THE BIOCHEMICAL EFFECTS OF ASCORBIC ACID CO-ADMINISTRATION WITH ANTIMALARIAL DRUG IN PLASMODIASIS

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Received *August 21, 2023;* ***Revised*** *August 30, 2023;* ***Accepted*** *September 01, 2023*

ABSTRACT

Malaria is a threat to the lives of below age five children and pregnant women living in sub-Saharan Africa. Oxidative stress is a key factor in malaria pathogenesis and artemisinin-based combination therapy with Vitamin C may protect human host against the toxicity of free radicals. In this study, we examined the alteration in biochemical indices and antioxidant enzymes gene expression in the bone marrow cells of Plasmodium berghei infected mice treated with artemether-lumefantrine and ascorbic acid. Five groups of six mice each categorized as basal control, untreated, ascorbic acid, artemether-lumefantrine and artemether-lumefantrine + ascorbic acid were used for this study. Biochemical assays and analysis of antioxidant enzyme gene expression were carried out. Artemether-lumefantrine co-administration with ascorbic acid resulted in complete parasite clearance day three post-infection; this same group had a non-significant increase ($p>0.05$) in superoxide dismutase activity and a significant decrease ($p<0.05$) in the malondialdehyde and hydrogen peroxide (H_2O_2) concentration of the liver when compared with the artemether-lumefantrine group. Similar trend was observed for H_2O_2 level in erythrocyte lysate. The levels of expression of catalase, Cu, Zn-superoxide dismutase and glutathione peroxidase genes in the P. berghei infected mice treated with artemether-lumefantrine plus Vitamin C were up-regulated compared with the group treated with lone artemether-lumefantrine. This study has shown that artemether-lumefantrine co-administration with ascorbic acid may be beneficial in P. berghei infected mice because total parasite clearance, decrease in oxidative stress markers and up-regulation in antioxidant enzyme gene expression were observed three days post treatment.

Keywords: Artemisinin-based combination therapies, Albino mice, Liver function enzymes, Malaria, Vitamin C

INTRODUCTION

Malaria is a threat to the lives of children below five years of age and pregnant women who are living in endemic countries especially in sub-Saharan Africa. Malaria is a disease that is caused by *Plasmodium* parasite and five species have been identified to infect humans namely *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi*. *P. falciparum* is the deadliest and it is responsible for the highest percentage of parasite infection in the tropics. World Health Organization (WHO) reported a steady reduction in global malaria cases (238 – 229 million) and deaths (736,000 – 409,000) between 2000 and 2019 (WHO, 2020). However, Nigeria remains the worst-affected country in the WHO African region in cases of infection (27%) and mortality (31%) (WHO, 2022).

The reduction in the rate of malaria infection is associated with a combination of factors one of which is the use of artemisinin-based combination therapies (ACTs). In the WHO African region, the recent first-line treatment for *P. falciparum* malaria includes the use of artemether-lumefantrine, artesunate-amodiaquine and dihydroartemisinin-piperaquine (WHO, 2020). Artemether-lumefantrine combination is used effectively in Nigeria to treat malaria and from a literature survey; there is no evidence of confirmed resistance to lumefantrine in Africa (WHO, 2020). The possible mode of actions of artemether-lumefantrine involves the reaction of artemether (a methyl ether derivative of dihydroartemisinin) endoperoxide bridge with Fe^{2+} in haem that is produced from haemoglobin breakdown to generate free radicals which alkylate and inactivate nearby proteins, while lumefantrine (an arylaminocarbinol) interacts with harmful haem in the food vacuole thereby preventing their bio-crystallization into harmless haemozoin and hence produce dangerous reactive oxygen species (ROS) which attack parasite's cell membrane (Warhurst *et al*., 2001).

During malaria infection there is elevation of ROS and reactive nitrogen species (RNS) that are caused by the parasite metabolic activity and exacerbated immunological response of the host (Vasquez *et al*., 2021). The increase in free radicals is countered by endogenous antioxidants like glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx) (Pham-Huy *et al*., 2008). However, in untreated individuals, plasmodiasis causes a significant decrease in these antioxidant enzymes with a corresponding increase in malondialdehyde level (Ezzi *et al*., 2017; Ojezele *et al*., 2017).

