Oxidative Stress in Biochemical, Seminological and Histological Alterations Due to Acute Administration of Intramuscular Artemether in Mice.

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ABSTRACT: Artemether is an antimalarial which is effective in the treatment of multidrug resistant malaria. Limited information is available on role of oxidative stress in tissue toxicity during administration of artemether. Therefore selected biochemical, seminological and histological changes following acute administration of artemether were evaluated using animal model. Artemether was administered to groups 1-4 for five consecutive days after which the mice were sacrificed on day 6. Hepatotoxicity, neurotoxicity, nephrotoxicity and toxicity of testes were assessed. There was increased lipid peroxidation in the four organs as indicated by increased malondialdehyde and antioxidants (reduced glutathione, catalase and superoxide dismutase) in groups given artemether when compared with control. In artemether treated groups, there were dose-dependent histopathological effects. Artemether induced oxidative stress which resulted in reduced sperm quality, lipids and lipoprotein levels as well as defective histology of the animal tissues.
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1. Introduction

A gradual progression of resistance to quinine and other antimalaria drugs by *plasmodia* has become a concern in many parts of the world leading to search for newer effective, affordable and readily available antimalaria drugs. Only few new antimalaria drugs appeared in the last few years, for instance artemether (Paluther) for the treatment of severe malaria and chloroquine - proguanil combination (savarine) for malaria chemoprophylaxis (Ambrose (1998). Since 1979, several derivatives of artemisinin have been synthesized and studied in China. Artemether and dihydroartemisinin tablets have all proved rapidly effective and these drugs have now replaced chloroquine and quinine for the treatment of malaria in China (Li GQ, 1994)

Artemether (methyl ether of dihydroartemisinin) is derived from artemisinin (Quinghaosu), an extract of the plant Artemisia annua and it is a highly effective drug for the treatment of *P. falciparum* malaria. Artemether is generally believed to have an excellent safety profile; however, concern about adverse effects still remains. Earlier on, we have reported toxicity of artemether in specific tissues using animal models (Li GQ et al., 1994; Adekunle et al., 2009; Adekunle et al, 2009).The concern about adverse effects may be associated with the fact that all artemesinin analogues possess endoperoxide bond in their molecule which has been reported to be involved in the generation of free radicals. Excess free radicals have been identified as being potentially cytotoxic and clastogenic (Adekunle et al., 2010) on major organs in the body and have been implicated in the etiology of over 100 diseases including hypertension and hypercholesterolemia (Gabriele et al., 2000). Also, artemisinin and its analogues such as artemether which are current choice of antimalaria drugs have been reported to induce oxidative stress (Brewer et al., 1998). In this study, we investigated the possible role of oxidative stress in the mechanism of toxicity of artemether using biochemical markers of oxidative stress.

2.0 Materials and Methodology

Male albino mice weighing between 35-37grams and fed with standard commercial feed and free of parasitic infections were used for the experiment. The animals were kept in well ventilated Animal House. Drinking water was made freely available for them. The animals were divided into four different groups as follows: groups 1 and 2 contained 30 uninfected mice each; groups 3 and 4 contained 20 *P. yoelli* infected albino mice each. In group 3 and 4, 0.2ml of parasitized red blood cells (58.98% parasitaemia) was injected intraperitoneally into each mouse. On the 3rd day after parasite infection (i.e. +3), the levels of malaria parasitemia in the infected mice were assessed by estimating the parasite numbers/µl of blood. Administration of artemether commenced when level of parasitaemia was confirmed in mice in groups 3 and 4 (72 hours). Mice in groups 2 and 3 received 1.2mg/kg body weight of artemether drug. The drug was administered intramuscularly for 5 consecutive days and at the same time of the day. On the other

