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Effect of Malarial Treatments on Biochemical Parameters and Plasma pH of Mice Infected with *Plasmodium berghei*

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Abstract: Mice are considered a comparable genetic model to humans and it is well established that mice also exhibit natural differences in susceptibility to malarial infection. The study aimed to determine and compare the effects of artesunate, artesunate+amodiaquine combination, amodiaquine and quinine on biochemical parameters such as liver catalase, plasma glutathione peroxidase, lipid peroxidation, total plasma proteins and plasma pH in the course of a malaria infection. Thirty male albino mice of eight weeks were randomly divided into 6 groups based on the specific antimalarial drug administered and two groups served as control and parasitized untreated groups respectively. The parasite used was a chloroquine-sensitive strain of *Plasmodium berghei* NK 65, inoculated into mice and observed for twelve days, followed by four days of antimalarial drug administration. Plasma sample was obtained and assay was done for lipid peroxidation, glucose concentration, plasma pH and liver catalase. Plasma pH was significantly lower ($p<0.05$) in the antimalarial groups compared to the Control and Parasitized untreated groups. Plasma glucose was significantly lower ($p<0.05$) in the parasitized untreated and antimalarial compared to the control group. Liver catalase was significantly higher ($p<0.05$) in the Parasitized untreated and antimalarial groups compared to the control group. Lipid peroxidation revealed different results within the antimalarial groups. Artemisinin and its combination with amodiaquine resulted in lowered plasma glucose and greater degree of lipid peroxidation, hence an index of suspicion should be put on possibility of hypoglycaemia with the use of ACTs.

Key words: Malaria, pH, lipid peroxidation, catalase activity, parasite, hypoglycaemia

INTRODUCTION

Malaria is an enormous public health problem worldwide and kills one to two million people every year, mostly children residing in Africa (Yoshida *et al*., 2010). Furthermore, malaria is the most lethal parasitic disease in the world, annually affecting approximately 500 million people mostly in African sub-Saharan countries (WHO, 2000b; Snow *et al*., 2005).

Malaria infection in humans and animals is caused by the parasite *Plasmodium*. Several species of *Plasmodium* have the ability to cause malaria in animals, including rodents. The most commonly Plasmodia species used to infect laboratory mice are *Plasmodium chabaudi* and *Plasmodium yoelii.*

P. berghei and *Plasmodium vinckei* mice are considered a comparable genetic model to humans: There is a high degree of genomic conservation, this is up to 99% (Pennacchio, 2003) and it is well established that mice also exhibit natural differences in susceptibility to malarial infection (Greenberg *et al*., 1954).

A prime event in malaria infection is increased production of highly reactive oxygen species (ROS) as part of the host defense (Wozencraft *et al*., 1984). Peripheral phagocytes can be activated by Plasmodium components *in vitro* to generate ROS (Kharazmi *et al*., 1987). Increased production of ROS in the whole blood was observed in *P. vinckei* infected mice (Stoker *et al*., 1984) and in patients with acute falciparum malaria (Descamps-Latscha *et al*., 1987). Circulating plasma lipids are therefore exposed to the oxidant stress and are vulnerable to ensuing lipid peroxidation. Thus, malaria infection has been found to be associated with lipid peroxidation accompanying reduction in anti-oxidant capacity (Idonije *et al*., 2011).

Malaria can cause metabolic acidosis via erythrocyte destruction, this will result in severe anaemia and low oxygen levels. Lactic acid will build up as a consequence with a drop in blood pH. Metabolic acidosis has emerged as a central feature of severe malaria. This is the most important independent predictor of a fatal outcome in both adults and children.

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Treatment of malaria involves supportive measures as well as specific anti-malarial drugs. Successful chemotherapy depends largely on the ability to exploit metabolic differences between pathogens and the host (Onyesom and Agho, 2011). The malaria parasite has developed resistance to drugs used in the therapy of malaria, except the artemisinins.

