Effects of Four Different Classes of Artemisinin - based Combination Therapies on Antioxidant System of Malaria-Infected Albino Mice

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Keywords: malaria, *plasmodium,* antioxidant, artemisinin-based combination therapies.

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ABSTRACT

Effects of four classes of artemisinin-based combination therapies (ACTs) on the antioxidant system of malaria-infected mice was undertaken. Thirty-six, three months old male and female albino mice, weighing 25-31g were randomly divided into six groups of six mice per group. Group 1 animals served as normal control. Group 2 animals served as positive control; were infected with Plasmodium berghei without being treated with ACTs. The test groups 3, 4, 5 and 6 animals were infected with Plasmodium berghei and treated with artesunate-amodiaquine, artemether-lumefantrine, rtesunate-mefloquine, and dihydroartemisinin-piperaquine phosphate respectively, at therapeutic doses. At the end of the three days treatment, the animals were sacrificed using chloroform anesthesia and the blood samples were collected by cardiac puncture for analyses, using standard methods. Artesunate-amodiaquine indicated a significant ($p<0.05$) increase in the concentration of serum superoxide dismutase, catalase, glutathione peroxidase, iron, and vitamin C, when compared with the positive control. Artemether-lumefantrine indicated a significant increase in the level of serum superoxide dismutase, catalase, glutathione peroxidase, zinc, iron and serum vitamin C when compared with the positive control. Artesunate-mefloquine indicated a significant ($p<0.05$) increase in serum superoxide dismutase, catalase, glutathione peroxidase, iron, vitamin C, and vitamin A concentrations, when compared with the positive control. Dihydroa- rtemisinin-piperaquine phosphate treated group indicated a significant increase in the concentrations of serum superoxide dismutase, catalase, vitamin C and vitamin A but decreased significance ($p<0.05$) in the level of copper when compared with the positive control. Hence, the overall result revealed that treatment of malaria-infected mice with ACTs ameliorates the antioxidant system in parasitized mice and therefore may help in improving or restoring health status under such conditions.

Keywords: malaria, *plasmodium,* antioxidant, artemisinin-based combination therapies.

Classification: DDC Code: 616.07, LCC Code: RB170

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I. INTRODUCTION

Malaria infection in humans and animals is caused by *Plasmodium* parasites. Several *Plasmodium* species have the ability to cause malaria in animals. *Plasmodium 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).

The malaria parasite has developed resistance to drugs, used in the therapy of malaria, except the artemisinins. Several authors have discussed the implications of free radicals through oxidative stress in the physiopathogenesis of malaria (Silva, 2011; Gomes, 2011). This involvement may be related to the pathogenic mechanisms triggered by the parasite (Potter *et al.,* 2005), as well as free radical production (Keller *et al.,* 2004) and antioxidant defenses (Sohail *et al.*, 2007), in host cells to abate the

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infection. Recent studies suggest that the generation of reactive oxygen and nitrogen species (ROS and RNS) associated with oxidative stress play a crucial role in the development of systemic complications caused by malaria. Malaria infection induces the generation of hydroxyl radicals ($OH^{\bullet}$) in the liver, which most probably is the main reason for the induction of oxidative stress and apoptosis (Guha *et al.,* 2006). A potential source of free radical production in this disease is the host's hemoglobin molecule, since the parasite uses this molecule as a source of amino acids for its own nutrition during the erythrocytic stage of the disease, resulting in the liberation of large amounts of circulating heme. By having Fe^{2+}-associated groups, these heme groups are able to induce intravascular oxidative stress, causing changes in erythrocytes and endothelial cells and facilitating the internalization of the parasite in tissues such as the liver and brain. A free radical species, which appears to be involved in this disease, is nitric oxide (NO) (Cabrales *et al.,* 2011). However,its role is still controversial. Some researchers claim that cerebral malaria is probably an unfortunate consequence of high amounts of NO production to promote the death of the parasites (Maneerat *et al.,* 2000).

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 acidosis. Artemisinins produce fast relapse 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-based combination therapies (ACTs). Artemisinin is a saturated endoperoxide lactone molecule and has been used by the Chinese for two millennia as a folk remedy against fever (Mpiana *et al.*, 2007). Also, the ACTs combine 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 ACTs, for example, pyrimethamine and proguanil, inhibit the tetrahydrofolic acid cycle and thus eliminate an important cofactor for DNA synthesis. Artemisinin and its derivatives also exert their antimalarial effects by production of carbon-centered radicals. However, the effects of four different classes of artemisinin-based combination therapies on the antioxidant systems are yet to be researched on. Hence, this study is aimed at discovering the class(es) of Artemisinin-based Combination Therapies that maintain the optimal antioxidant status.