Oxidative stress is a key factor in malaria pathogenesis, and it appears that supplementing artemisinin-based combination therapy with an adjuvant (Vitamin C) could help in protecting parasite host against the toxicity of ROS whose levels surge in plasmodiasis. In treating malaria infection, some health workers simultaneously administer Vitamin C with ACTs to minimize the effect of oxidative stress. For example, a survey conducted by Omole and Oamen (2010) on the rational use of ACTs among health practitioners in south-west Nigeria showed that out of 200 of the health officials that completed the questionnaire, 173 recommended Vitamin C co-administration with ACTs. However, there is a lack of consensus in the reports obtained so far from ascorbic acid co-administration with ACTs for the treatment of plasmodiasis. Some investigations revealed that ascorbic acid acts synergistically with glutathione to mop up haem-mediated oxidative stress (Li *et al*., 2006). However, in the absence of glutathione in *Plasmodium* infected red blood cell, ascorbic acid works as pro-oxidant through interaction with Fe^{2+} to increase free radical levels in the form of hydrogen peroxide or hydroxyl radical and accentuates the haemolytic mechanisms in malaria (Mendiratta *et al*., 1998; Li *et al*. 2006). Hallberg *et al*. (1989) showed that Vitamin C can increase the bioavailability of non-haem iron through the conversion of Fe to Fe^{2+} for intestinal absorption; this Fe^{2+} has been shown to be indispensable for the development of the ring and trophozoite stages of *P. falciparum* into schizont (Marva *et al*., 1992). Furthermore, Ganiyu *et al*. (2012) and Ojezele *et al*. (2017) reported that Vitamin C co-administration with artemether/artesunate may

be counter productive, but Ekeh *et al*. (2019) reported enhanced activity of artemether antimalarial efficacy when it was combined with ascorbic acid and zinc.

Research that had assessed the effects of co-administration of Vitamin C with antimalarial drugs mainly evaluated percentage parasitaemia and some biochemical parameters in the serum of animal model. There is a need for more investigations especially at genetic levels to ensure clarity as well as to contribute current and reliable data to existing information on ascorbic acid concurrent administration with ACTs. Basically, the novelty of this research is the measurement of the level of expressions of antioxidant enzyme genes (catalase gene, glutathione peroxidase gene and CuZn-superoxide dismutase gene) that are dependent on NRF2 (nuclear erythroid 2-related factor 2) signaling pathway in the bone marrow cells of *P. berghei* infected mice. Therefore, we examined the changes in antioxidant gene expression profiles in the bone marrow cells and alterations in biochemical indices in *P. berghei* infected mice treated with artemether-lumefantrine and ascorbic acid supplementation.

MATERIALS AND METHODS

Drugs and Chemicals: The RPMI (Roswell Park Memorial Institute) 1640 was obtained from Lonza Pharma and Biotech, Belgium and methanol was gotten from Sigma-Aldrich, Germany. Other reagents used were of analytical grade.

Experimental Animals: A total of thirty (30) Swiss albino mice, all male weighing 18.5 – 22.5 g were used for the study. The experimental animals were bred and supplied by the Nigerian Institute of Medical Research (NIMR), Lagos State, Nigeria. The mice were housed in a well-ventilated metal cage and catered for at a temperature of 22 ± 1°C and relative humidity of 60 % in a 12-hour light/dark cycle, with food and water provided *ad libitum*. The animals were acclimatized for one week before the commencement of the experiment. The experimental protocol received ethical approval from the Institutional Ethics Review Committee of Michael and Cecilia Ibru University (Ethics Approval Number: MCIU/ETH/20/06) in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 (United Kingdom, 1986) and associated guidelines, EU Directive 2010/63/EU for animal experiments (European Commission, 2010).

Parasites and Inoculation: *P. berghei* NK65 (chloroquine sensitive) maintained in mice was obtained from the National Institute of Medical Research (NIMR), Lagos, Nigeria. Blood from a single donor mouse with parasitaemia 25% was obtained through cardiac puncture into heparinized tube and was diluted with isotonic saline solution. Aliquot of 0.2 mL containing standard inoculums of 1×10^7 *P. berghei* infected erythrocytes was administered intraperitoneally to each mouse where necessary. Parasitaemia was estimated by making a thin blood smear obtained from the periphery of the cut mice tail. The samples were stained with a 10% solution of Giemsa stain in phosphate buffer (pH 7.2) and the slides were read with a light microscope under ×100 magnification with oil immersion. The percentage parasitaemia for each animal was determined by counting the number of red blood cells infected with *P. berghei* and uninfected and final estimation was done using the formula: % Parasitaemia = Number of parasitized erythrocytes / Total number of erythrocytes x 100.

Dosage of Artemether-lumefantrine and Ascorbic Acid: Drugs were dissolved in 0.2 ml of the vehicle after appropriate calculation using the mice's body weights in line with OECD (2023) guidelines. Dosage (mg) = [Average weight of mice (g) × Dose (mg)] / 1000 g.

Experimental Design: The Rand () function in Microsoft Excel was used to randomly distribute thirty (30) male Swiss albino mice into 5 groups of 6 mice each. On the first day of the experiment (termed 'day 0'), all mice were injected intraperitoneally with standard inoculums of *P. berghei containing* 1×10^7 infected erythrocytes except for the animals in the control group. Treatment commenced

seventy-two (72) hours post-infection for the experimental animals. Treatment was done orally twice daily for three consecutive days. Percentage parasitaemia level was monitored daily before and after treatment by thin blood film microscopic examination. The experimental plan of this study is shown in Table 1.