Artemisinin is a saturated endoperoxide lactone molecule and has been used by the Chinese for 2 millenniums as a folk remedy against fever (Mpiana *et al*., 2007). Also, the artemisinins produce fast recrudescence when used alone due to their short half-lives. Due to this and to forestall resistance they are used in combinations with other antimalarials, a combination known as Artemisinin Combination Therapies (ACTs).

ACTs combines artemisinin derivatives with other antimalarials, including quinoline compounds, such as amodiaquine and mefloquine. The quinolines act mainly by inhibiting hematin polymerization, thus intoxicating the parasite with the ferriprotoporphyrin groups generated by hemoglobin degradation (Vennerstrom *et al*., 1999). Other antimalarials used in ACT, for example, pyrimethamine and proguanil, inhibit the tetrahydrofolic acid cycle and thus eliminate an important cofactor for DNA synthesis. Despite the arsenal of drugs available for malaria treatment, the disease remains a worldwide public health problem.

Various antimalarials have been shown to influence the biochemical environment within and around the Plasmodium infected erythrocytes with variable outcomes (Iyawe and Onigbinde, 2009). Specifically, quinolones like amodiaquine and chloroquine can increase free radical generation and worsen lactic acidosis.

Artemisinin and its derivatives also exert their anitmalarial effects by production of carbon-centered radicals. However, the effect of artemisinin compounds and their combinations on various biochemical parameters have neither been evaluated nor compared to some of the older drugs. Thus, the overall aim of this present study is to determine and compare the effects of Artesunate, Artesunate+Amodiaquine combination, Amodiaquine and Quinine on biochemical parameters such as liver catalase, plasma glutathione peroxidase, lipid peroxidation, total plasma proteins and plasma pH in the course of a malaria infection.

MATERIALS AND METHODS

Thirty male albino mice of eight weeks were obtained from the Laboratory Animal Department, College of Medicine, University of Lagos. The animals weighed 25-30 g, were housed in clear polypropylene cages lined with wood chip beddings, fed on standard mice pellet diet and had access to water *ad libitum*. Animals were kept under standard conditions of temperature 27-30°C, with 12 h light/dark cycle and were randomly divided into 6 groups of 6 mice each.

Artesunate, amodiaquine, artesunate+amodiaquine combination and quinine were all purchased from Evans Medical Plc, Agbara, Lagos. Nigeria. These drugs were registered and unexpired by the National Agency for Food and Drug Administration and Control and were administered dissolved in distilled water orally with the aid of a stainless metallic feeding cannula (Oreagba and Ashorobi, 2006) for four days.

The parasite used was a chloroquine-sensitive strain of *P. berghei* NK 65 (Oreagba *et al*., 2008) maintained in mice, from the National Institute of Medical Research, Yaba, Lagos, Nigeria. Although *Plasmodium berghei* is generally used in rodent model for malaria (Ene *et al*., 2008), mice model was used in this study because of the high susceptibility of mice to *P. berghei* infection compared to laboratory rats and hamsters, which are less susceptible (Kellick-Kendrick, 1978). The susceptibility of mice to *P. berghei* infection is equally supported by the study of Pavia (1983).

The parasite was subsequently passaged into fresh mice, which served as donor mice in this study. 1 mL of parasitized blood was obtained from an infected mouse and diluted in 5 mL freshly prepared phosphate buffer solution (PBS). It was then assumed that 1 mL of the parasitized blood contained 5×10^9 RBC mL^{-1} infected erythrocytes (Agomo, 1990) thus, 1 mL of blood in 5 mL PBS (pH 7) contained 100, 000, 000 infected erythrocytes. Subsequently, 0.1 mL inoculum (Adejuwon and Adejuwon, 2005) of this dilution was then injected intraperitoneally into each mouse. Summarily, a standard dose of 10^4 parasitized red blood cells (RBC) was inoculated intraperitoneally (Iyawe *et al*., 2006).