hand, mice in groups 1 and 4 were injected with equal volume of diluents (corn oil) containing no drug. Mice in group 1 were not infected and were not given arthemeter and they served as control group. On the 6th day after drug administration commenced, all mice were sacrificed after being fasted overnight and blood samples collected by cardiac puncture after immobilization by cervical dislocation method. The samples were centrifuged at 300rpm and the supernatant decanted into labeled sample bottles and stored until analyses were carried out. The liver, brain, kidney and testes were quickly excised and immediately placed on a blotting paper to remove the blood. Samples of organs were immediately rinsed in 1.15% of potassium chloride solution to remove the hemoglobin. The organ samples were homogenized in aqueous potassium phosphate buffer (0.1M, pH 7.4) in volumes of four times the weight of samples using a Teflon homogenizer. The resultant homogenates were centrifuged at 10,000g for 20 minutes to obtain the post-mitochondrial supernatant fraction (PMF). The PMF was decanted into sample bottles and stored at -80oC prior to use. The tissue homogenates of the organs were used to assay for the markers of oxidative stress which are malondialdehyde, catalase, superoxide dismutase and lipids.

2.1 Histological Study

For histological study, samples of the organs (brain, liver, kidney and testicle) with no blood stain were placed in 10% formalin solution in appropriately labeled sample bottles. The organs were later removed and fixed in Bouin's fluid for 24 hours. After fixation, the tissues were dehydrated through ascending grades of ethanol. Thereafter, it was cleared in xylene and finally embedded in paraffin wax. Using a rotary microtome, specimens were sectioned at 5µm and sections were mounted on clean slides and stained with haematoxylin and eosin.

2.2 SPERM ANALYSES

Immediately after the testicles were removed from each mouse, the sperm specimens were collected by aspiration from the epididymis. This involved making an incision in the caudal of right ductus deference of the testicle. Two drops of semen were placed on the microscope slide and two drops of warm 2.9% sodium citrate were added. This was then covered with the cover slip and examined under the microscope using x 40 objective with reduced light. Sperm counts, motility, viability and morphology were carried out using the new improved Neubeur's haemocytometre counting chamber.

2.3 ANALYSIS OF BIOCHEMICAL PARAMETERS

2.3.1 Assessment of lipid peroxidation

Lipid-peroxidation was assessed by measuring the thiobarbituric acid reactive (TBAR) products using the procedure of (Varshney and Kale 1990) and expressed as micromolar of malondialdehyde (MDA) / g tissue.

2.3.2 Determination of Superoxide Dismutase (SOD) Activity.

The level of superoxide dismutase (SOD) activity was determined by the method of (Mistra and Fridovich, 1972).

2.3.3 Determination of Catalase activity

Catalase activity was determined according to the method of (Sinha, 1972).

2.3.4 Determination of Reduced Glutathione (GSH)

The total sulfhydryl groups, protein-bound sulphydryl groups and free sulphydryl groups (such as GSH) in biological samples can be determined using Ellman's reagent, 5,5'-; dithiobis-(2- nitrobenzoic acid) (DTNB) as described by (Sedlak and Lindsay 1968).

2.4 Statistical Analysis

Results obtained are presented as mean ±SD. Analysis of variance was used to compare values among groups. Comparison between groups was carried out using the Bonferoni multiple comparison method. Significance was determined at $p \leq 0.05$.

3.0 RESULTS

3.1 Table 1: Effects of artemether on sperm characteristics among the different groups.

Groups	Group1(control)	Group 2	Group 3	Group 4
Sperm counts (1x 10^6)	77.50±17.77	^{x}60.00±13.75	$^{X\#}$16.80±14.45	^{x}34.88±15.20
Sperm motility (%)	83.35±7.23	^{x}71.56±8.49	$^{X\#}$35.07±26.22	^{x}51.30±34.30
Sperm morphology (%)	16.94±3.48	^{x}27.02±5.00	$^{X\#}$63.38±26.03	^{x}42.28±14.92
Sperm Viability (%)	86.63±6.71	^{x}79.50±7.68	$^{X\#}$39.91±35.50	^{x}54.26±36.89

Significant at $^{x}p<0.05$ when compared with control group (group 1); significant at $^{x\#}p<0.05$ when compared with infected but untreated group (group 4).