Sample Collection: Mice were fasted overnight (12 hours), thereafter; they were anaesthetized with chloroform vapour for the collection of blood through the aorta, 24 hours post-drug treatment. The Serum was obtained from blood samples by centrifugation at 2000 rpm for 10 minutes. Potter-Elvehjem homogenizer was used to homogenize I g of harvested organs (liver and kidney) in 5 mL of ice-cold 0.1 M phosphate buffer. The supernatant obtained was centrifuged at 12,000 g for 15 minutes at 4°C to obtain tissue homogenates.

Biochemical Assays: Using Randox test kits the serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were estimated following the method of Reitman and Frankel (1957). Albumin and total protein levels were determined according to the method of Grant *et al*. (1987) and Tietz (1995) respectively. Agappe diagnostic test kits were used to estimate total bilirubin (Walter and Gerard, 1970), direct bilirubin (Aman *et al*., 2007), urea (Weatherburn, 1967) and creatinine (Allen and Michalko, 1982). For the antioxidant status and levels of oxidative stress assessment, Misra and Fridovich (1972) method was used to estimate superoxide dismutase (SOD) activity, Buege and Aust (1978) method was used to determine the level of malondialdehyde (MDA) formed and the protocol of Wolff (1994) was used to deduce the level hydrogen peroxide (H_2O_2) present.

Gene Expression Study

Harvesting bone marrow of mice: Bone marrow cells were harvested from the femurs by flushing out the marrow with RPMI 1640 supplemented with 10% fetal bovine serum using a 26-gauge needle into a sterile Eppendorf tube. After pelleting the bone marrow of the mice by centrifugation it was resuspended in RNA*later* (Sigma-Aldrich Germany) before RNA isolation.

Total RNA isolation, cDNA conversion and PCR amplification: The isolation of total RNA, conversion of cDNA and its PCR amplifications, as well as agarose gel electrophoresis, were carried out using the method of Ebohon *et al*. (2020). Primer3 software was used to design the Primers targeting the coding segment junction of the gene (Ye *et al*., 2012). All oligonucleotide synthesis was done by Inqaba Biotec, Pretoria, South Africa. The primers for the genes of interest and loading control [catalase, superoxide dismutase-1 (CuZn-SOD), glutathione peroxidase-1 (GPx-1) and β-Actin] were presented in Table 2. In-gel amplicon bands images captured on camera were processed on Keynote platform. Gel density quantification was done using Image-J software (Rueden *et al*., 2017). Each point represented relative expression [(test gene band intensity/ internal control band intensity) x 100] plotted using Numbers Software (Mac OSX Version).

Statistical Analysis: Statistical package for social science (SPSS) for Windows, Version 16.0 (SPSS Inc., Chicago, IL, USA) was used to carry out all statistical analysis. The results obtained were expressed as Mean ± SEM. One way analysis of variance (ANOVA) was used to determine significant differences between the groups and post hoc multiple comparison test was done using Tukey's HSD (honest significant difference). Statistical significance was declared when P value was less than 0.05.

RESULTS

Percentage Parasitaemia of *Plasmodium berghei* Infected Mice Pre- and Post-Treatment with Artemether-Lumefantrine, Ascorbic Acid and Their Combinations: The group infected with *P. berghei* without treatment showed a progressive increase in mean percentage parasitaemia.

Table 1: Experimental design of *Plasmodium berghei* infected mice treated with ascorbic acid, artemether-lumefantrine and their combination

Groups	Experimental plan
Basal control	The uninfected group received orally 0.2 mL of vehicle (olive oil) two times daily for three days in succession.
Untreated	The group was infected with *P. berghei* and received orally 0.2 mL of vehicle two times daily for three days in succession.
Ascorbic acid	The group infected with *P. berghei,* received an oral dose of 2.73 mg/kg of ascorbic acid in 0.2 mL of distilled water twice daily for three days in succession.
Artemether-lumefantrine	The group was infected with *P. berghei*, treated with an oral dose of 1.45 mg/kg of artemether and 8.73 mg/kg of lumefantrine in 0.2 mL of the vehicle twice daily for three days in succession.
Artemether-lumefantrine and Ascorbic acid	The group was infected with *P. berghei* and co-administered artemether (1.45 mg/kg) lumefantrine (8.73 mg/kg) with ascorbic acid (2.73 mg/kg) twice daily for three days in succession.

