligands of the manganese ions are 3 histidine side-chains; an aspartate side- chain, and a water molecule or hydroxyl ligand, depending on the Mn oxidation state (respectively II and III) (Borgstabl *et al*., 1992)

• **Nickel — prokaryotic:** This has a hexamerie structure built from right- handed 4-helix bundles, each containing N-terminal hooks that chelate a Ni ion. The Ni-hook contains the motif His-Cys-X-XPro-Cys-Gly-X-Tyr; it provides most of the interactions critical for metal binding and catalysis and is, therefore, a likely diagnostic of NiSODs Barondeau *et al*., 2004 and Wuerges *et al.*, 2004).

Three forms of superoxide dismutase are present in humans, in all other mammals, and most chordates. SODI is located in the cytoplasm, SOD2 in the mitoehondria, and SOD3 is extracellular. The first is a dinier (consists of two urnts), whereas the others are tetramers (four subunits). SOD 1 and SOD3 contain copper and zinc, whereas SOD2, the mitochondrial enzyme, has manganese in its reactive centre. The genes are located on chromosomes 21, 6, and 4, respectively.

In higher plants, superoxide dismutase enzymes (SODs) act as antioxidants and protect cellular components from being oxidized by reactive oxygen species (ROS) (Alseher *et al.,* 2002). ROS can form as a result of drought, injury, herbicides and pesticides, ozone, plant metabolic activity, nutrient deficiencies, photo inhibition, temperature above and below ground, toxic metals, and UV or gamma rays (Smirnoff and Nicholas, 1993) and (Raychaudhuri and Xeng, 2008). Specifically, molecular 0_2 is reduced to 0_2 (an ROS called superoxide) when it absorbs an excited electron released from compounds of the electron transport chain. Superoxide is known to denature enzymes, oxidize lipids, and fragments DNA (Smirnoff and Nicholas, 1993). SODs catalyzes the production of 0_2 and $H_2{}^0$ from superoxide (0_2^-), which results in less harmful reactants.

SOD out-competes damaging reactions of superoxide, thus protecting the cell from superoxide toxicity. The reaction of superoxide with non-radicals is spin forbidden. In biological systems, this means its main reactions are with itself (dismutation) or with another biological radical such as nitric oxide (NO) or with a transition-series metal. Superoxide is one of the main reactive oxygen species in the cell. Consequently, SOD serves a key antioxidant role. The physiological importance of SODs is illustrated by the severe pathologies evident in mice genetically)' engineered to lack these enzymes. Mice lacking SOD2 die several days after birth, amid massive oxidative stress (Li *et al*., 1995). Mice lacking SODI develop a wide range of pathologies, including hepatocellular carcinoma, (Elehuri *et al.,* 2005) au acceleration of age-related muscle mass loss, (Muller *et al*., 2006). Mice lacking SOD3 do not show any obvious defects and exhibit a normal lifespan, though they are more sensitive to hypertoxic injury (Sentman *et al.,* 2006).

In recent years, it has become more apparent that in mice, the extracellular superoxide dismutase (SOD3, ecSOD) is critical in the development of hypertension (Lob *et al.,* 2010). In other studies, diminished SOD3 activity was linked to lung diseases such as Acute

Respiratory Distress Syndrome (ARDS) or chronic obstructive pulmonary disease (COPD) (Young *et al.*, 2006; Gangitly *et al.*, 2009; Gongora *at al.*, 2008).

Superoxide dismutase is also not expressed in neural crest cells in the developing fetus. Hence, high levels of free radicals can cause damage to them and induce dysraphic anomalies (neural tube defects). SOD has powerful anti-inflammatory activity. For example, SOD is highly effective in treatment of colonic inflammation in experimental colitis. Treatment with SOD decreases reactive oxygen species generation and oxidative stress and, thus, inhibits endothelial activation and indicates that modulation of factors that govern adhesion molecule expression and leukocyte-endothelial interactions. Therefore, such antioxidants may be important new therapies for the treatment of inflammatory bowel disease (Segui *et al.*, 2004). Similarly, SOD has multiple pharmacological activities. E.g. it ameliorates cis-platinum-induced nephrotoxicity in rodents (McGinness *et al.*, 1978). As "Orgotein" or "ontosein", a pharmacologically-active purified bovine liver SOD; it is also effective in the treatment of urinary tract inflammatory disease in man (Marberger *et al.*, 1974).

SOD may reduce free radical damage to skin—for example, to reduce fibrosis following radiation for breast cancer. Superoxide dismutase is known to reverse fibrosis, perhaps through reversion of myofibroblasts back to fibroblasts (Vozenin-Brotons *et al.*, 2001).

2.6 Catalase

Catalase is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the decomposition of hydrogen peroxide to water and oxygen (Chelikani *et al.*, 2004). It is a very important enzyme in reproductive reactions. Likewise, catalase has one of the highest turnover numbers of all enzymes; one catalase molecule can convert millions of molecules of hydrogen peroxide to water and oxygen each second (Goodsell, 2004). Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long (Boon *et al.*, 2005). It contains four porphyrin heme (iron) groups that allow the enzyme to react with the hydrogen peroxide. The optimum pH for human catalase is approximately 7 (Maehly and Chance, 1954), and has a fairly broad maximum (the rate of reaction does not change appreciably at pHs between 6.8 and 7.5) (Aebi, 1984). The pH optimum for other catalases varies between 4 and 11 and optimum temperature also varies depending on the

species. As hydrogen peroxide enters the active site, it interacts with the amino acids asparagine at position 147 and His at position 74, causing a proton (hydrogen ion) to transfer between the oxygen atoms. The free oxygen atom coordinates, freeing the newly formed water molecule and Fe(IV)=O. Fe(1V)=O reacts with a second hydrogen peroxide molecule to reform Fe(III)-E and produce water and oxygen (Boon *et al.,* 2005). The reactivity of the iron center may be improved by the presence of the phenolate ligand of Tyr357 in the fifth iron ligand, which can assist in the oxidation of the Fe (III) to Fe (IV). The efficiency of the reaction may also be improved by the interactions of His74 and Asn147 with reaction intermediates (Boon *et al.,* 2005). In general, the rate of the reaction can be determined by the Michaelis-Menten equation (Maass, 1998). Catalase can also catalyze the oxidation, by hydrogen peroxide, of various metabolites and toxins, including formaldehyde, formic acid, phenols, acetaldehyde and alcohols.

Hydrogen peroxide is a harmful byproduct of many normal metabolic processes; to prevent damage to cells and tissues, it must be quickly converted into other, less dangerous substances. To this end, catalase is frequently used by cells to rapidly catalyze the decomposition of hydrogen peroxide into less-reactive gaseous oxygen and water molecules (Gaetani *et al.,* 1996). The true biological significance of catalase is not always straightforward to assess. Mice genetically engineered to lack catalase are phenotypically normal, indicating this enzyme is dispensable in animals under some conditions (Ho *et al;* 2004). A catalase deficiency may increase the likelihood of developing type 2 diabetes (Laszlo *et al.,* 2001) and (Laszlo, 2008). Some humans have very low levels of catalase (acatalasia), yet show few ill effects. The predominant scavengers of H_2O_2 in normal mammalian cells are likely peroxiredoxins rather than catalase. Human catalase works at an optimum temperature of 37°C, (Aebi, 1984) which is approximately the temperature of the human body. In contrast, catalase isolated from the hyperthermophile archaea *Pyrobaculum calidifontis* has a temperature optimum of 90°C (Amo *et al.,* 2002). Catalase is usually located in a cellular, bipolar environment organelle called the peroxisome. Peroxisomes in plant cells are involved in photorespiration (the use of oxygen and production of carbon dioxide) and symbiotic nitrogen fixation (the breaking apart of diatomic nitrogen (N_2) to reactive nitrogen atoms). Hydrogen peroxide is used as a potent antimicrobial agent when cells are infected with a pathogen. Catalase-positive pathogens, such as *Mycobacterium tuberculosis*, *Legionelia pneumophila,* and *Campylobacter jejuni*, make catalase to deactivate the peroxide radicals, thus allowing them to survive unharmed within the host (Srinivasa *et*

al., 2003). Catalase contributes to ethanol metabolism in the body after ingestion of alcohol, but it only breaks down a small fraction of the alcohol in the body.