3.2 Table 2: Oxidative effects of artemether on the kidney of the mice in the different groups.

parameters	Group1 (control)	Grp 2	Grp 3	Grp 4
MDA (µmol/g tissue)	421.98±99.3	^{x}540.34±141.9	$^{X\#}$596.50±327.2	^{x}468.69±79.1
GSH (iu/g tissue)	1.28±0.43	1.29±0.09	$^{X\#}$4.00±5.41	1.29±0.17
Total cholesterol (mg/dl)	77.57±7.04	52.69±2.70	65.19±10.70	64.39±11.63
Triglyceride (mg/dl)	56.63±34.47	36.67±23.36	39.84±24.21	38.90±19.46
Total protein (g/dl)	4.19±2.05	3.72±1.12	3.57±1.49	3.88±1.36

Significant at $^{x}p<0.05$ when compared with control group (group 1); significant at $^{x\#}p<0.05$ when compared with infected but untreated group (group 4). MDA: Malondialdehyde; GSH- reduced glutathione.

3.3 Table 3: Oxidative effects of artemether in tissue homogenates of testes of mice in the different groups

Parameters	Group 1	Group 2	Group 3	Group 4
MDA (µmol/g tissue)	430.84±262.2	$^{X\#}$892.73±59.9	$^{X\#}$811.44±164.4	^{x}661.52±332.0
GSH (iu/g tissue)	1.04±0.18	$^{X\#}$4.80±3.90	$^{X\#}$1.18±0.15	0.87±0.06
Catalase (iu/g tissue)	2.66±1.38	^{x}3.25±1.89	2.56±1.71	^{x}9.85±15.44
Superoxide dismutase (iu/g tissue)	1.40±0.70	^{x}3.20±4.11	^{x}2.14±1.01	^{x}4.47±4.29
Total cholesterol (mg/dl)	77.26±15.42	73.17±28.44	67.68±5.34	68.58±23.92
Triglyceride (mg/dl)	78.83±40.28	73.49±40.54	37.31±21.50	82.72±45.83
Total proteins (g/dl)	3.14±1.78	2.57±1.15	2.19±1.62	2.50±1.22

Significant at $^{x}p<0.05$ when compared with control group (group 1); significant at $^{x\#}p<0.05$ when compared with infected but untreated group (group 4). SOD- superoxide dismutase.

3.4 Table 4: Oxidative effects of arthemether in tissue homogenates of liver of mice in the different groups

Parameters	Group 1	Group 2	Group 3	Group 4
MDA (µmol/g tissue)	332.09±55.3	^{x}496.03±194.4	^{x}530.56±336.7	^{x}564.37±291.0
GSH (iu/g tissue)	0.98±0.26	^{x}1.26±0.25	^{x}1.41±0.28	^{x}1.18±0.32
Catalase (iu/g tissue)	2.50±0.48	^{x}3.03±0.75	^{x}4.27±1.32	^{x}3.75±1.76
Superoxide dismutase (iu/g tissue)	2.07±0.67	1.60±0.44	1.93±1.27	2.66±1.21
Total cholesterol (mg/dl)	80.35±4.53	77.68±4.58	75.99±10.15	73.65±3.49
Triglyceride (mg/dl)	50.69±24.96	71.89±32.49	51.14±17.40	58.37±10.64
Total proteins (g/dl)	2.91±0.62	3.76±1.09	3.17±1.05	3.91±2.18

Significant at $^{x}p<0.05$ when compared with control group (group 1);

3.5 Table 5: Oxidative effects of artemether in tissue homogenates of brain of mice in the different groups

Parameters	Group 1	Group 2	Group 3	Group 4
MDA (µmol/g tissue)	454.76±169.9	^{x}580.16±141.18	^{x}716.51±198.3	^{x}770.18±140.6
GSH (iu/g tissue)	1.06±0.05	^{x}1.36±0.06	^{x}1.25±0.20	^{x}1.20±0.21
Catalase (iu/g tissue)	2.90±0.90	^{x}3.25±1.61	^{x}12.30±21.81	^{x}14.23±24.12
Superoxide dismutase (iu/g tissue)	3.14±1.42	2.00±0.67	^{x}4.01±2.13	^{x}3.66±2.98
Total cholesterol (mg/dl)	95.37±4.72	101.32±2.84	96.36±5.05	95.19±11.40
Triglyceride (mg/dl)	65.54±31.40	59.13±33.42	50.57±28.85	46.36±33.91
Total proteins (g/dl)	3.06±0.53	3.57±0.41	3.38±1.42	2.65±0.35

Significant at $^{x}p<0.05$ when compared with control group (group 1).