Table 2: Primer Sequences used in the molecular study of *Plasmodium berghei* infected mice treated with ascorbic acid, artemether-lumefantrine and their combination

Target genes	Accession numbers	Primer sequences	Temperature (°C)	Amplicon Size (bp)
Catalase	NM_012520.2	Forward (5'-3'): TCACCTGAAGGACCCTGACA Reverse (5'-3'): TCCATCTGGAATCCCTCGGT	55	103
CuZn-SOD	NM_017050.1	Forward (5'-3'): TTTTGCTCTCCCAGGTTCCG Reverse (5'-3'): GGTTCACCGCTTGCCTTCT	60	133
GPx-1	NM_030826.4	Forward (5'-3'): ATCAGTTCGGACATCAGGAGA Reverse (5'-3'): TCACCATTCACCTCGCACTT	59	124
β-Actin	NM_031144.3	Forward (5'-3'): CTGGCTCCTAGCACCATGAA Reverse (5'-3'): CGCAGCTCAGTAACAGTCCG	61	192

CuZn-SOD = Superoxide dismutase -1, GPx-1 = Glutathione peroxidase – 1

There was an initial decrease in the group administered only ascorbic acid on day 2 but a surge in mean parasitaemia was seen on day 3 during treatment. Complete parasite clearance occurred on day 2 post-infection of the group administered artemether-lumefantrine alone. Although, the group administered artemether-lumefantrine and ascorbic acid combination had a similar pattern with lone artemether-lumefantrine but total elimination of the parasite from their blood was observed day 3 during treatment (Figure 1).

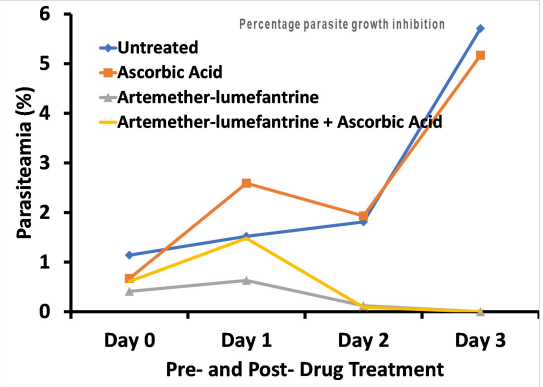

Figure 1: Effects of ascorbic acid, artemether-lumefantrine, and their combination on parasite clearance in *Plasmodium berghei* infected mice

Alteration in Body Weight and Organ-To-Body Weight Ratios of *Plasmodium berghei* Infected Mice Treated with Ascorbic Acid, Artemether-Lumefantrine and Their Combination: The *P. berghei* infected mice administered only ascorbic acid had an increase in the liver-to-body weight and spleen-to-body weight ratios compared with untreated mice. Organ body-to-weight ratio of artemether-lumefantrine and its combination with ascorbic acid treated groups were comparable with the control (Table 3).

Changes in Liver and Kidney Function Parameters of *Plasmodium berghei* Infected Mice Treated with Ascorbic Acid, Artemether-Lumefantrine and Their Combination: The mean levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, total protein, total bilirubin, direct bilirubin, creatinine and urea for artemether-lumefantrine plus Vitamin C were not significantly different

(p>0.05) from the artemether-lumefantrine group (Table 4). Although, there was a non-significant decrease in direct bilirubin, and an increase in albumin in the group administered artemether-lumefantrine plus ascorbic acid compared with the mice treated with artemether-lumefantrine alone. Also, the urea and creatinine concentration increase significantly in the untreated group compared to lone artemisinin-lumefantrine treatment and its combination with ascorbic acid (Table 4).

Changes in SOD Activity, MDA and H_2O_2 Levels in the Liver, Kidney and Erythrocyte Lysate of *Plasmodium berghei* Infected Mice Treated with Ascorbic Acid, Artemether-Lumefantrine and Their Combination: The group treated with artemether-lumefantrine plus ascorbic acid had a non-significant increase (p>0.05) in SOD activity of the liver and erythrocyte lysate when compared with the lone artemether-lumefantrine group (Table 4). A significant decrease (p<0.05) was observed in the MDA concentration and H_2O_2 level of the liver in *P. berghei* infected mice treated with artemeter-lumefantrine plus ascorbic acid when compared with lone artemether-lumefantrine (Table 4). This same trend was observed for the H_2O_2 level in the erythrocyte lysate.

Changes in Gene Expression of Catalase, Glutathione Peroxidase (GPx-1), and Cu, Zn-Superoxide Dismutase (CuZn-SOD) Enzymes in the Bone Marrow Cells of *Plasmodium berghei Infected* Mice Treated with Ascorbic Acid, Artemether-Lumefantrine and Their Combination: In Figures 2a, b and c, the genes coding for catalase, Cu, Zn-superoxide dismutase (CuZn-SOD) and glutathione peroxidase (GPx-1) in the untreated group were down-regulated compared with the basal control and the other treatment groups. The levels of expression of catalase mRNA, CuZn-SOD mRNA and GPx-1 mRNA in the *P. berghei* infected mice treated with artemether-lumefantrine plus Vitamin C were highly up-regulated compared with the group treated with lone artemether-lumefantrine.