Catalase is used in the food industry for removing hydrogen peroxide from milk prior to cheese production. Another use is in food wrappers where it prevents food from oxidizing (Hengge, 1999). Catalase is also used in the textile industry, removing hydrogen peroxide from fabrics to make sure the material is peroxide-free. A minor use is in contact lens hygiene - a few lens-cleaning products disinfect the lens using a hydrogen peroxide solution.

2.7 Fructose in Semen

Fructose makes up 99% of the reducing sugar present in semen. This sugar is produced in seminal vesicles and its presence may indicate an obstruction proximal to these glands. Although a fructose test is NOT part of a routine semen analysis, the clinician may want to measure this in cases of azoospermia secondary to obstruction of the ejaculatory ducts or absence of the vas deferens fructose is usually absent. When azoospermia is caused by failure of the testes to produce sperm, fructose is present. Measuring fructose levels can thus help the clinician determine the cause of pH is often more useful in this regard. The procedure for determining the amount of fructose in semen involves heating semen in a strong acid in the presence of resorcinol. Fructose gives a red color to this solution when present.

2.7.1 Fructose Level

Regarding the level of fructose in the semen, WebMD lists normal as at least 3mg/ml. WHO specifies a normal level of 13umol per sample. Absence of fructose may indicate a problem with the seminal vesicles.

2.8. pH

WHO criteria specify normal pH as 7.2-7.8 acidic ejaculate (Lower PH value) may indicate one or both of the seminal vesicles are blocked. A basic ejaculate (higher pH value) may indicate on infection. A pH value outside of the normal range is harmful to sperm.

2.9. Antioxidant Vitamins

It has been reported that antioxidants such as Vitamins A, C and E would provide protection against the oxidative stress induced by malaria infection (Adelekan *et al,* 1997).

An antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation reactions can produce free radicals which in turn start chain reactions in the cell causing damage or death to the cell. Antioxidant terminates these chain reactions by removing free radicals intermediates and inhibit other oxidation reactions. The three major antioxidants vitamins are beta carotene (Vitamin A), Vitamin C and Vitamin E. They are obtained from fruits and vegetables, eggs, legumes, nuts etc.

The human body has developed several antioxidant strategies to protect itself from reactive oxygen species (ROS) damage. This allows for normal oxidative metabolism to occur without damaging the cells, while still allowing for normal ROS- mediated cellular responses such as destruction of infectious pathogens and intracellular signaling oxidative stress occurs when the production of ROS overwhelms the antioxidant defense mechanisms leading to cellular damage. Seminal plasma and sperm themselves are well endowed with array of protective antioxidants (Fujii *et al.,* 2003). Non enzymatic antioxidants present within the semen include: ascorbic acid (vitamin), α-tocopherol (Vitamin E), glutathione, amino acids, albumin, carnithine, carotenoids, flavenoids, urate and prostasomes. These agents principally act by directly neutralizing free radicals activity chemically enzymatic antioxidants also present within the semen are superoxide dismutase (SOD) and catalase which are in active the superoxide anion and peroxide radicals by converting them into water and oxygen. A substantial number of researchers have reported a significant reduction in non-enzymatic antioxidant activity in seminal plasma of infertile compared with fertile men (Smith *et al.,* 2006; Gaetani *et al.,* 1996).

Antioxidants contained within seminal plasma obviously helpful for preventing sperm oxidative attack following ejaculation. However, during spermatogenesis and epididymal storage, the sperm are not in contact with seminal plasma antioxidants and their own intrinsic antioxidant capacity for protection. Seminal plasma antioxidants may help minimize ejaculated sperm oxidative stress; they have no capacity to prevent oxidative damage initiated upstream at the level of the testis and epididymis.

CHAPTER THREE

MATERIALS AND METHODS

3.1: Materials

Spectrophotometer (Spec 20D, Techmel and Techmel, USA), Centrifuge (80D, Serico, China), Refrigerator (HTF 319, Haier Thermocool, Japan), Laboratory Incubator (T-9052 Techmel and Techmel, USA), Microscope (B240-5, Toledo, Switzerland), Waterbath (HH-6, Guohua China), soxhlet apparatus (corning USA) rotary evaporator (Buchi R.-210 Hana china laboratory mill (kenwood limited hertford shire, U.K)and analytical balance (Metteler toledo, USA) (Glasswares) (Pyrex, MBL, Germany). Stock TCA- TBA- HCI reagents (composed of 15g of trichloroacetic acid 0.375g of thiobarbituric acid and 0.25N hydrochloric acid), Sodium salt, chloroform, Giemsa stain, ethanol, formaldehyde, peroxide, tetraoxosulphate (vi) acid, sodium hydroxide, phosphate buffer, carbonate buffer, epinephrine, Sodium carbonate, adrenaline solution, EDTA, Ellman reagent, Haematoxylin and Eosin. These chemicals were AnalaR Grade, supplied by BDH, Chemicals, Poole, England. Mice Swiss (BALB/c albino strain, FBMS, DELSU, Abraka) Kits for glucose, fructose and testosterone assays, were supplied by Randox Laboratories, Ardmore, England).

3.2 Experimental Animals

Adult albino male mice about eight weeks old, weighing between 22g-27g were obtained from the Laboratory Animal Centre, LAC, Faculty of Basic Medical Sciences, FBMS, Delta State University, DELSU, Abraka, Nigeria, where they were kept under storage at standard room temperature and pressure. They were fed on growers' mash obtained from Top Feeds Flour Mill, Sapele, Delta State, and were also given water *ad libitum*. The animals were housed in cages constructed of stainless steel materials and maintained under12 hours light and 12 hours dark cycle. The albino mice used in this study were maintained in accordance with the guidelines approved by the *Animal Ethnical Committee, Delta State University, Abraka, Delta State, Nigeria.* Six (6) *Plasmodium berghei* infected (donor) mice were obtained from the Nigerian Institute of Medical Research, Yaba, Lagos, Nigeria.

3.2.1: Inoculation of Experimental Animals

The parasite (*Plasmodium berghei)* was obtained from the Nigerian Institute of Medical Research Yaba, Lagos. The albino mice were infected with parasites by obtaining parasitized blood from the cut tail tip of the infected (donor) mice. The inoculum was prepared by phosphate buffered saline (PBS). Then, 0.1ml of infected blood was diluted in 0.9 ml phosphate buffered saline, PBS, pH 7.2. The mice were inoculated with 0.1ml parasitized suspension containing about 12,000 parasites. Parasitaemia was assessed by the film made by collecting blood from the cut tail tip of the infected mice and stained with Giemsa stain which was later view under the microscope (TH- 9845, Serico, China) at x40 magnification.