The level of parasitaemia (parasite count) was observed under the microscope using Giemsa-stained thin blood films (WHO, 2000a). Parasite count of animals in each group was determined at days 3, 6, 9 and 12. Antimalarial drug administration commenced after the 12th day for four days duration. At the end of the treatment procedures, mice were anaesthetized with chloroform, blood samples were collected by using heparinized capillary tubes from the ocular orbit into heparinized bottles; centrifuged at 3000 g for 15 min to obtain plasma.

The plasma samples obtained was stored on ice and its assay used for determination of plasma pH using a pH meter (SevenMulti™ S40-professional pH meter); Malondialdehyde concentration was measured following

the method of Niehaus and Samuelsson (1968), Glutathione peroxidase was measured as described by Moron *et al.* (1979); Glutathione-s-transferase (GST) activity measured using Cayman's glutathione S-transferase assay kit; Cholesterol level using enzymatic colorimetric method; Glucose concentration using glucose oxidase method (Hugget and Nixon, 1957); Superoxide dismutase concentration using assay method described by Sum and Zigma (1978) and total plasma protein level was estimated according to the Gornall *et al.* (1949) method. The liver was isolated and homogenized in PBS with ground glass using mortar and pestle for estimation of catalase activity using colorimetric method as described by Sinha (1972).

Results were expressed as Mean±SEM. The significant differences between groups were analyzed statistically by One-way ANOVA (Analysis of Variance), followed by Student's unpaired t-test. Differences were considered statistically significant at $p<0.05$.

RESULTS

The parasite density (Parasite count/µL) 3 days after inoculation was 11.768.4±1551.89; 6 days after inoculation was 26.590±2001.2; 9 days after inoculation was 63.278±3123.6; and 12 days after inoculation was 75.525.33±2805.94. Parasite density as expected was significantly lowest ($p<0.05$) 3 days after inoculation and increased significantly till the 12th day after inoculation (Fig. 1).

Plasma pH was significantly lower ($p<0.05$) in the Artesunate+Amodiaquine (7.10±0.1); Artesunate (7.08±0.1) and Amodiaquine (7.11±0.1) groups compared to the Control (7.44±0.2) and Parasitized untreated (7.36±0.1) groups. However, there was no significant difference in plasma pH level in Quinine group (7.22±0.1) compared to Control and Parasitized untreated groups (Fig. 2).

Plasma glucose level (mg dL^{-1}) was significantly lower ($p<0.05$) in the Parasitized untreated (22.57±0.3); Artesunate+Amodiaquine (27.67±0.2); Artesunate (25.97±0.3) and Quinine (24.27±0.2) groups compared to the Control (33.74±0.1) and Amodiaquine (39.32±0.1) groups. This is presented in Table 1.

Liver Catalase (µmol/min/mg) was significantly higher ($p<0.05$) in the Parasitized untreated (48.8±2.1) and Amodiaquine (38.0±2.0) groups compared to the control (28.7±2.1); artesunate+amodiaquine (14.1±1.2) and Artesunate (13.1±0.7) groups. However, there was no significant difference in liver Catalase in Quinine group (28.8±1.9) compared to Control group (Table 1).

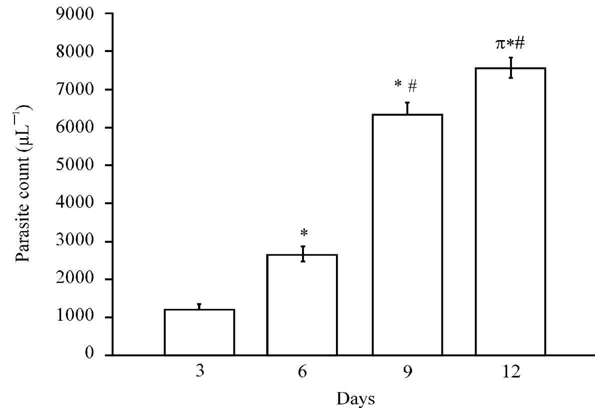

Fig. 1: Parasite count in 3, 6, 9 and 12 days after inoculation. *$p<0.05$ vs. 3 days, $^{\#}p<0.05$ vs. 6 days, $^{\pi}p<0.05$ vs. 9 days