DISCUSSION

In the present study, evidence that the artemisinin-based combination therapy was effective for the treatment of malaria and the role of Vitamin C co-administration was provided. Also, information on changes in animal body weight and organ to body weight ratio as well as changes in biochemical indices and gene expression in *P. berghei* infection in experimental animals were also given.

The observed increase in mean parasite count in the untreated group and *P. berghei* infected mice treated with only ascorbic acid in the experimental animals has been established in previous research (Ganiyu *et al*., 2012; Ojezele *et al*., 2017). Though the exact mechanism by which ascorbic acid promoted rapid growth of *P. berghei* is not clear. However, Hallberg *et al*. (1989) showed that Vitamin C could increase the availability of Fe^{2+} for the development of the ring and trophozoit stages of *P. falciparum* into schizont (Marva *et al*., 1992). The total parasite clearance observed in lone artemether-lumefantrine treated group was expected because it is an established antimalarial drug whose efficacy is known (Elhassan *et al*., 1993). However, very few parasites were seen in the erythrocytes of mice treated with artemether-lumefantrine combined with ascorbic acid, but these parasites' counts were insignificant and could not survive day 3 during treatment. These data are in agreement with previous research in which total parasite clearance was reported in *P. berghei* infected mice treated with either artemether or artesunate co-administered with ascorbic acid (Ganiyu *et al*., 2012; Ojezele *et al*., 2017).

The co-administration of ascorbic acid with artemether-lumefantrine may be a good adjunct therapy for the control of plasmodiasis because the *P. berghei* infected mice placed on this remedy did not show a deleterious increase in the organ-to-body weight ratio and the result obtained was similar to lone artemether-lumefantrine treatment. Several studies have shown splenomegaly and hepatomegaly in mice with untreated malaria infection (Adachi *et al*., 2001).

Table 3: Changes in mean body weight and the organ-to-body weight ratio of controls and *Plasmodium berghei* infected mice treated with ascorbic acid, artemether-lumefantrine, and their combination

Parameters	Basal Control	Untreated	Ascorbic acid	Artemether-lumefantrine	Artemether-lumefantrine + ascorbic acid
Body weight Pre-infection	25.60 ± 1.22	24.90 ± 1.70	25.10 ± 0.99	25.70 ± 1.49	28.30 ± 0.60
Body weight Post infection	26.50 ± 0.73	22.44 ± 1.64	20.63 ± 1.10	22.50 ± 2.57	24.25 ± 0.75
Liver/body weight	0.049 ± 0.002	0.090 ± 0.003	0.087 ± 0.010	0.058 ± 0.005	0.062 ± 0.003
Spleen/body weight	0.010 ± 0.001	0.025 ± 0.002	0.027 ± 0.002	0.014 ± 0.002	0.021 ± 0.001
Kidney/body weight	0.016 ± 0.001	0.016 ± 0.001	0.016 ± 0.000	0.015 ± 0.002	0.015 ± 0.000

Data represent mean ± SEM (n = 5/group), Means were not significant (p>0.05)

Table 4: Changes in liver function parameters, kidney function indices and antioxidants status of control and *Plasmodium berghei* infected mice treated with ascorbic acid, artemether-lumefantrine and their combination