3.2.2: Animal Grouping

A total of 10 mice were used for this study. They were separated into 2 groups of 5 mice per group as follows:

Group 1: Control Group (uninfected) was inoculated with phosphate buffered saline (PBS).

Group 2: The Experimental Group (infected), was inoculated with *Plasmodium berghei* (Strain NK65; obtained from the Department of Parasitology, Nigerian Institute of Medical Research, NIMR, Yaba, Lagos State, Nigeria).

3.2.3: Animal Sacrifice and Sample Collection

On the 7^{th} day after establishing parasitaemia, the mice were fasted overnight and sacrificed the next morning having anaesthesized the animals in a chamber saturated with chloroform (AnalaR Grade, BDH Chemicals, Poole, England).

Testes, semen and blood specimens were collected and processed as required for analysis.

3.2.4 Preparation of Testicular Homogenate

One gram (1g) of the fresh testis was blended and homogenized in 49ml of normal saline. The homogenate was then centrifuged (80D Serico, China) at 12000xg for 15minutes at room temperature (28-30^{o}C) to obtain the supernatant. The supernatant was transferred into another plain sterile bottle and kept frozen until required for biochemical assay.

3.3: Semen Analysis

The caudal epidermis was dissected out and an incision (about 1mm) was made in the caudal epidymis and sperm fluid was then squeezed out to the microscope slide.

Epidymal Sperm: was assessed by calculating motile spermatozoa per unit area and was expressed as percent motility.

Sperm Viability: The sperm viability was determined using eosin (Raji *et al*, 2003). Some portion of the semen was diluted and centrifuged at 15,00xg for 7mins in order to collect the semen plasma for biochemical assays.

Measurement of Volume: Normal semen was thick and viscous but it became liquefied within 60 minutes due to a fibrinolysin in the fluid. When liquefied, the volume of fluid in milliliters was measured using a small graduated cylinder. Normal specimens: Usually 2 ml or more.

Measurement of pH:

Using a narrow range pH paper (e.g. pH 6.4-8.0) a drop of liquefied semen was spread on the paper.

After 30 seconds, pH was recorded. pH of normal semen should be pH 7.2 or more within 1 hour of ejaculation. When the pH is over 7.8 this may be due to infection. When the pH is below 7.0 and the semen is found to contain no sperm, this may indicate dysgenesis (failure to develop) of the vas deferens, seminal vesicles or epididymis.

Estimation of the Percentage of Motile and Viable Spermatozoa

- One drop of well-mixed liquefied semen was placed on a slide and covered with a 20 x 20 mm or 22x 22 mm cover glass. Drop falling from a 21 g needle is equivalent to a volume of 10 -15p.
- The specimen was focus using l0x objective. The condenser iris was sufficiently close to give good contrast. To Ensure the spermatozoa were evenly distributed.
- The 40x objective was used to examine several fields to assess motility, i.e. whether excellent (rapid and progressive) or weak (slow and non-progressive). A total of 100 spermatozoa was and noted out of the hundred how many were motile. The percentage was recorded in motile and non-motile.

Normal motility: Over 50% of spermatozoa were motile within 60 minutes of ejaculation. The spermatozoa remain motile for several hours. When more than 60% of spermatozoa are non-motile, examine an eosin

preparation to assess whether the spermatozoa were viable or non-viable. Presence of cells in semen: Report when more than a few leucocytes (pus cells) or red cells are present. When pus cells are seen, examine a Gram stained smear for bacteria.

Viability: One drop (10 -15µl) of semen was mixed with 1 drop of 0.5% eosin solution on a slide. Dissolve 0. 1g of eosin in 20m1 of fresh physiological saline. After 2 minutes examine the preparation microscopically. The l0x objective was used to focus the specimen and the 40x objective to count the percentage of viable and non-viable spermatozoa. Viable spermatozoa remain unstained, nonviable spermatozoa stain red. **Normal viability:** 75% or more of spermatozoa should be viable (unstained). A large proportion of non-motile but viable spermatozoa may indicate a structural defect in the flagellum.

Perform a Sperm Count: A graduated tube or small cylinder was used to dilute the semen 1 in 20 as follows:

The tube or cylinder was tilled to ml mark with well- mixed liquefied semen. And sodium bicarbonate-formalin was added to dilute the fluid to the 20m1 mark, and it was well mixed.

- A Pasteur pipette was used to fill an Improved Neubauer ruled chamber with well-mixed diluted semen. And the spermatozoa were settled within 3-5 minutes.
- Using the 10 objective with the condenser iris closed sufficiently to give good contrast, count the number of spermatozoa in an area of 2 sq mm, i.e. 2 large squares.

Note: The total area of an Improved Neubauer and a Bürker ruled chamber is 9sqmm, i.e. 9 large squares.

- The number of spermatozoa was calculated in 1m1 of fluid by multiplying the number counted by 100000. Normal count: 20 x 10 spermatozoa 1m1 or more. Counts less than 20 x 10 are associated with male sterility.

Morphology of Spermatozoa

The preparation for normal spermatozoa was examined using the 40x objective. And the l00x objective was used to confirm abnormalities.

100 spermatozoa were counted to estimate the percentage showing normal morphology and the percentage that appear abnormal (Macleod 1952).

3.4 Blood Assay

The collected blood was allowed to clot and then, centrifuged at 1200xg for 5min at room temperature (28-31^{o}C). The supernatant (serum) was decanted into bijou bottle and stored frozen until required for assay.

3.5 Biochemical Assay of Testicular Homogenate, Semen and Serum

3.5.1 Fructose Assay

The assay method documented by WHO (2010) for the examination and processing of human semen was adopted. This involves photometric test for quantifying fructose in human seminal plasma.

The semen sample was liquefied at room temperature. Total semen (plasma) volume was measured with a sterile syringe. Then, 100µl of semen seminal plasma was pipetted into separate test-tubes. Also, 100µl of the fructose standards was pipetted into a test-tube and treated like a semen sample. Thereafter, 0.5ml of 50ml TCA solution was added and mixed. It was centrifuged for 10 minutes at 100g.Then, 20µl of supernatant/standard from 50ml TCA solution was pipetted into an empty Eppendorf tube and 20µl of purified water was pipetted into an empty Eppendorf tube which served as blank sample. Also, 200µl of 25ml concentrated HCl was added to each of the tubes and 20µl of 3ml indole was added into each of the tubes. Tubes were closed and incubated for 30 minutes at 37^{0}C in a water-bath. Thereafter, 200µl of 25ml NaOH was added to stop the colour reaction and 200µl of sample was pipetted into an empty well and results read at 480nm in a plate reader.

3.5.2 Malondiadehyde Assay

The method of Halliwel and Gutteridg, (1999) was adopted to estimate lipid peroxidation in terms of thiobabituric acid reactive species (TBARS) using malondialdehyde (MDA) as a standard.