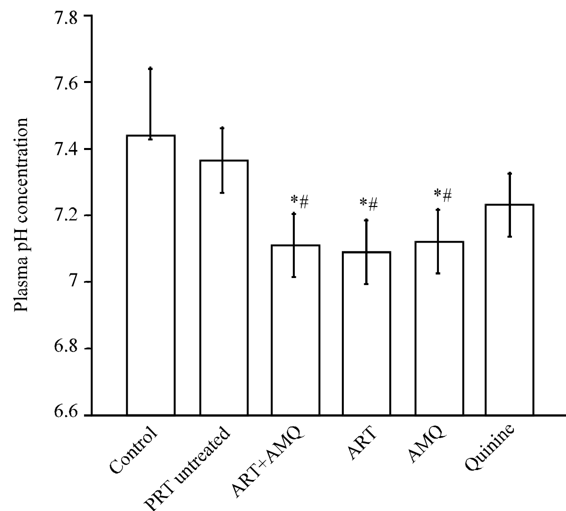

Fig. 2: Plasma pH concentrations in control, parasitized untreated, artesunate-amodiaquine (ART+AMQ), artesunate (ART), amodiaquine (AMQ) and quinine treated groups, *$p<0.05$ vs. control, $^{\#}p<0.05$ vs. parasitized untreated

Plasma glutathione peroxidase concentration (µmol mL^{-1}) was not significantly different in all the treated groups compared to the mice in the Control group (Table 1).

Plasma Malondialdehyde (nmol/mL) was significantly higher ($p<0.05$) in the artesunate+amodiaquine (9.96±1.1) and Artesunate (12.65±3.0) groups compared to the Control (7.00±1.0); Parasitized untreated (5.52±0.3); Amodiaquine (3.64±1.1) and Quinine (5.52±0.1) groups (Table 1).

Table 1: Biochemical parameters level in plasma and liver catalase activity level

	Control	Parasitized untreated	ART+AMQ	ART	AMQ	Quinine
Plasma glucose Level (mg dL^{-1})	33.74±0.1	22.57±0.3$^{£\#}$	27.67±0.2$^{£\mu a\alpha\#}$	25.97±0.3$^{£a\#}$	39.32±0.1$^{£\mu a\#}$	24.27±0.2$^{£}$
Liver catalase (μmol/min/mg)	28.70±2.1	48.80±2.1$^{£\mu *\alpha\#}$	14.10±1.2$^{£}$	13.10±0.7$^{£}$	38.00±0.7$^{£\mu *\#}$	28.80±1.9$^{\mu *}$
Plasma glutathione peroxidase (μmol mL^{-1})	0.620±0.05	0.620±0.04^{ns}	0.650±0.04^{ns}	0.58±0.04^{ns}	0.580±0.04^{ns}	0.5900±0.01^{ns}
Plasma malondialdehyde (nmole mL^{-1})	7.000±1.0$^{\mu}$	5.520±0.3$^{\mu}$	9.960±1.1$^{\mu}$	12.65±3.0$^{\mu}$	3.640±1.1$^{\mu}$	5.520±0.1$^{\mu}$
Plasma SOD (min mg^{-1})	111.17±0.3	68.40±0.5$^{£\mu a\#}$	100.59±0.2$^{£\mu a\alpha\#}$	43.23±0.1$^{£}$	60.00±0.4$^{£\mu\#}$	50.15±0.3$^{£\mu}$
Plasma GST (μmol mL^{-1})	468.75±8.7	15.63±2.3$^{£}$	62.63±7.2$^{£\mu a\alpha}$	18.75±3.4$^{£}$	31.25±5.6$^{£}$	56.25±4.6$^{£\mu a\alpha}$
Plasma cholesterol (mg dL^{-1})	51.41±0.5	62.71±0.3$^{£}$	75.14±0.2$^{£a}$	80.23±0.5$^{£\mu a\#}$	107.91±0.8$^{£\mu a\#}$	75.71±0.4$^{£a*}$
Total plasma protein (g L^{-1})	54.60±2.8$^{\alpha}$	44.40±1.7$^{\alpha}$	52.80±1.9$^{\alpha}$	52.70±0.8$^{\alpha}$	55.10±1.3$^{\alpha}$	53.00±2.8$^{\alpha}$