II. MATERIALS AND METHODS

2.1 Materials

P-Alaxin (dihydroartemisinin-piperaquine phosphate), Artequin(artesunate-mefloquine), Lumartem(artemether-lumefantrine) and Camosunate(artesunate-amodiaquine), were purchased from Contour Pharmacy, Abak. The four classes of ACTs were ground to a powdered form, mixed with distilled water and administered as an aqueous suspension. Standard recommended therapeutic doses (according to the weight of the experimental animals) of each artemisinin-based combination drug suspension were administered orally to the different groups of the experimental animals for three days. The ACTs were administered orally *via* an oral carnula. New stock solutions of the drugs were prepared each day of the administration. The assay kits were obtained from Fortress Diagnostics Limited, Unit 2C Antrim Technology Park, Antrim, BT41 1QS (United Kingdom).

2.2 Experimental Design and Treatment of Animals

A total of thirty six (36) albino mice (male and female) ranging from 25-31g by weight, were divided into six different groups of six per group. Group 1 were uninfected with the parasite and were given normal rat pellets and water. Group 2 were infected with the parasite but untreated.

Group 3 were infected with the parasite and then treated with 1.42 mg/kg body weight of Artesunate and 4.29mg/kg body weight of Amodiaquine twice a day for three days. Group 4 were infected with the parasite and then treated with 1.14mg/kg of Artesunate body weight and 6.86mg/kg body weight of Lumefantrine twice a day for three days. Group 5 were infected with the parasite and then treated with 2.86mg/kg body weight of Artesunate and 3.57mg body weight of mefloquine once a day for three days. Group 5 were infected with the parasite and then treated with 1.71mg/kg body weight of dihydroartemisinin and 13.71mg/kg body weight of Piperaquine Phosphate once a day for two days followed by the administration of two thirds of the dosage on the third day.

2.3 Estimation of Serum Antioxidant Enzymes

Estimation of superoxide dismutase (SOD) activity was carried out according to the method of Marklund *et al.,* (1974). Estimation of catalase (CAT) activity was carried out according to the method of Deisseroth and Dounce, (1970). Estimation of glutathione peroxidase (GPx) activity was carried out according to the method of Paglia *et al.*, (1967). Estimation of reduced glutathione (GSH) activity was carried out according to the method of Chinard *et al.,* (1954).

2.4 Estimation of Antioxidant related Minerals

The concentration of serum Zinc was assayed using the method of Saito *et al.,* (1982). Serum Iron (Fe) concentration was assayed using the method of Henry, (1984). Copper was assessed using direct measurement on an atomic absorption spectrophotometer.

2.5 Estimation of Antioxidant Vitamins

The determination of serum vitamin C was carried out using the method of Rutkowski *et al.,* (1998). The determination of serum vitamin E was carried out using the method of Rutkowski *et al.,* (2005). Determination of serum vitamin A was carried out using the method of Rutkowski *et al.,* (2006).

III. RESULTS

Table 1: Four classes of ACTs and their Percentage Parasitaemia Clearance

Group Clearance	Pre-Treatment	Post-Treatment	% Parasitaemia
NC	0.00±0.00	0.00 ±0.00	0.00
PC	17868.00±3030.41	56261.29±5565.44	---
AA	25368.00 ±4987.35	71.46±41.95	99.72
AL	12924.00 ±3382.21	188.41±107.08	98.54
AM	29022.00±6283.26	885.51±467.59	96.95
DP	17688.00±2486.93	294.13±108.70	98.34

NC=control, PC=positive control, AA=artesunate amodiaquine, AL=artemether lumefantrine, AM=artesunate mefloquine, DP=dihydroartemisinin piperaquine phosphate n =6