To assay for MDA, 1.0ml of sample was used and Blank and was left empty. Then, 2.0ml of TCA- TBA- HCI reagents was added to sample but 3.0ml of it was added to Blank. The solution was heated for 15 minutes in boiling water-bath (100^{o}C) to form a pink colour

product which has maximum absence at 532nm. After cooling, it was centrifuged to remove flocculent precipitated at 1000g for 10 minutes. Thereafter, absorbance of the sample was read at 535nm against the blank. The malondiadehyde concentration of sample was then calculated using extinction co-efficient of $1.56x10^5 m^{-1} cm^{-1}$(Halliwel and Gutteridge, 1999)

3.5.3 Catalase Assay

The activity of catalase was determined in the tissue homogenates by the method of Cohen *et al*. (1972).

Principle

In the assay, excess potassium permanganate is added and then residual unreacted permangantate is measured spectrophotometrically. It has been shown that the decomposition of hydrogen peroxide by catalase follows first order kinetics (Haber and Weiss, 1934).

Procedure

This was carried out by pipetting 1.0ml phosphate buffer into a reference cuvette. Then, 2.0ml of sample was added into the reference cuvette and test cuvette respectively. Enzymatic reaction was initiated by adding 1.0ml of cold 10mM H_2O_2 into the test cuvette and mixing thoroughly. To stop the reaction, 7 ml of 0.1NKMnO4 was added within 30s and thoroughly mixed. The spectrophotometer standard was prepared by adding 7 ml of 0.1 N KMnO4 to a mixture of 5.5 ml of 0.05 N phosphate buffer, pH 7 and 1 ml of 6 N H2SO4. The reaction was carried out in an ice-water bath (0-2^oC) and after exactly 3 minutes, the substrate concentration was measured at 240nm.

Calculation of Activity

The mathematical determination of the activity was done using the formula below:

$$K = \frac{S_o}{S_3} \times \frac{2.3}{t}$$

Where K = First order rate constant

t = time interval over which the reaction is measured (viz 3 mins)

S_o = Substrate concentration at zero time

S_3 = Substrate concentration at 3 minutes

3.5.4 Superoxide Dismutase (SOD) Activities

This was determined according to the method of Misra and Fridovich (1989).

Having set the spectrometer at 420nm, it was adjusted to zero with a blank made up of 3.0ml of distilled water. Then, 0.2ml of distilled water was added to the reference tube, while, 0.2ml of the appropriate enzyme extracts were added to appropriate labelled test tube. To each of these was added 2.5ml of the carbonate buffer, followed by equilibration at room temperature, 0.3ml of 0.3nM adrenaline solution was added and allowed to mix. Then absorbance was read at 420nm.

3.5.5 Glucose Assay

Test kit and samples (Serum) were stabilized at room temperature. Test tubes were labelled as Blank, Standard and Sample. To each of the test tubes, 1000µl reagent was pipetted. Then, 10µl of standard solution was pipetted into test tube labelled Standard followed by 10µl of sampled pipetted into test-tube labelled Sample.

Samples in the tubes were mixed and incubated for 10minutes at 37^{o}C. Absordance of standard and the sample were measured against the reagent blank at 546nm within 60 minutes. Normal glucose range: Serum: 75-115mg/dl.

3.5.6 Testosterone Assay

Serum and testicular testosterone were estimated by enzyme linked immunosorbent assay (ELISA). The procedure was as described by the manufacturer of the kit (Randox Laboratory, Limited, UK). About 10µl each of serum and testicular samples was added to appropriately labelled microlitre wells and 100µl enzyme conjugated detection antibody was also added to the wells. Also, 5µl of mice anti-testosterone reagent was added to each well. The same procedure was performed for the standard as well as experimental serum and testicular samples. They were incubated at 37^{o}C for 90 minutes. The wells were washed 3 times with deionized water to remove unbound antibodies. Thereafter, 100µl TMB reagent was added and well incubated at room temperature for 20 minutes for the colour to develop. Then, 100µl of HCl was added to the various wells to stop further development of colour. Absorbance was read at 450nm using ELISA machine and deionized water served as blank. Testosterone normal range 280-1,100ng/dl.

3.5.7 Gluthathione Peroxidase Assay (GPX)

The estimation of glutathione peroxidase was described by Haefman *et al* (1974).Two suitable cuvettes labelled Test and Blank were prepared. Then, 2.10ml of deionized water was pipetted, into each tube- Test and Blank. Then, 0.32ml reagent A (Buffer) each pipetted into Test and Blank followed by 0.16ml of reagent B (H_2O_2) into the Test and 0.10ml of the same reagent B (H_2O_2) pipetted into the Blank. Equal amount (0.32ml) of reagent C (parogallol) each pipetted into Test and Blank, respectively.

They were mixed by inversion and equilibrated for 10 minutes at 20^oC in a spectrophotometer. It was monitored until it became constant by using a suitable thermostated spectrophotometer. Then, 0.10ml of reagent A (Buffer) was added to blank, while, 0.10ml of reagent D (enzyme solution) was added to Test. It immediately after mixing by inversion, increase in A420 for approximately 5 minutes was recorded. The maximum linear rate was used to obtain A420/20seconds for both the test and sample. Normal range for GPx: 0-8µM units/min

3.5.8 Reduced Glutathione (GSH) Assay

The semen reduced glutathione level was estimated using improved method reported by Beutler *et al,* (1963). About 2ml of 5% trichloroacetic acid (TCA) was added to the sample. Also, 0.5ml semen was added to the sample and mixed. The mixture was allowed to stand for 5 minutes at room temperature and centrifuged for 10minutes at 400rpm.

Thereafter, 0.05ml serum (supernatant) was added to sample, while, 0.5ml of Ellman's reagent (DTNB) was added to reagent Blank, Standard and Sample. Also, 3.0ml phosphate buffer each added to Blank, Standard and Sample, while, 0.05ml standard (GSH) was added to Standard followed by 0.05ml distilled water added to the reagent blank. Absorbance was taken at 412nm. Normal value for GSH : 0-16µM.

3.5.9 Acid Phosphatase (ACP) Assay

The determination of acid phosphatase is by enzymatic colorimetric method as described by (Tietz, 1976).

The inhibitors of ACP, such as tartrate, flouride, EDTA, oxalate and citrate should be avoided in sample preparation. Serum, plasma, urine, semen and cell culture media can be

assayed directly. Cells ($1x10^5$) or tissue (10mg) can be homogenized in 100µl assay buffer then, centrifuged to remove insoluble materials at 13, 000g for 3 minutes. Then, test sample was added directly into 96 well plates, bringing total volume to 80µl with assay buffer. Addition of the same amount of sample into separate wells, brought volume to 80µl. Then, 20µl stop solution was added and mixed well to terminate ACP activity in the sample. Thereafter, 50µl of pNPP substrate solution was added to each well containing the test samples and background controls. Then mixed well and incubated for 60 minutes at 25ºC, while protected from light. It was diluted with 40µl of the 5mMpNPP solution with 160µl assay buffer to generate ImMpNPP standard. Then, 0, 4, 8, 12, 16, 20 were added µl into 96-well plate in duplicate to generate 0,4,8,12,16, 20µl with assay buffer. Then, 10 µl of ACP enzyme solution was added to each well containing the pNPP standard and Mixed well. The ACP enzyme will convert pNPP substrate to an equal amount of colour.

p-Nitrophenol (pNP). The reaction was incubated for 60 minutes at 25ºC, protected from light. All reactions were stopped by adding 20µl stop solution into each standard and sample reaction except the sample background control reaction. Then, the plate was gently shaken and absorbance was measured at 405nm in a micro plate reader.