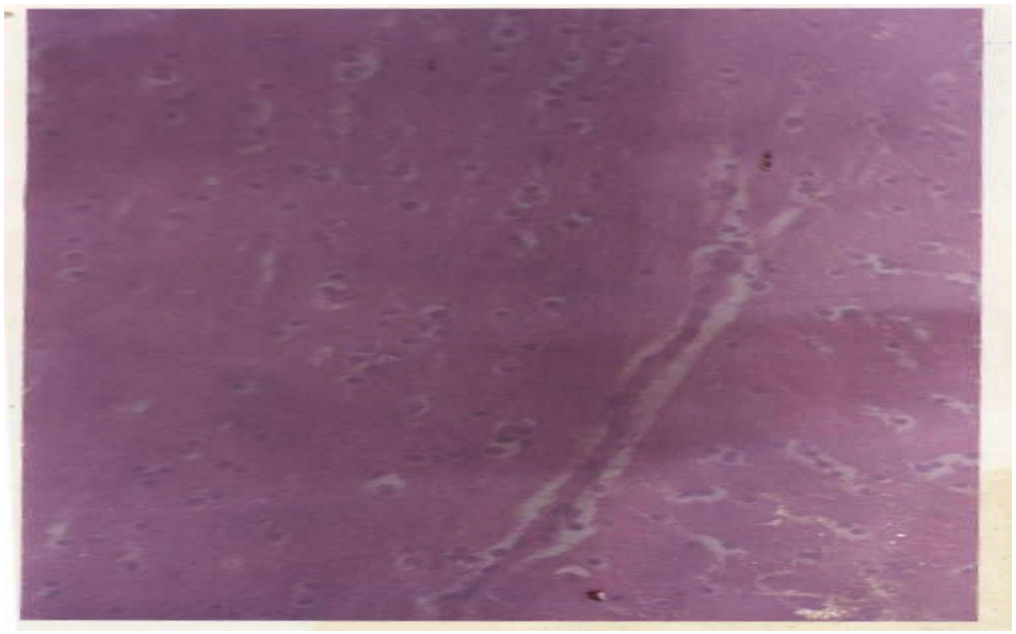

Figure 1. Cross section of brain of mice treated with artemether for 5 days. Stains: haematoxylin and eosin. Magnification=400.There was cellular necrosis and hypertrophy.