Parameters	Basal Control	Untreated	Ascorbic acid	Artemether-lumefantrine	Artemether-lumefantrine + ascorbic acid
ALT (U/L)	10.16 ± 1.05^{a}	30.9 ± 6.30^{b}	9.80 ± 2.10^{a}	9.32 ± 0.72^{a}	10.35 ± 0.69^{a}
AST(U/L)	84.52 ± 1.18^{a}	104.54 ± 4.38^{b}	96.46 ± 6.25^{ab}	93.19 ± 4.24^{ab}	92.61 ± 5.55^{ab}
Total Protein (g/dL)	2.65 ± 0.03^{a}	3.07 ± 0.21^{ab}	3.61 ± 0.06^{ab}	3.20 ± 0.37^{ab}	3.79 ± 0.45^{b}
Albumin (g/dL)	1.41 ± 0.07^{a}	1.28 ± 0.02^{a}	2.37 ± 0.18^{b}	2.28 ± 0.11^{b}	2.31 ± 0.19^{b}
Total bilirubin (mg/dL)	0.19 ± 0.03^{a}	0.54 ± 0.02^{c}	0.45 ± 0.04^{b}	0.23 ± 0.02^{a}	0.26 ± 0.02^{a}
Direct bilirubin (mg/dL)	0.12 ± 0.04^{a}	0.43 ± 0.03^{b}	0.19 ± 0.02^{a}	0.18 ± 0.03^{a}	0.17 ± 0.04^{a}
Urea (mg/dL)	11.62 ± 0.41^{ab}	16.07 ± 1.20^{c}	12.75 ± 1.81^{bc}	8.13 ± 1.54^{a}	9.79 ± 0.76^{ab}
Creatinine (mg/dL)	0.35 ± 0.00^{ab}	0.46 ± 0.07^{c}	0.32 ± 0.03^{ab}	0.24 ± 0.11^{a}	0.29 ± 0.03^{ab}
SOD Liver (unit/g protein)	0.21 ± 0.02^{b}	0.12 ± 0.02^{a}	0.16 ± 0.04^{a}	0.17 ± 0.01^{a}	0.18 ± 0.01^{a}
SOD kidney (unit/g protein)	0.70 ± 0.06^{b}	0.32 ± 0.05^{a}	0.70 ± 0.04^{b}	0.57 ± 0.018^{ac}	0.40 ± 0.05^{a}
SOD RBC (unit/g protein)	0.11 ± 0.00^{a}	0.03 ± 0.00^{a}	0.14 ± 0.01^{a}	0.17 ± 0.00^{a}	0.18 ± 0.00^{a}
MDA Liver (Mole/mg protein)	0.31 ± 0.06^{a}	0.34 ± 0.01^{a}	0.30 ± 0.07^{a}	0.55 ± 0.12^{b}	0.38 ± 0.10^{a}
MDA Kidney (Mole/mg protein)	0.95 ± 0.31^{a}	1.08 ± 0.06^{a}	0.75 ± 0.09^{a}	1.08 ± 0.44^{a}	0.68 ± 0.13^{a}
MDA RBC (MDA/mg protein)	0.13 ± 0.00^{a}	0.20 ± 0.00^{b}	0.14 ± 0.01^{ab}	0.17 ± 0.01^{ab}	0.17 ± 0.00^{ab}
H_2O_2 liver (µM)	6.46 ± 0.33^{a}	10.10 ± 0.29^{b}	6.53 ± 0.28^{a}	8.26 ± 0.67^{b}	6.69 ± 0.17^{a}
H_2O_2 kidney (µM)	2.27 ± 1.22^{a}	4.74 ± 0.68^{b}	2.82 ± 0.23^{a}	4.85 ± 0.26^{b}	4.94 ± 0.17^{b}
H_2O_2 RBC (µM)	4.15 ± 0.14^{a}	5.86 ± 0.49^{b}	5.69 ± 0.13^{b}	5.30 ± 0.03^{b}	3.73 ± 0.13^{a}

Data represent mean ± SEM (n = 5/group). Values in the same row with different lowercase letters represent a significant difference between means at p<0.05. AST=aspartate aminotransferase; ALT=alanine aminotransferase; SOD = superoxide dismutase; MDA = malondialdehyde

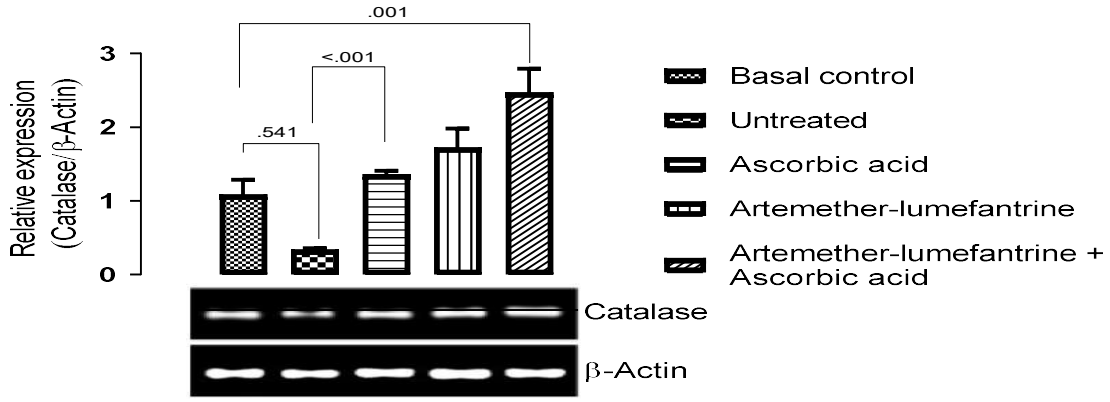

Figure 2a: Response of catalase gene expression in bone marrow cells of *Plasmodium berghei* infected mice to ascorbic acid, artemether-lumefantrine and their combination treatment represented with densitometry analysis and mRNA gel image of catalase gene. ***Key:*** *β-Actin was used as the loading control, Values are mean ± SEM, n = 5 mice/group*