3.6.0 Statistical Analysis

The data was analyzed using Student's T- Test. The Statistical Package for Social Sciences (version 20.0) was used for the analysis. The results were expressed as Mean ± Standard Deviation (SD).

CHAPTER FOUR

RESULTS

The results obtained from the study on the changes in testicular activities induced by *Plasmodium berghei* malarial parasite in experimental mice are shown in Tables 4.1-4.2 and Figures 4.1-4.10.

4.1 Biochemical Assay

From Table 4.1, malarial infection significantly (P<0.05) reduced the activities of SOD, GP_x and levels of GSH in testis of experimental mice. In semen, however, only CAT activity was significantly reduced. MDA values indicate significant oxidative damage to both testis and semen, when compared with control, and this affected the ability of testis to synthesize testosterone.

Table 4.1: Changes in testicular and Seminal Antioxidants in *P. berghei* Malarial Infected Mice Infected Mice.

	CONTROL (n=5)		Experimental (n= 5)	
Antioxidants	**Testis**	**Semen**	**Testis**	**Semen**
SOD (mmol/mL/min)	1.03±0.16[a]	1.07±0.15[a]	0.07±0.03[b]	1.44±0.13[a]
CAT (µmol/mL/min)	12.35±1.68[a]	12.30±2.10[a]	11.00±0.96[a]	6.63±0.65[b]
GP_x (µmol/mL/min)	6.10±0.84[a]	4.97±0.83[a]	2.26±0.74[b]	9.23±0.72[a]
Acid phosphatase activity (Iµ/L)	0.54±0.22[a]	0.32 ± 0.02[b]	0.60±0.16[a]	0.48±0.72[b]
GSH (µM)	12.62±1.64[a]	13.94±1.57[a]	1.38±0.24[b]	18.50±1.67[a]
Testosterone (ng/mL)	0.15±0.19[a]	0.10±0.36[a]	0.05±0.26[b]	0.02±0.13[b]
MDA (µM)	44. 10 ±1.31[a]	34.12±6.48[a]	61.84±14.55[b]	64.21±9.88[b]

Data are expressed as Mean ± SD for n=5 mice per group. Values that bear another superscript in a row differ significantly (p<0.05).SOD= superoxide dismutase, CAT= catalase, GPx= glutatthione peroxide, GSH= reduce glutathione,MDA= malondialdehyde

4.2 Semen Analysis

Table 4.2 shows the analysis of semen from *P. berghei* malarial infected mice. Evidence from semen analysis (Table 4.2) indicates that malarial infection in experimental mice produced non viscous semen with reduced volume and fructose/glucose contents, having significantly ($P< 0.05$) reduced sperm cell motility and count with high rate of structural abnormalities, when compared with control features.

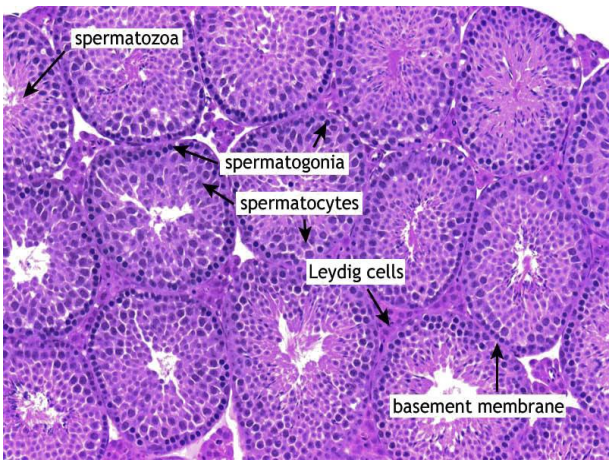
Table 4.2Semen Analysis of Mice Infected with *P. berghei* infected

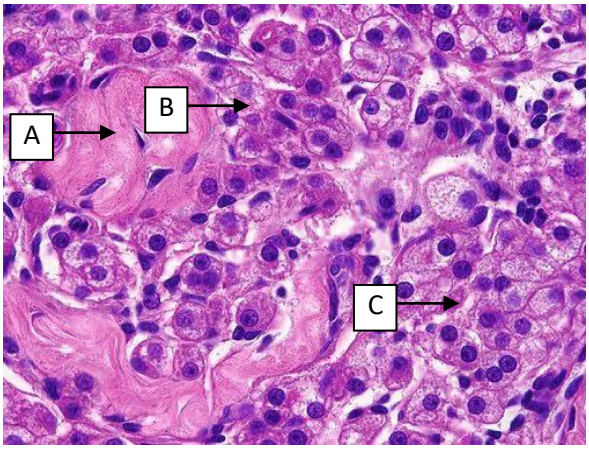
Biomarkers *(Infected)*	**Control (n= 5)**	**Experimental (n = 5)**
	Semen quality	
Semen volume (mL)	0.50±0.07[a]	0.25±0.04[b]
Semen viscosity	moderate	none
Semen fructose (mg/ejaculate)	2.32± 0.16[a]	1.24±0.12[b]
Glucose (mg/dl)	92.73±6.53[a]	59.28± 7.31[b]
	Spermatozoa viability	
Sperm count (million/mL)	25.00±2.10[a]	5.00±0.16[b]
Sperm motility (%)	70.00±10,00[a]	20.00±5.00[b]
Abnormal sperm heads (%)		
• Headless	10.00±0.50	15.00±2.50
• Hookless heads	3.00±0.20	5.00±1.50
• Round headed	2.50±0.50	2.00±1.30
• Amorphous headed	1.00±0. 10	5.00±1.20
Abnormal sperm tails (%)		
• Coiled tails	2.0±0. 10	5.00±1.40
• Double tails	1.5±0.15	5.00±0.50
• Tailess	5.0±1.50	10.00±2.50
Total sperm abnormality (%)	25.00±3.05 [a]	48.00±10.90[b]

Data are presented as mean ± SD for n=5 mice per group. Values that bear another superscript on a row differ significantly at p<0.05.

4.3 Histopathological Analysis of Testes

Figures 4.1a and 4.1b show the histological features of the testes for control and the experimental infected with *p. berghei. Microstructural features of testicular cells were normal for the control mice, but distorted for the P. berghei infected animals. Magnification x40 (H&E Stain).*





A B

Figures 4.1A-B: **Effect of *P. berghei* on Histological Structure of Mice Testes.** (*Haematoxylin and eosin X 40)*

Key: A= Control B= Experimental (Infected with *P. berghei)*

4.4: Micro-Morphological Analysis of Spermatozoa

Figure 4.3A-B below shows the morphological features of sperm cells obtained from the control (uninfected) mice. Micro-morphological cell features indicate normal hook head, and well outlined tail.