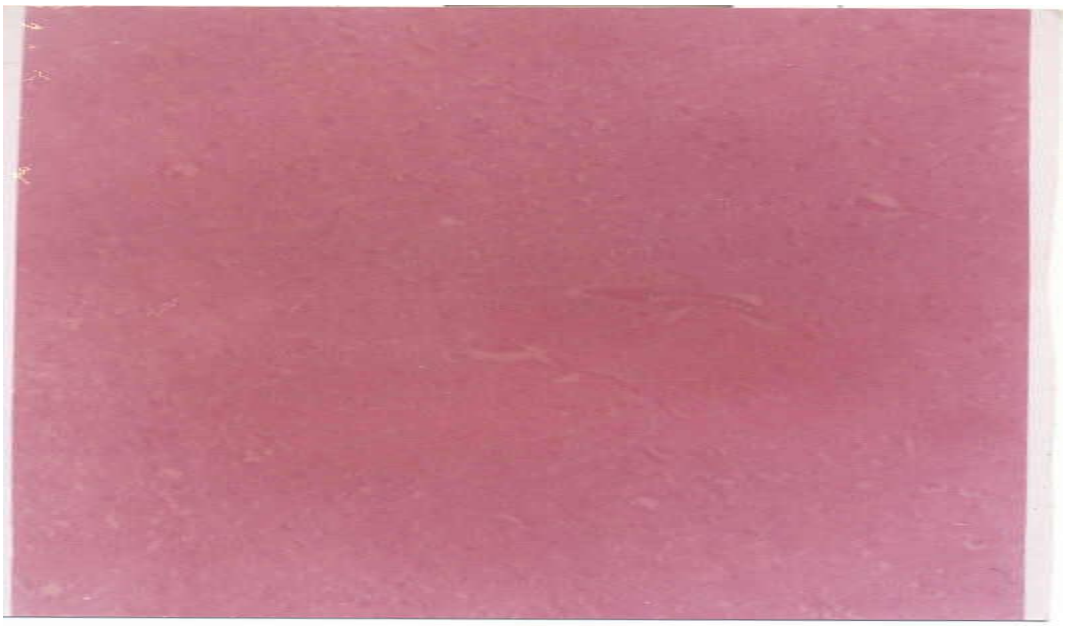

Figure 2. Cross section of brain of control mouse. Stains: haematoxylin and eosin. Magnification: 400x. The neurons and the glial cells appeared normal. There was no cytoarchitectural disruption.

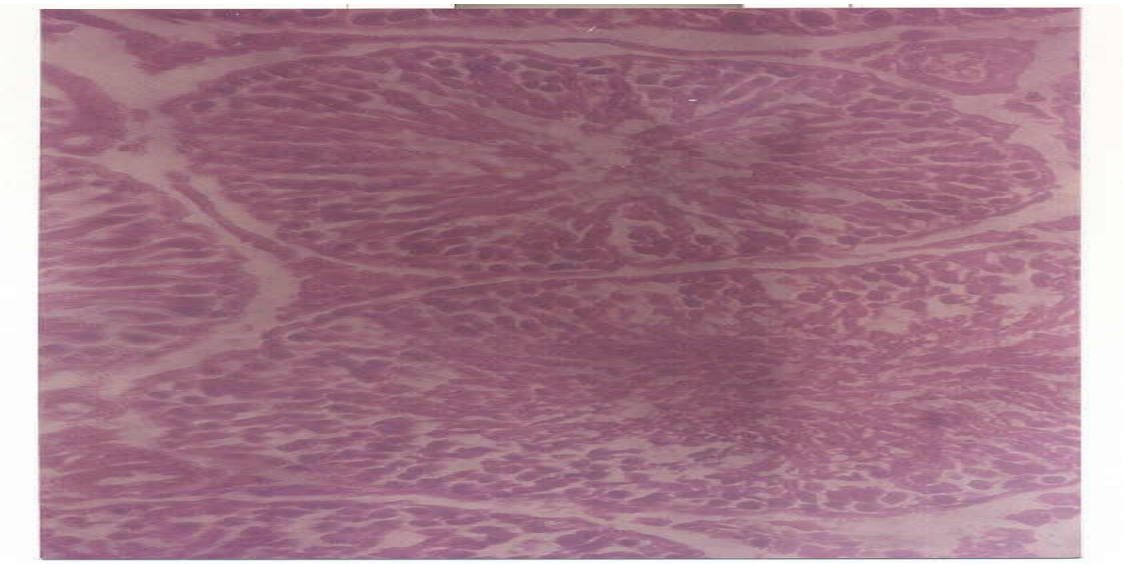

Figure 3. Cross section of testicle of control mouse. Stains:haematoxylin and eosin. Magnification: 400x. The seminiferous tubule, interstitial leydig cells and seminiferous epithelium appeared normal