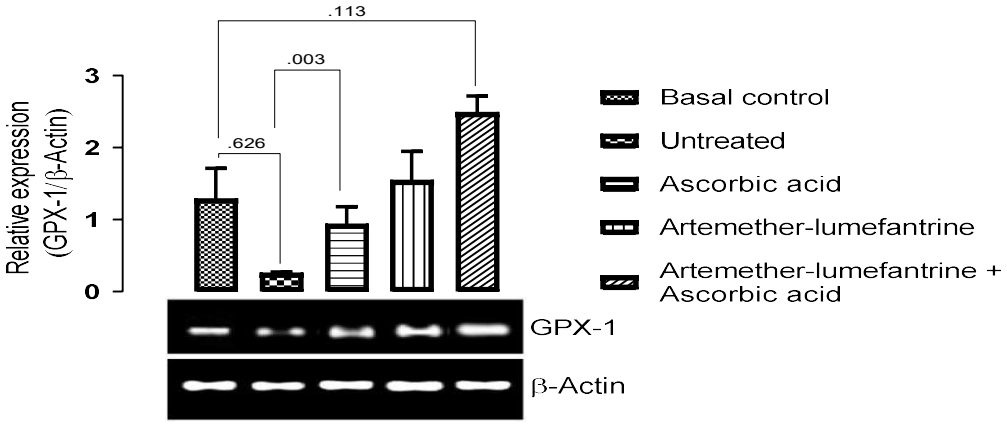

Figure 2b: Response of glutathione peroxidase (GPx-1) gene expression in bone marrow cells of *Plasmodium berghei* infected mice to ascorbic acid, artemether-lumefantrine and their combination treatment represented with densitometry analysis and mRNA gel image of GPx-1 gene. ***Key:*** *β-Actin was used as the loading control, Values are mean ± SEM, n = 5 mice/group*

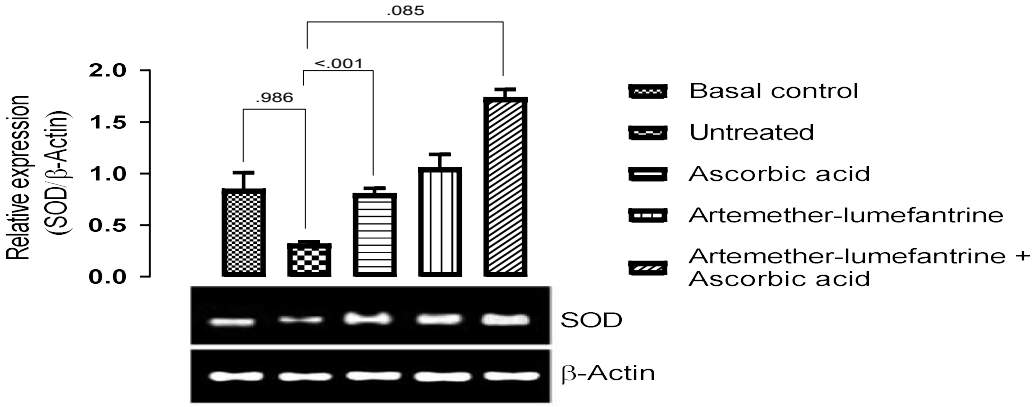

Figure 2c: Response of CuZn-superoxide dismutase (CuZn-SOD) gene expression in bone marrow cells of *Plasmodium berghei* infected mice to ascorbic acid, artemether-lumefantrine and their combination treatment represented with densitometry analysis and mRNA gel image of CuZn-SOD gene. ***Key:*** *β-Actin was used as the loading control, Values are mean ± SEM, n = 5 mice/group*

This enlargement has been linked to inflammatory stimuli caused by *Plasmodium* parasite and its haemozoin pigment accumulation in the respective tissues (Vanderberg and Frevert, 2004).

The normal activity of AST and ALT in all the treated groups especially the mice administered a combination of artemether-lumefantrine, and ascorbic acid suggested that there was both parasite clearance and quenching of free radicals in the experimental animals. However, the normal levels of ALT and AST activity recorded for ascorbic acid treated *P. berghei* infected mice are associated with the antioxidant ability of Vitamin C in scavenging ROS generated by the parasite and the natural immune response of the host rather than its ability to cause parasite inhibition since there was a significant increase in parasitaemia (Wang *et al*., 2015; Ojezele *et al*., 2017). The increase in the total protein estimated for all the *P. berghei* infected mice whether treated or untreated could have been caused by inflammatory responses of the kidney and liver as well as haemolysis of the red blood cells (Roman *et al*., 2009; Iyawe and Onigbinde 2010; Adebayo *et al*., 2018). The non-significant decrease in direct bilirubin and an increase in albumin in the group administered artemether-lumefantrine plus ascorbic acid compared with the mice treated with artemether-lumefantrine alone suggest protective effects of ascorbic acid in concurrent administration with the antimalarial drug used in this investigation. The benefit of ascorbic acid co-administration with antimalarial drug may be its effective scavenging of free radicals that surge in malaria infection; these reactive oxygen species are known to be deleterious to the synthetic function of the liver and the membrane of red blood cells (Ojezele *et al*., 2017).