Table 2: The Effect of Four Classes of ACTs on Antioxidant Enzyme Activities

Groups	SOD (U/ml)	CAT(umol/min/ml)	GPX (U/L)	GSH(mMol)
NC	34.24 ± 1.32	43.13 ± 0.65	20.17 ± 0. 54	1.74±0.27
PC	21.34 ± 0.68^{a}	5.83 ± 1.17a	6.95 ± 0.42^{a}	1.40±0.02
AA	30.08± 1.46^{a,b}	37.35 ± 2.15^{a,b}	20.14 ± 0.89^{b}	1.57±0.10
AL	60.1 ± 0.65^{a,b,c}	41.25 ± 1.41^{b,c}	21.04 ± 0.88^{b}	1.69±0.16
AM	41.15±2.01^{a,b,c,d}	40.06 ± 0.89^{b}	12.76±0.65^{a,b,c,d}	1.57±0.08
DP	33.32±1.23^{b,d,e}	31.95±0.69^{a,b,c,d,e}	6.17±0.39^{a,c,d,e}	1.64±0.05

NC=control, PC=positive control, AA=artesunate amodiaquine, AL=artemether lumefantrine, AM=artesunate mefloquine, DP=dihydroartemisinin piperaquine phosphate

Data are presented as Mean ± Standard Error of Mean. a = significantly different from Group 1 ($p < 0.05$); b = significantly different from Group 2 ($p < 0.05$); c = significantly different from Group 3 ($p < 0.05$); d= significantly different from Group 4 ($p < 0.05$), e = significantly different from Group 5 ($p < 0.05$).

Table 3: Antioxidant Minerals Concentrations

Groups	Copper (umol/l)	Zinc (umol/l)	Iron (uM)
NC	37.22 ± 0.58	14.57 ± 0.32	14.57 ± 1.45
PC	36.02 ± 0.64	11.59 ± 0.34[a]	11.59 ± 1.26[a]
AA	30.21 ± 0.34[a,b]	12.46 ± 0.43[a]	12.46 ± 0.72[b]
AL	36.30 ± 0.71[c]	15.67 ± 0.90[b,c]	15.67 ± 0.35[a,b,c,d]
AM	32.37 ± 0.35[a,b,c,d]	13.09 ± 0.34[d]	13.09 ± 0.62[b]
DP	27.10± 0.64[a,b,c,d,e]	12.17 ± 0.59[a,d]	12.17 ± 0.76[a,c,d]

NC=control, PC=positive control, AA=artesunate amodiaquine, AL=artemether lumefantrine, AM=artesunate mefloquine, DP=dihydroartemisinin piperaquine phosphate

Data are presented as Mean ± Standard Error of Mean. a = significantly different from Group 1 ($p < 0.05$); b = significantly different from Group 2 ($p < 0.05$); c = significantly different from Group 3 ($p < 0.05$); d= significantly different from Group 4 ($p < 0.05$), e = significantly different from Group 5 ($p < 0.05$).

Table 4: Antioxidant Vitamin Concentrations

Groups	Vitamin C (ug/ml)	Vitamin E (ug/ml)	Vitamin A (uM)
NC	72.92 ± 1.78	0.005 ± 0.00	0.43 ± 0.06
PC	9.06 ± 1.19[a]	0.007 ± 0.00[a]	0.34 ± 0.06
AA	57.04 ± 1.03[a,b]	0.005 ± 0.00[b]	0.04 ± 0.01[a,b]
AL	20.76 ± 0.46[a,b,c]	0.005 ± 0.00[b]	0.29 ± 0.04[c]
AM	51.00 ± 0.75[a,b,c,d]	0.005 ± 0.00[b]	0.74 ± 0.08[a,b,c,d]
DP	28.80 ± 1.23[a,b,c,d,e]	0.007 ± 0.00[a,c,d,e]	0.61 ± 0.05[a,b,c,d]

Data are presented as Mean ± Standard Error of Mean. a = significantly different from Group 1 ($p < 0.05$); b = significantly different from Group 2 ($p < 0.05$); c = significantly different from Group 3 ($p < 0.05$); d= significantly different from Group 4 ($p < 0.05$), e = significantly different from Group 5 ($p < 0.05$).