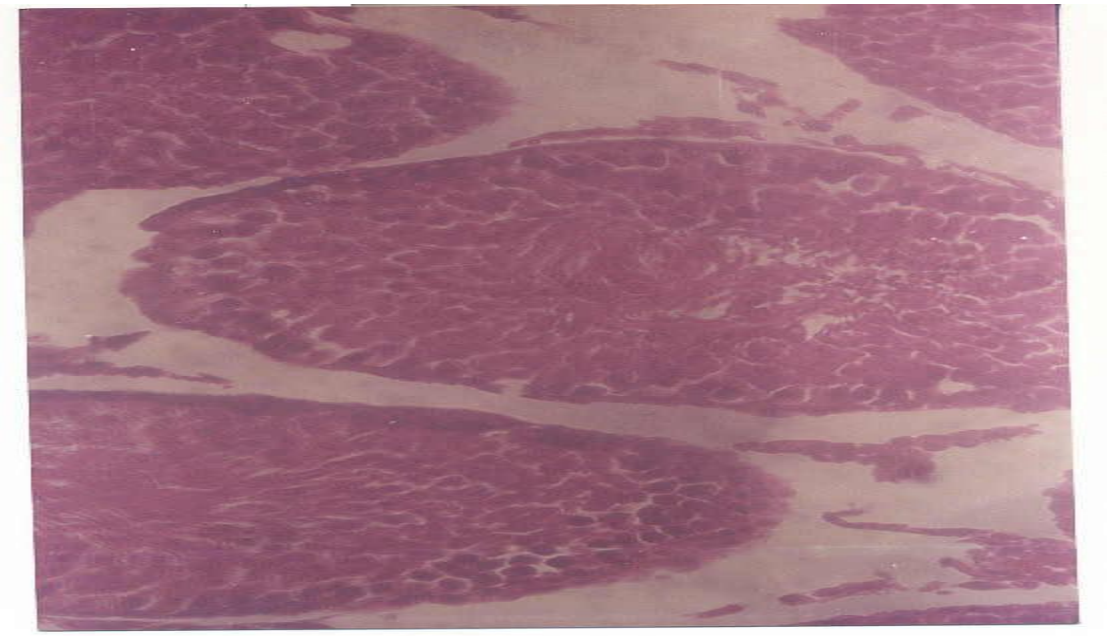

Figure 4. Cross section of testicle of mouse given arthemether for 5 days. Stains:haematoxylin and eosin. Magnification: 400x. There was degeneration of interstitial leydig cells. Focal atrophied seminiferous tubule was observed. There were degenerations of seminiferous epithelium and interstitial leydig cells.

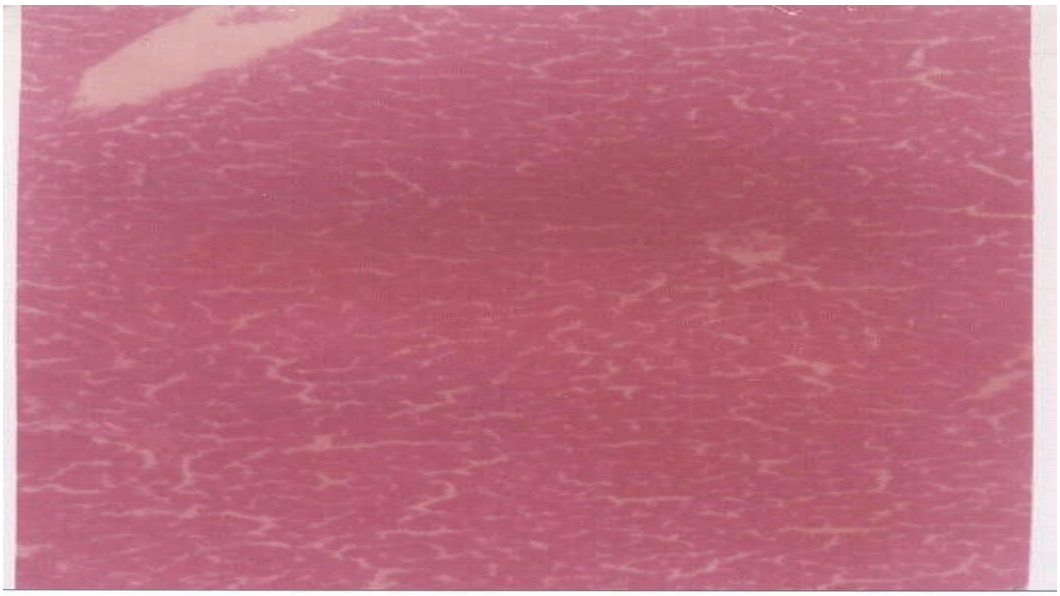

Fig. 5: A cross section of the hepatocytes of control mouse. Stains: Haematoxylin & eosin, Magnification: x400. The hepatocytes appear normal.