In this investigation, the observed increase in urea and creatinine in untreated mice infected with *P. berghei* has earlier been reported (Somsak *et al*., 2013). It was proposed that elevation in kidney function parameters in serum during malaria infection is related to renal injury caused by the adhesion of parasite-infected red blood cells to the endothelial surface of the kidney and aggressive immunological response to oxidative haemozoin pigment and other metabolic products associated with parasite infection (Elias *et al*., 2012). This interaction causes modification in the renal vascular endothelial thereby decreasing oxygen supply to its cells (Clark and Cowden, 1999) resulting in an overall decrease in its excretory function and significant rise in serum urea and creatinine. The decrease in serum urea and creatinine in treated groups may be either due to the inhibition of ROS generation (Al-Obaidi and Taylor-Robinson, 2017) or suppression and elimination of parasite or a combination of both (Elias *et al*., 2012).

The destruction of infected erythrocytes and the release of haemozoin pigment could have contributed to the observed oxidative stress in untreated *P. berghei* infected mice. This result conformed to an increase in free radicals in the parasitized untreated mice reported by Iyawe and Onigbinde (2009) after they investigated the impact of *P. berghei* and chloroquine on the haematological and antioxidants indices in mice. The digestion of haemoglobin in the acidic food vacuole of the parasite releases haematin which is a pro-oxidant that generates ROS like superoxide radical ($^{\bullet}O^{-}_{2}$) that requires cellular SOD to dismutate it to hydrogen peroxide which is then reduced to molecular oxygen and water by catalase (Arinola *et al*., 2008). In untreated malaria infection, the system experiences an in balance between free radical concentration and antioxidant status due to continuous bombardment of the system with ROS produced from haemoglobin degradation by the parasite and exacerbated immunological response.

As seen in this study, an excess of ROS generated over the physiological limit resulted in the depletion of cellular antioxidant (SOD) and an increase in the serum and tissue malondialdehyde levels as well as H_2O_2 concentration in untreated mice. However, the infected mice treated with artemether-lumefantrine plus ascorbic acid had a non-significant increase in SOD activity in the liver compared to the grouped administered lone artemether drug. In addition, infected mice treated with combined therapy had a significant decrease in MDA in the liver and a reduction in

H_2O_2 concentration in both the liver and erythrocyte lysate respectively compared to the infected mice treated with lone antimalarial drug. Previous literature report (Ojezele *et al.*, 2017) agreed with the outcome of this investigation and the decrease in MDA and H_2O_2 concentrations observed could have been the capacity of the exogenous antioxidant (ascorbic acid) to act synergistically with reduced glutathione to mop up free radicals (Hallberg *et al.*, 1989; Ojezele *et al.*, 2017). This suggests that ascorbic acid may confer a homeostatic advantage on tissues during plasmodiasis when it is used to supplement antimalarial drugs (artemether-lumefantrine).

In malaria infection the levels of ROS and RNS exceed their physiological thresholds (Al-Obaidi and Taylor-Robinson, 2017) causing oxidative stress. This free radical imbalance may have been responsible for the down-regulation in the expression of genes coding for the first-line antioxidants (CuZn-SOD, catalase and GPx-1) seen in untreated *P. berghei* infected mice. Currently, there exist limited research perspectives on the reason for the up-regulation in the expression of genes coding for these enzymes after treatment with therapeutic doses of Vitamin C, artemether-lumefantrine and their combination. We observed more increase in the expression of the genes coding for CuZn-SOD, catalase and GPx-1 in the artemether plus Vitamin C treated mice compared with lone artemether group. The increase in their expressions could be associated with the antioxidant action of Vitamin C (Xu *et al.*, 2020) acting in synergy with artemether-lumefantrine to up-regulate a large battery of antioxidant enzymes through the activation of NRF2/NQO1/HO-1 pathway (Liu *et al.*, 2018; Xu *et al.*, 2020). Nuclear erythroid 2-related factor 2 (Nrf2) is transcription factor that interacts with antioxidant response element (ARE) and thereby regulates the genetic expression of cellular antioxidants like SOD, catalase, GPx, quinone oxidoreductase 1 (NQO1) and heme oxidase-1 (HO-1) (Petri *et al.*, 2012). Our observation correlated with the investigation of Xu *et al*. (2020) who reported marked elevation in Nrf2 mRNA and protein with enhanced binding of quinone oxidoreductase 1 and heme oxidase-1 loci of antioxidant response element leading to increase production of other cellular antioxidants in a rat model suffering from oxidative stress injury and treated with a high dose of Vitamin C.

Conclusion: The potency of artemether-lumefantrine was not compromised when combined with ascorbic acid at a therapeutic dose to treat malaria infection in mice. Furthermore, artemether-lumefantrine co-administration with ascorbic acid caused a decrease in oxidative stress parameters and up-regulated the expression of antioxidant genes.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance from the laboratory staff of Michael and Cecilia Ibru University and technical support from Dr. I. O. Omotuyi.

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