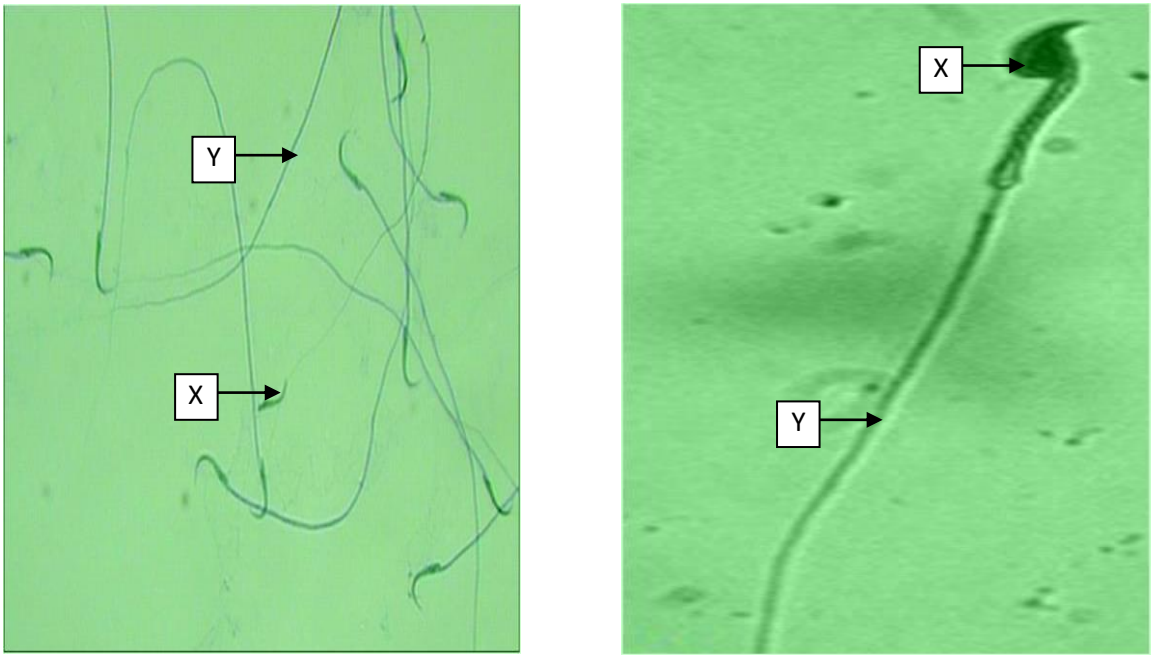
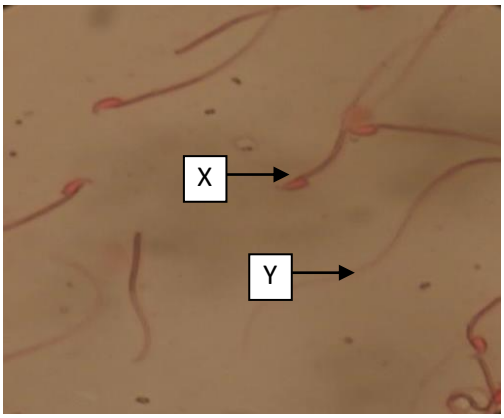


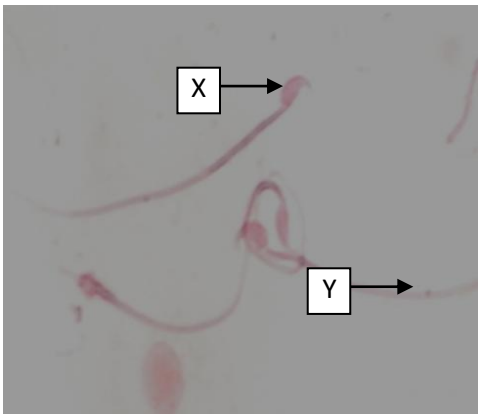
Figure 4.2A-B: Morphological Features of Spermatozoa Obtained from Uninfected Mice *Magnification x40 (H & E stain).*

Key: X= Hook Head, Y= Tail

Figures 4.3A-F show morphological features of spermatozoa from *P. berghei* infected mice, indicating various kinds of abnormality. Morphological characteristics of spermatozoa from the *P. berghei* infected (Group 2) mice (group 2) indicate round-headed spermatozoa (Fig.4.3A), headless spermtozoa (Fig 4.3B), coiled and double tailed spermatozoa (Fig. 4.3C). hookless spermatozoa (Fig.4. 3D), amorphous-headed spermatozoa (Fig 4.4E) round – headed and amorphous spermatozoa (Fig.4.3F). Magnification = x40 (H & E stain)



A



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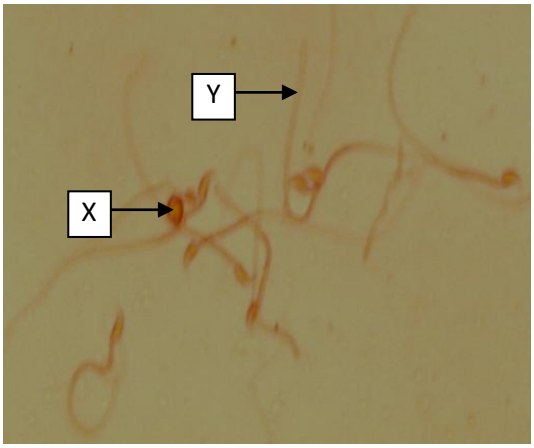
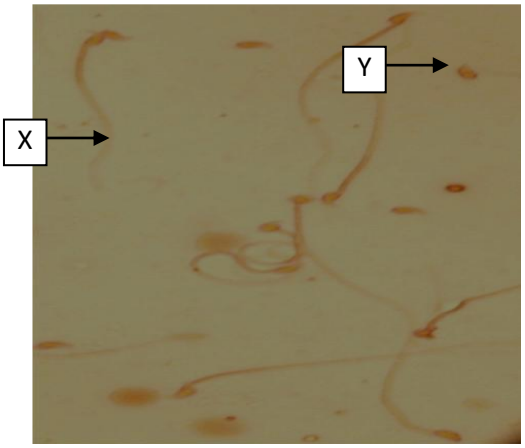
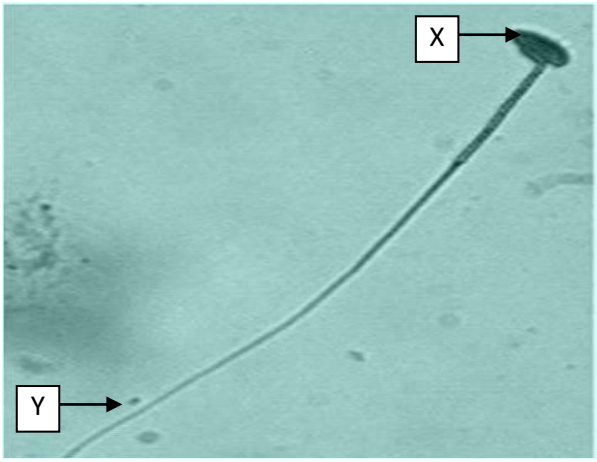


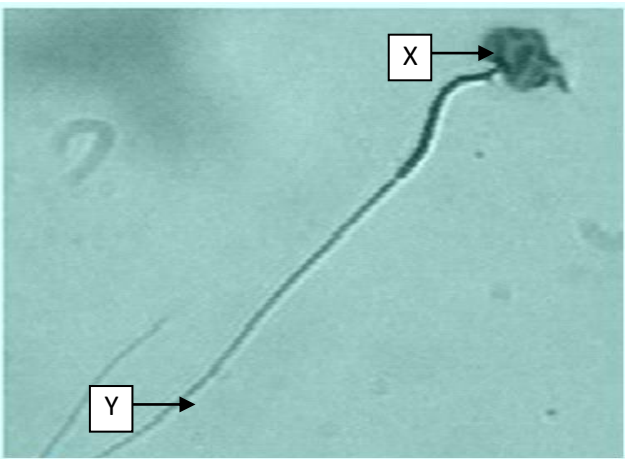
D



C



E



F

Figure 4.3A-B: Effect of *P.* berghei on the Morphological Features of Spermatozoa Mice *Magnification x40 (H & E stain).*

Key X= Head, Y = Tail

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

Results from this study indicates testicular damage- as judged by acid phosphatase activity and malondialdehyde values (Table 4.1) and complimented by the histological features (Figure 4.2) which impacted the tissue's ability to synthesize testosterone and secrete fructose whose productions were also reduced in the *P. berghei* infected mice when compared with control. Previous studies have demonstrated sere necroospermia, oligozoospermia and ozoospermia associated with malaria infection (Singer *et al.,* 1987).