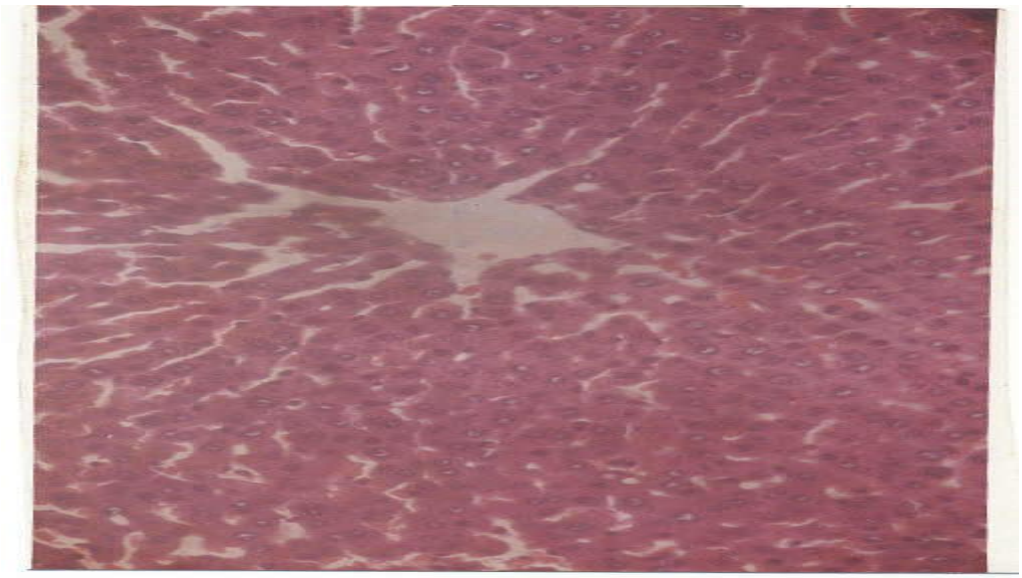

Fig. 6: A cross section of the hepatocytes of mouse after administration of arthemerher for 5 days. Stains: Haematoxylin & eosin, Magnification: x400. There was degeneration of the hapatocyes.

1. Discussion

In this study, artemether significantly ($p<0.05$) affected the sperm characteristics which included sperm counts, motility, morphology and viability. The implication is that there are less live sperm cells in groups given artemether when compared with control. Also, there is less movement by the sperm cells in groups given artemether when compared with control group. Artemether administration caused reduction in the number of sperm cells with normal morphology and viability as shown in table 1. The toxic effects on sperm characteristics may be ascribed to the degeneration of interstitial leydig cells and atrophy of the seminiferous tubules caused by free radicals generation induced by artemether (fig. 4). Free radicals have been shown to cause cellular injury and have been implicated in the aetiology of many pathological incidences such as atheriosclerosis,

isceamia etc. The structure of artemether possesses endoperoxide bond which when degraded generates free radicals. Comparing the tissue of uninfected mouse and not treated with artemether (fig. 3), with that of mouse given artemether (fig.4), it is glaring that artemether caused degeneration of the interstitial leydig cells and atrophy of seminiferous tubules. The increased catalase and superoxide dismutase levels in the tissue homogenates of testes of uninfected treated mice are indications that there was lipid peroxidation via generation of free radicals. Elevated levels of reduced glutathione may be in response to increased free radicals. The generation of free radicals may be responsible for the observed degeneration of interstitial leydig cells. The results showed adverse effects of *P. yoelli* and artemether on reproductive indices which include sperm counts, motility, morphology and viability, testicular testosterone and testicular weight. Decreases in sperm motility, sperm count and viability in mice infected with plasmodium berghei berghei have been reported by (Raji et al., 2006). Other previous studies have also shown adverse effects of drugs on reproductive indices (Raji et al., 2006; Borovskaya, 2000). These adverse effects are connected to generation of free radicals during metabolism of the drugs and also during *P. yoelli* infection.