$^{£}p<0.05$ vs. Control: $^{\mu}p<0.0$ 5 vs. ART: $^{a}p<0.05$ vs. PRT untreated: $^{\alpha}p<0.05$ vs. AMQ: $^{*}p<0.05$ vs. ART+AMQ: $^{\#}p<0.05$ vs. quinine: ns: Not significant

Plasma Superoxide dismutase concentration (min/mg) was significantly higher ($p<0.05$) in mice in the Control group (111.17±0.3) compared to mice in the Parasitized untreated (68.4±0.5); Artesunate+Amodiaquine (100.59±0.2); Artesunate (43.23±0.1); Amodiaquine (60.0±0.4) and Quinine (50.15±0.3) groups (Table 1).

Plasma glutathione-S-transferase concentration (μmol/mL) was significantly higher ($p<0.05$) in mice in the Control group (468.75±8.7) compared to mice in the Parasitized untreated (15.63±2.3); artesunate+amodiaquine (62.63±7.2); Artesunate (18.75±3.4); amodiaquine (31.25±5.6) and quinine (56.25±4.6) groups (Table 1).

Plasma cholesterol concentration (mg dL^{-1}) was significantly lower ($p<0.05$) in mice in the Control group (51.41±0.5) compared to mice in the Parasitized untreated (62.71±0.3); artesunate+amodiaquine (75.14±0.2); Artesunate (80.23±0.5); amodiaquine (107.91±0.8) and Quinine (75.71±0.4) groups (Table 1).

Total plasma protein (g L^{-1}) was significantly lower ($p<0.05$) in mice in the parasitized untreated group (44.4±1.7) compared to mice in the Control group (54.6±2.8); artesunate+amodiaquine (52.8±1.9); artesunate (52.7±0.8); amodiaquine (55.1±1.3) and quinine (53.0±2.8) groups (Table 1).

DISCUSSION

There was a progressive increase in level of parasitaemia as the days (duration) progressed from day 3 to 12 in the infected mice (Fig. 1). This is in line with the view that parasitaemia increases progressively after inoculation or infection until the point of death in the absence of suitable treatment (Trampuz *et al*., 2003; Breman *et al*., 2001).

The results on plasma glucose reflect an interesting effect of antimalarials on glucose metabolism in infected mice. Plasma glucose was significantly higher ($p<0.05$) in both control and amodiaquine groups compared to the other groups. This could be due in part to the fact that during malaria parasite infection, glucose is rapidly taken up across the parasite plasma membrane through a facilitated hexose transporter and is in turn metabolized through the process of glycolysis (Woodrow *et al*., 1999). This is accompanied with approximately 100-fold increase in glucose utilization when compared with uninfected erythrocytes thus causing a profound hypoglycaemia if untreated (Anders and Dekant, 1998). The results also show that amodiaquine might have no significant effect on glycolysis because plasma glucose was significantly higher ($p<0.05$) in the amodiaquine group compared to that in the control group (Table 1). This could be due to a fall in glycolysis activity within the cells of mice in this group, with a resultant decrease in formation and production of lactic acid.