IV. DISCUSSIONS

Artemisinin-based combination therapy (ACT) has been adopted as a strategy to mitigate multidrug resistance to antimalarial monotherapies. ACT combines the rapid and effective, but rather short plasma half-life antimalarial action of an artemisinin derivative with a longer acting partner drug (Kavishe *et al.*, 2017). The level of parasitaemia (parasite count) was observed under the microscope using Giemsa-stained thin blood films (WHO, 2000) as presented on Table 1. The result showed a high percentage clearance in the four treatment groups, after being administered with the ACTs. Additionally, this finding indicated that the ACTs generally have a high percentage clearance on parasitaemia when compared with the positive control. However, the group treated with Artesunate-Amodiaquine indicated the highest percentage clearance. This is consistent 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). The Artemisia annua plant (Artemisia) is known to be the most ancient antimalarial treatment, having been used in China for over 2000 years. It contains

artemisinin, a substance which eliminates the blood-stage parasites more rapidly than any other drug and works well against *Plasmodium falciparum* species that are resistant to other drugs. This drug produces free radicals in contact with iron, a common metal in the body, especially within erythrocytes (Grahame-Smith, 2004).

The effects of the four classes of ACTs on the antioxidant enzymes are presented on Table 2. Superoxide dismutase (SOD) catalyzes the breakdown of the superoxide anion into oxygen and hydrogen peroxide. The groups treated with Artesunate-Amodiaquine, Artemether-Lumefantrine, Artesunate-Mefloquine and dihydroartemisinin-piperaquine phosphate indicated increased significance in the activity of superoxide dismutase (SOD) when compared to the positive control. This is as a result of reduced parasitaemia in the ACT-treated groups. This discovery agrees with Erel *et al.* (2001) who reported that the number of platelets and the activities of antioxidant enzymes-superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px)-in patients with *vivax* malaria were reduced.

Catalases are enzymes that catalyze the conversion of hydrogen peroxide to water and oxygen, using either an iron or manganese cofactor. It was observed that the groups treated with Artesunate-Amodiaquine, Artemether-Lumefantrine, Artesunate-Mefloquine, and Dihydroartemisinin-Piperaquine Phosphate increased significantly in the activity of serum catalase when compared with the positive control. Patients with acute uncomplicated *Plasmodium falciparum* or *Plasmodium vivax* malaria have been reported to have lower catalase activity than healthy controls but a higher SOD activity (Seixas *et al.*, 2009).

Glutathione peroxidase (GPx) is an important antioxidant enzyme that catalyzes the reduction of organic and inorganic hydroperoxides to water in oxygen-consuming organisms, using glutathione as an electron donor (Kang *et al.*, 2014). Glutathione peroxidase (GPx) is a selenium dependent and lipid peroxide-scavenging enzyme that effectively reduces lipid peroxides with the concomitant oxidation of glutathione. It is an enzyme containing four selenium-cofactors that catalyzes the breakdown of hydrogen peroxide and organic hydroperoxides. This finding indicated a significant increase in the activity of glutathione peroxidase in groups treated with Artesunate-Amodiaquine, Artemether-Lumefantrine, and Artesunate-Mefloquine, when compared with the positive control. This finding is in consonance with Bhatttacharya *et al.* (1987) who detected a decreased GPX activity in patients with vivax malaria. Additionally, Erel *et al.* (2001) showed that the number of platelets and the activities of antioxidant enzyme-glutathione peroxidase (GPx) in patients with *vivax* malaria were reduced.

Reduced glutathione (GSH) plays an important role in protecting cells against oxidative stress and toxic agents. The concentration of GSH in the positive control indicated no significant difference when compared with the groups treated with the four different classes of ACTs. This result is not consistent with that of Abubakar *et al.* (2016) who reported a significant reduction in the level of GSH in children infected with *Plasmodium falciparum* malaria.

The effects of the four classes of ACTs on the antioxidant minerals are presented on Table 3. Trace metals, including Zn and Cu, are directly involved in metabolic processes critical to cell differentiation and replication (Davachi *et al.*, 2009). Zinc (Zn) and Copper (Cu) are the essential elements that play a crucial role in the immune system. These trace elements act as cofactors for antioxidant enzymes involved in the destruction of toxic free radicals produced in the body. The serum levels of antioxidants vary in many diseases including malaria. These alterations are part of defense strategies of organisms and are induced by different cytokines (Faryadi *et al.*, 2003). Some studies relating micronutrient status and malaria infection reported low plasma levels of certain micronutrient in acute malaria infection (Alonso, 2004).