Furthermore, artemether induced lipid peroxidation in the tissue of the kidney as shown by significant ($p<0.05$) elevation of malondialdehyde (MDA) in mice given given artemether when compared with control mice (table 2). Free radicals are generated normally in the system by physiological processes as well as pathological processes and their generations elicit syntheses of antioxidant agents. However, if there is inbalance in oxidant/antioxidant status, oxidative stress occurs leading to peroxidation of membrane lipids. Malondialdehyde is one of the endproducts of oxidized lipids and is used as a marker of lipid peroxidation. Elevated MDA in this study is an evidence that artemether induced lipid peroxidation in the kidney tissue of the artemether treated mice. This was further supported by significant ($p<0.05$) elevation of enzymic antioxidantsmeasured (reduced glutathione). Enzymic antioxidants are known to rise in the system to mop up free radicals generated by pro-oxidant agents.

Study of the status of hepatic oxidative stress and antioxidant defence indices in this work showed that artemether treatment induced oxidative stress which was evidenced by increased level of malondialdehyde. Increased concentration of malondialdehyde is an evidence of increased lipid peroxidation which causes inpairment of membrane functions and inactivation of membrane bound receptorsand enzymes (Halliwell and Gutteridge, 1989). Also there was concomitant increase in reduced glutathione activity in the artemether treated mice and this could be ascribed to increased pro-oxidant activity which generates free radicals and consequently induced the synthesis of reduced glutathione to mop up the free radicals. The increased concentration of malondialdehyde observed in *P. yoelli* infected mice when compared with uninfected and untreated mice suggests prooxidative activities due to the *P. yeolli* in the infected mice. However, this pro-oxidant activity which causes generation of reactive oxygen species induced the increased production of antioxidative agents such as catalase and superoxide dismutase observed in this study. Reduced levels of superoxide dismutase were observed in infected mice treated with artemether and untreated infected mice when compared with control mice. Generally, when there is generation of free radicals, there is a concomitant increase in antioxidative agents such as superoxide dismutase. However, whenever the generation of reactive oxygen species overwhelms the host's antioxidative defense system, antioxidants depletion occurs. This may explain the reduction in concentrations of superoxide dismutase observed in untreated infected and treated infected mice.

Observation in this study showed degeneration of brain cells confirming that the brain was one of the affected organs. Photomicrograph of the brain showed neurodegeneration indicating that the drug has had toxic effects on the brain cell. Administration of artemether in this study caused degeneration of the neuronal cells, reduction in the population of glial cells thereby making the cells to be scanty in number. The scanty population of the glial cells caused increased ratio of the interstitial space to cell to be more pronounced. In early Chinese studies of organ distribution of intramuscular artemether, it was observed that the most exposed organs are liver, brain, kidneys, (China Cooperative Research Group, 1982a) implying that the brain is a major site of action of the drug. It has been postulated by other workers (Petras et al., 2000 and 1997) that in vitro artemisinin derivatives including artemether are toxic to neuronal cells by a mechanism which may be similar to its antimalaria mechanism. In this mechanism, in which case the iron produced as a result of the degradation of red blood cells by the malaria parasite, reduces the endoperoxide bond in artemisinin generating high-valent ironoxo species resulting in the generation of reactive oxygen radicals which damage the parasite thus leading to its death. Therefore, since the brain also contains sizeable concentration of iron, it may be postulated that this iron content reacted with the peroxide bond in artemether drug, generating free radicals that destroy the neuronal cells, as observed in this study. (Hein and White, 1993) also reported that

heme potentiates the toxicity of artemisinin derivatives to neuronal cells in-vitro. (Fishwick and Edwards, 1995) demonstrated that artemisinin has cytotoxic effects on cells containing high iron concentration. The reported neurotoxic effect of artemether in this study is corroborated by findings of artemether neurotoxicity by Brewer et. al (Brewer et al., 2000). The free radicals generated by the degradation of the endoperoxide bond contained in the artemether may cause lipid peroxidation in the membrane of the cells of the brain leading to degeneration of these cells. The increased level of superoxide dismutase, catalase and reduced glutathione observed in the brain tissue homogenates of artemether treated mice in this study is an indication that synthesis of these antioxidants was induced by the increased production of free radicals from the metabolism of artemether.

The study showed mild adverse effects of artemether at therapeutic dose in the various organs assessed. This further showed that the mechanism of toxicity may be via free redical generation resulting in oxidative stress. Extensive clinical studies are required to determine the level of adverse effects in humans.

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