The decreased level of testosterone observed in the *plasmodium berghei* infected mice maybe due to parasite-induced oxidative damage to the testes which altered hormonal signaling. Testosterone is an important male hormone that regulates normal sperm development. So, low level eventually results to infertility. Therefore malaria suppresses testostrerone amounts in the testes of infected mice. High production of reactive oxygen species (ROS) and hence, oxidative stress associated with malaria parasite infection has been reported to reduce testosterone production (Klein, 2004).

Also, the significant increase in the level of seminal MDA in *P. berghei* infected mice suggests oxidative stress in the semen. This can be attributed to the large amount of reactive oxygen species generated during malarial infection and lack of sufficient defense response since other antioxidants were spared. So, evidence suggests that sperm cell membrane went through rapid lipid peroxidation and this observation is in agreement with other studies that show elevated level of MDA in sperm membrane which results in defective sperm function, motility, reduced sperm quality and infertility (Pudy *et al.,* 2004).

Furthermore, fructose in seminal plasma is produced by the seminal vessicles and it is essential for sperm motility. It is also the main source of energy for the spermatozoa, while, staying in the semen (Ozgokmen *et al.,* 2001). Results show decreased in fructose level (Table 4.2) with significantly reduced sperm count (Table 4.2) and motility in the *Plasmodium berghei* infected mice when compared with the control mice. This implies that malarial infection may affect production or utilization of seminal fructose which in turn affects motility of spermatozoa, and this may cause temporary infertility. In addition, low

fructose level observed in the semen of infected mice is a characteristic of ejaculatory duct obstruction, bilateral congenital absence of the vas deference, partial retrograde ejaculation and androgen deficiency (WHO 2010). Therefore, mice infected with *Plasmodium berghei* if untreated may result in male infertility.

Biological system protect itself against the damaging effects of activated species by the actions of free radical scavengers and chain terminator enzymes such as SOD, CAT, GSH and GPx system (Kurata *et al.,* 1993). The results of this study show reduced antioxidant activity of SOD, CAT, GPx and GSH in the semen of mice infected with *Plasmodium berghei*. This could lead to inversion of *Plasmodium berghei* in the semen which exerts oxidative stress within the semen with concomitant decrease in antioxidant enzymes (Clark *et al.,* 1989), which is expressed by high lipid peroxidation (MDA) value.

Reduced glutathione (GSH) acts as primary line of defense to cope with deleterious effects of reactive oxygen species (Bradley and Nathan, 1984). Glutathione protectS the cellular system against toxic effect of lipid peroxidation. Drastic reduction in the level of reduced glutathione observed in the testis of malarial infected mice could indicate its increased utilization due to oxidative stress. Increased in the level of semen reduced glutathione is an indication of protection against toxic effect of lipid peroxidation.

Moreover, increase in reactive oxygen species (ROS) and decrease in antioxidants has been reported in malaria patients (Agarwal *et al.,* 2014), the alterations in the major antioxidants of the erythrocytes may result in the release of ACP (Kaeoket *et al.,* 2008). The increase in testicular and semen acid phosphatase (ACP) activity levels (Table 4.1) in mice infected with *Plasmodium berghei* may be due to oxidative stress and this could be used as additional investigation in the diagnosis of malaria.

Furthermore, reactive oxygen species or free oxygen radicals are normally generated by sertoli cells that cause alterations in the cellular structures and indices of morphological changes in spermatids during spermatogenesis (Aitkene *et a.l,* 2010). From the results on microscopic morphology of the spermatozoa infected with *P. berghei* (Figs 4.5-4.10), there were abnormal spermatozoa features–spermatozoa without head, speimatozoa with double and coiled tail and spermatozoa with hookless head, when compared with the malarial uninfected spermatozoal features which and that appeared normal and healthy. The results complement other studies that reported decreased motility and abnormal morphology of

spermatozoa from animals exposed to reactive oxygen species, bansal, (Bilaspuri, 2010; Potts *et al.,* 2000; and Kaeoket *et al,* 2008).

Low fructose level observed in the semen of infected mice as shown in the result (Table 4.2) is in agreement with (WHO, 2010) which states that low fructose level is a characteristic of ejaculatory duct obstruction, bilateral congenital absence of the vas deference, partial retrograde ejaculation and androgen deficiency which may result in infertility in male mice.

The elevated level of MDA as seen in this study (Table 4.1) is in agreement with other studies which show that elevated level of MDA in sperm membrane result in defective sperm function, motility, reduced sperm quality and infertility (Pudy *et al,* 2004). This result is also in agreement with Davey *et al* (2005), since the degree of peroxidation observed in this study can be estimated by the amount of malondiadehyde in testis and semen.

The low level of SOD activity observed in this study is in agreement with the other studies which confirmed that normal level of superoxide dismutase enzymes act as antioxidant and protect cellular components from being oxidized by ROS (Alseher *et al*., 2002).

The reduced antioxidant levels observed in this result is in agreement with other studies. The inversion of *Plasmodium berghei* exerts oxidative stress within the semen of mice infected with *Plasmodium berghei* malarial parasite with concomitant decreased in antioxidant enzymes (Clark, *et al.,* 1989) which is expressed by high lipid peroxidation value (MDA-a maker of damaged lipid membrane).

5.2 Conclusion

Results show that antioxidant defense capacity in the semen is weak, hence, high degree of abnormalities of sperm cells. Altogether, *P. berghei* malarial parasite infection induced oxidative damage to the testes and this affected testicular function which reduced semen quality, sperm viability and the capacity to produce fructose and testosterone.

5.3 Recommendations

The impact of antioxidant supplement in the treatment regimen of malaria, on testicular function should be explored in further studies

5.4 Contributions to Knowledge

1. The study demonstrates that malarial infection compromises antioxidant defense capacity in both testis and semen of experimental mice**.**
2. The weak antioxidant defense capacity affected the ability of the testes to produce enough quantities of testosterone and sperm fructose.

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APPENDIX 1
STATISTICAL FORMULAE AND SYMBOLS

i. Mean

$$\text{mean } (\bar{x}) = \frac{\sum x}{n}$$

where $\bar{x}$ mean value

$\sum$ = summation

n = number of sample

x = individual sample

ii. STANDARD DEVIATION (SD)

Standard Deviation (SD) = $\sqrt{\frac{\sum (x - \bar{x})}{n-1}}$

Where

N= number of values

X = number of each score

$\overline{X}$ = mean value

iii. STUDENTS t- TEST

$$t = \frac{x_1 - x_2}{\sqrt{\frac{S_1^2}{n_1} \times \frac{S^2_2}{n_2}}}$$

Where x1 = Mean of first set of values

x_2 = Mean of second set of value

s_1 = Standard deviation of first set of values

s_2 = Standard deviation of second set of values

n = total number of values in first set

n_2 = total number of values in second test.