Copper (Cu) plays a critical role in the normal biochemical function of the body. It is found as a component of many enzymes and is also used for biological electron transport (Jain, 2006). The groups treated with Artesunate-Amodiaquine, Artesunate-Mefloquine and Dihydroartemisinin -Piperaquine Phosphate indicated a significant reduction in the concentration of serum copper when compared with the positive control. This is not consistent with the report that children with acute malaria infection have very low plasma copper concentration and this is inversely related to the acute phase proteins, C-reactive protein (Nussenblatt and Semba, 2002).

Zinc is a micronutrient that acts as a cofactor for several enzymes, most importantly those that regulate storage and metabolism of vitamin A (Macdonald, 2000). Zinc deficiency decreases the ability of the body to respond to infection, affecting both cell mediated immune responses and humoral responses (Okochi and Okpuzor, 2005). There was no significant difference in the concentration of Zn when the groups treated with Artesunate-Amodiaquine, Artesunate-Mefloquine and Dihydroartemisinin-Piperaquine Phosphate were compared with the positive control. However, there was a significant elevation in the concentration of Zn in the group treated with Artemether-Lumefantrine when compared with the positive control. This is in conformity with Brown *et al.* (1993) who reported a significant decrease in serum level of zinc in pregnant women infected with malaria parasite, where infection has been found to have an effect on the plasma level of zinc. This might be due to the redistribution of zinc from plasma to lymphocytes and liver during the acute phase response. Plasma zinc concentrations have been found to vary inversely with malaria parasitaemia and may preferentially protect against more severe malaria with high levels of parasitaemia (Gouado *et al.*, 2007).

Iron deficiency affects nearly two million people globally and results in over five hundred million cases of anemia (Nyakeriga *et al.*, 2004). The burden of both iron deficiency and malaria fall primarily on preschool children, apart from pregnant women (Nyakeriga *et al.*, 2004). This finding showed a significant increase in the concentration of Fe in the groups treated with Artesunate-Amodiaquine, Artemether-Lumefantrine and Artesunate-Mefloquine when compared with the positive control. This result agrees with Otitoju *et al.* (2012) who reported a marked decrease in the serum iron level of malaria infected children when compared to the value obtained for control.

Vitamin C (Ascorbic Acid) is a water soluble vitamin that acts as an antioxidant, especially protecting the immune system cells from free radicals generated during their assault on invaders (Wintergerst, *et al.,* 2006). There was a significant increase in the concentration of Vitamin C in the groups treated with Artesunate-Amodiaquine, Artemether-Lumefantrine, Artesunate-Mefloquine, and Dihydroartemisinin-Piperaquine Phosphate, when compared with the positive control. This finding is consistent with the report that the plasma concentration of antioxidants is significantly decreased in chronic and acute malaria infections (D'Souza *et al.*, 2006; Mohammad, 2012).

Vitamin E (α-Tocopherol) is a potent antioxidant. There was a significant decrease in the concentration of serum vitamin E in groups treated with Artesunate-Amodiaquine, Artemether-Lumefantrine and Artesunate-Mefloquine when compared with the positive control. This finding is not in line with the investigation of Nmorsi *et al.* (2007) and Das *et al.* (1996), where they documented that children with both severe and mild malaria had significant lower plasma vitamin E concentrations than the control group without malaria.

Vitamin A is an essential nutrient required for maintaining immune function, playing an important role in the regulation of cell-mediated immunity and in humoral antibody responses, suggesting that it could play a role in protection against malaria (Semba, 1998). There was a significant increase in the concentration of serum vitamin A in the groups treated with Artesunate-Mefloquine and Dihydroartemisinin -Piperaquine Phosphate. This is consistent with

the report that malaria-infected persons have low vitamin A concentration (Galloway *et al.*, 2000; Metzger *et al.*, 2001). However, there was a significant decrease in the concentration of vitamin A in the group treated with Artesunate-Amodiaquine when compared with the positive control. This result is not in line with Ekeanyanwu *et al.*, (2009) who reported that vitamin A concentration in *Plasmodium falciparum* infected children was significantly lower than in the control subject.

V. CONCLUSIONS

This research paper discussed the effects of four different classes of artemisinin-based combination therapies on antioxidant system of malaria-infected albino mice, and the overall results revealed that, artemisinin-based drugs demonstrate a high level of parasitaemia clearance on the infected mice, due to the potent nature of the antimalarial drugs, and generally ameliorate the antioxidant system in such condition.

VI. CONFLICTS OF INTERESTS

The authors declare no conflicts of interest regarding the publication of this paper.

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