The results on the other antimalarials except quinine could be due to the emptying of red blood cell cytoplasmic content into the plasma after lipid peroxidation of the cell membrane because of cleavage of the artemisinin endoperoxide bridge resulting in lactate production. The result on quinine could be peculiar because the drug has the ability to concentrate in the acidic food vacuole of the parasite where it inhibits the activity of the enzyme haem polymerase that is responsible for the conversion of haem to haemozoin, which automatically causes the death of the malaria parasite, thus plasma glucose level in the quinine group was not significantly different to that of the parasitized untreated group (Table 1).

Malaria often results in metabolic acidosis, mainly because the parasite while destroying red blood cells efficiently causes severe anaemia which in turn causes low oxygen tension. Thus, Lactate production builds up within the cell to eventually cause a drop in pH, which in turn could develop into metabolic acidosis. This drop in plasma pH was observed in all the treated groups (Fig. 2) in varying degrees. Reduction in plasma pH below 7.4 could result in fatal outcomes due to metabolic acidosis (Arjen *et al*., 2005). Unfortunately, all the antimalarials used in this study were unable to restore the plasma pH to the control level. This could be due to the fact that metabolic acidosis due to malaria parasite infestation has multiple etiologies such as poor tissue perfusion and occlusion of microcirculation by parasites (Woodrow *et al*., 1999).

The liver catalase level was significantly higher ($p<0.05$) in the Parasitized untreated group compared to the other groups. This is in line with the view of Iyawe and Onigbinde, (2009) and also plausible because the presence of malaria parasites in liver cells induces hepatic cell catalase activities, probably through the mechanism of microsomal and electron transport chain production of superoxides (Iyawe and Onigbinde, 2009). The antimalarials were able to achieve the observed varying degree of liver catalase reduction probably through a feedback inhibition or oxidative inactivation of enzyme protein due to an excess reactive oxygen species generation (Pigeolet *et al*., 1990). Thus, excess hydrogen peroxide production from generation of free radicals by artemisinin derivatives due to cleavage of the endoperoxide bridge to cause subsequent destruction of the malaria parasite (Guha *et al*., 2006).

Plasma Glutathione Peroxidase was not significantly different in all the treated groups compared to the Control group (Table 1). This could be due to the fact that glutathione peroxidase is non-specific for hydrogen peroxide thus in mopping up hydrogen peroxide radicals, catalase levels must first be depleted before glutathione peroxidase is whipped into action (Chance *et al*., 1979).

Plasma malondialdehyde concentration was used as an index of lipid peroxidation. This was significantly higher ($p<0.05$) in the artesunate+amodiaquine and Artesunate groups compared to the other groups. Destruction of malaria parasites will normally produce free radicals; also, artemisinin compounds exert antimalarial effect by breaking up the endoperoxide bridge of malaria parasites hence the experimental groups treated with artemisinin probably had excess free radicals which overwhelmed the antioxidant system leading to higher lipid peroxidation among this group.

Both plasma Superoxide Dismutase (SOD) and glutathione-S-transferase (GST) concentrations were significantly higher ($p<0.05$) in the control group compared to all the other groups. This is in line with the view of Iyawe and Onigbinde (2009) and also reasonable because after malaria parasite infestation, infected erythrocytes produce reactive oxygen species in the parasite vacuole during haemoglobin digestion. This migrates from the parasite to the host cell cytosol (Marnett *et al*., 2003) to cause a significant decrease in SOD and GST activities.

As anticipated, total plasma protein was significantly lower ($p<0.05$) in mice in the Parasitized untreated group compared to all the other groups. This could probably be due to a corresponding drop in the constituent protein such as albumin in the parasitized untreated group. Areekul (1988) stated that during malaria parasite infestation, plasma albumin may decrease due to an increased transcapillary escape rate.

The results of this study showed that artemisinin and its combination with amodiaquine resulted in lowered plasma glucose and greater degree of lipid peroxidation, hence an index of suspicion should be put on possibility of hypoglycaemia with the use of ACTs.

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