APPENDIX II

REAGENT COMPOSITION, CONCENTRATIONS AND PREPARATIONS

Antioxidant	Composition	Concentration	Preparation
A.Super Oxide Dismutase Activity	Carbonate buffer	0.05M	0.214 of Na_2CO_3 and0.0372, of EDTA was dissolved in about 80ml of H_2O pH adjusted to 10.2 with 0.1m NaOH then the volume Made up to 100ml with Distilled water.
B. Malondialdehyde (MDA)	Stock TCA-TBA-HCl	-	15g of trichloroacetic acid, 0.375g of thiobarbituric acid and 0.25hydrochloric Acid were mixed. The solution mildly heated.
C. Catalase	(a)Phosphate buffer	50mM	0.13g, of Na_2 HPO_4, 0.019, of Na_2 PO_4 and 0.8g of Nacl were dissolved in about 80ml distilled water. The PH adjusted to 7.4with 0.1m NaOH and make up to 100ml distilled water.
	(b) Hydrogen peroxide solution	30mM	3.4ml of 30% hydrogen peroxide solution was diluted to 100ml using phosphate buffer.
D. Glutathione reductase	(a) Ellman's Reagen	0.5ml	0.0198g of 2-nitrobenzoic acid and1.0g of 1% sodium citrate are dissolved in 100ml of distilled water.
	(b) Phosphate buffer	3.0ml	3.44g of disodium hydrogen phosphate and sodium dihydrogen phosphate of 0.3725g were dissolved in 200ml of distilled water.
	(c) Trichloroacetic acid (TCA)	5%	5g of 5% TCA dissolved in 100ml of distilled H_20
E. Frutose	Trichloroacetic acid (TCA) solution	50ml	
	32% concentrated hydrochloric acid	25ml	
	Indole	3ml	
	Sodium hydroxide (NaOH)	0.5Ml	
	Fructose Standard (5mg/ml)	10ml	

SOD IN TESTIS (NORMAL SOD LEVEL=0.2-1.25ml/mm)

		Control Group (No disease with *P. berghei*)	
1	A		1.12
	B		0.91
	C		0.82
	D		1.13
	E		1.18
2.	A	Experimental group (Infected with *P. bergei*)	0.03
	B		0.07
	C		0.09
	D		0.09
	E		0.10

ACID PHOSPHATE IN TESTIS
(NORMAL ACID PHOSPHATE 0.3-0.8 U/L)

GROUP		**-ACID PHOSPHATE IN TESTIS** -U/L	
1	A	Control Group (No disease with *P. berghei*)	0.3
	B		0.4
	C		0.5
	D		0.7
	E		0.8
2	A	Experimental group (Infected with *P. bergei*)	0.4
	B		0.6
	C		0.5
	D		0.7
	E		0.8

CATALASE IN TESTIS
(NORMAL VALUE FOR CATALASE IS 10-15 MM)

GROUP-CATALASE IN TESTIS			(30 SEC)	(60sec)
1	A	Control Grou(No disease with *P. berghei*)	12.3	12.5
	B		13.4	14.7
	C		14.4	10.5
	D		10.3	12.6
	E		12.4	10.3
2	A	Experimental group (Infected with *P. bergei*)	10.1	10.3
	B		10.2	10.4
	C		11.3	11.5
	D		12.4	12.6
	E		10.5	10.7

GSH IN TESTIS
(NORMAL VALUE FOR GSH IS 0-16µM)

GROUP-GSH IN TESTIS			
1	A	Control Group(No disease with *P. berghei*)	10.1
	B		12.4
	C		13.5
	D		14.5
	E		12.6
2	A	Experimental group (Infected with *P. bergei*)	1.1
	B		1.2
	C		1.4
	D		1.5
	E		1.7

GP_x IN TESTIS
(NORMAL VALUE FOR GP_x IS 0-8 UM)

	GROUP-GP_x IN TESTIS	
1 A	Control Group(No disease with *P. berghei*)	5.5
B		5.3
C		6.4
D		7.4.
E		5.9
2 A	Experimental group (Infected with *P. bergei*)	2.0
B		2.1
C		2.2
D		3.5
E		1.5

MDA IN TESTIS
(NORMAL VALUE FOR MDA IS 0-50 µM)

	GROUP-MDA IN TESTIS	
1 A	Control Group(No disease with *P. berghei*)	45.1
B		42.3
C		44.5
D		43.2
E		45.4
2 A	Experimental group (Infected with *P. bergei*)	57.5
B		60.3
C		25.4
D		40.5
E		25.5

GROUP		TESTOSTERONE IN Testis (ng/ml)
1.		0.13
2.		0.16
3.	Control Group (No disease with *P. berghei*)	0.15
4.		0.14
5.		0.18
1.		0.06
2.		0.02
3.	Experimental group (Infected with *P. bergei*)	0.04
4.		0.06
5.		0.09

SEMEN ANALYSIS AND MORPHOLOGY

	C_1	C_2	C_3	C_4	C_5
Motility	50%	60%	60%	50%	70%
Non motility	20%	20%	15%	10%	15%
Headless	20%	20%	15%	20%	5%
Tailess	10%	10%	20%	10%	
Viscosity	moderate	moderate	moderate	moderate	moderate
Volume	0.5ml	0.5ml	0.6ml	0.5ml	0.4ml
Sperm count	$30x10^6$	$25x10^6$	20×10^6	22×10^6	$24x\ 10^6$

PB INFECTED SEMEN ANALYSIS										
Motility	10%		5%		10%		10%		5%	
non Motility	50%		60%		70%		60%		65%	
Headless	25%		30%		20%		20%		20%	
Tailess	15%		5%		10%		10%		10%	
Viscous	None		None		None		None		None	
Volume	0.3ml		0.2ml		0.1ml		0.5ml		0.1ml	
Sperm count	$5x10^6$		$7x10^6$		3×10^6		2×10^6		4×10^6	

SEMEN ANALYSIS Control Semen						
Glucosese	Fructose	SOD	CAT		GP_x	GSH
(Mg/dl)	(Mg/ejaculate)	(mmol/mL/min)	(µmol/mL/min)		(µM)	(µM)
95.2	2.11	1.22	10.11	10. 12	5.01	12.10
97.1	2.31	1.20	11.13	11.14	6.21	13.50
82.51	2.01	0.91	11.20	11.18	4.20	15.17
90.21	2.14	0.93	14.0	14.21	5.21	15.90
9861	2.31	1.11	15.00	14.90	4.20	13.01

***Plasmodium berghei* INFECTED SEMEN**						
Glouse	Fructose	SOD	CAT		GP_x	GSH
(mg/dl)	(mg/ejaculate)	(mmol/mL/min)	(µmol/mL/min)		(µM)	(µM)
55.20	1.4	1.28	15.91	15.82	8.57	17.21
65.70	1.3	1.38	16.50	16.70	8.91	18.11
68.21	1.2	1.42	17.20	17.40	15.14	19.25
56.21	1.9	1.62	16.00	16.21	10.21	20.11
51.10	1.6	1.52	17.20	17.30	8.68	17.81

	GROUP	TESTOSTERONE IN SERUM (mg/ml)
1.		0.15
2.	Control Group (No disease with *P. berghei*)	0.12
3.		0.13
4.		0.2
5.		0.11
1.		0.08
2.		0.07
3.		0.05
4.	Experimental group (Infected with *P. bergei*)	0.06
5.		0.05