Original Article

Oral administered ascorbic acid attenuated dihydroartemisinin anti-plasmodial activity and elicited hepatic injury in *Plasmodium berghei* strain Anka infected mice

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ABSTRACT

Objective: This study investigated the effects of oral co-administered ascorbic acid (AA) and dihydroartemisinin (DHA) on some hepatotoxic biomarkers and parasitaemia counts in *Plasmodium berghei* Anka strain infected mice for 7 d.

Methods: Twenty four male Swiss albino mice were randomly distributed into six groups; group I: "non-parasitized and non-treated"(nPnT), group II: "parasitized and non-treated"(PnT), group III: parasitized mice administered 5 mg/kg DHA, group IV: parasitized mice administered 5 mg/kg AA, group V: parasitized mice co-administered 5 mg/kg DHA+ 5 mg/kg AA and group VI: parasitized mice administered 25 mg/kg chloroquine (CQ) as standard.

Results: Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were significantly (P<0.05) reduced in AA, DHA and CQ treated mice compared with PnT mice respectively. In addition, parasitized mice co-treated with DHA+AA had elevated plasma ALT and AST activities compared with DHA treated mice. Further investigation showed that parasite count/μl blood in PnT, AA and AA+DHA were not significantly (P>0.05) different. However, DHA and CQ treated mice had significantly reduced parasite count/μl blood at P<0.05 and 0.01 compared to PnT.

Conclusion: Thus, data from this study indicated that the AA interferes with the anti-plasmodial activity of DHA and this condition could predispose the plasmodium infected individual to hepatic injury.

KEY WORDS: Ascorbic acid; dihydroartemisinin, *Plasmodium berghei*; hepatotoxicity

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INTRODUCTION

Malaria is an endemic public health challenge that is predominantly widespread in tropical and subtropical regions of the world particularly Sub-Saharan Africa [1]. In 2015, there were 214 million cases and 438 thousand deaths resulting from malaria. In addition, about 3.2 billion people are at risk of malaria infection [2]. This was partly due to increased incidence of multidrug resistant *Plasmodium falciparum* [3]. In response to this menace, World Health Organization recommended the use of artemisinin based combination therapy (ACT) with the advantage to rapidly reduce parasite biomass, rapid resolution of clinical symptoms and effective action against multidrug resistant *P. falciparum*. In addition, ACT reduces the rate of malaria transmission. Artemisinin-based therapy has significantly reduced mortality, particularly for children with severe malaria (>30%). Currently, overall world death rate from malaria during the past 15 years had reduced by 15% [4].

Despite this progress, some data had shown emerging resistance against artemisinin drugs [5]. This could partly be due to inadequate understanding of the mechanism of action of artemisinin and its derivatives. Artemisinin is a sesquiterpene lactones derived from the herb *Artemisia annua* Linn. Clarification of the chemical structure of the parent drug (artemisinin) led to the development of its semisynthetic derivatives including arteether, artesunate

and artemether with the sole purpose to enhance bioactivity of artemisinin [6]. Semisynthetic derivatives of artemisinin are metabolized by hepatic cytochrome P450 3A4/5 into dihydroartemisinin (DHA), which is highly toxic against *P. falciparum* [7]. Currently, DHA is now being chemically produced in large scale as a semi-synthetic derivative of artemisinin due to poor oral bioavailability of artemisinin [8].

The exact mechanism of action of artemisinin derivatives had remain unknown, however some evidence point to Fenton-type reaction, generation of reactive oxygen species, alteration of mitochondrial membranes and carbon centered radical molecules that modify proteins of plasmodium parasite [9,10]. Some studies had also shown that artemisinin derivatives possess anti-cancer property [7].

In malaria endemic regions, antimalarial drugs including chloroquine and dihydroartemisinin are often prescribed as monotherapy or in combined therapy by medical practitioners alongside other drugs including folic acid and vitamin supplements. This practice is not without the attendant cases of malaria recrudescence among malaria parasite-infected patients. Previous studies showed that intraperitoneal co-administration of artemisinin and vitamins, may affect the pharmacodynamics of artemisinin [11]. Another study showed evidence of counteractive

effect of intraperitoneal administration of lipid soluble artemether and water soluble ascorbic acid on the parasite clearance in infected mice [12]. However, the effect of ascorbic acid on the anti-plasmodial activity of dihydroartemisinin remains to be clearly shown. Therefore, this study was designed to evaluate the influence of orally co-administered ascorbic acid and dihydroartemisinin on some liver function biomarkers and parasite clearance in mice infected with *P. berghei* Anka strain.

MATERIALS AND METHODS

Animals

Twenty four male Swiss albino mice weighing between 15 - 22 g were obtained from the National Institute of Medical Research (NIMR), Yaba, Lagos, Nigeria. Mice were assigned into experimental groups, housed in propylene cages and maintained under standard laboratory conditions (25 ± 2°C) with dark / light cycle to acclimatize for 14 d. Animals were given commercial pellets (Pfizer Livestock Feeds, Lagos Nigeria) and distilled water. Animals were humanely handled in conformity with the standard operating procedures prescribed by the National Research Council [13] and ethical approval was obtained from the Babcock University Health, Research Ethics Committee.

Parasite and mice inoculation

NK65 strain of *Plasmodium berghei* was obtained from Malaria Laboratory, Department of Biochemistry, NIMR, Yaba, Lagos. Mice were inoculated intraperitoneally with 0.1 ml infected blood containing approximately 1×10^7 *P. berghei* parasitized red blood cells obtained from a donor mouse with 60% parasitemia count. Thin blood films were made by collecting blood from the tip of the mice tail 72 h after initial passaging. Subsequently, obtained blood was stained with Giemsa and the percentage of parasitized red blood cells determined by thin blood film microscopic examinations repeatedly every day for 7 d.

Estimation of parasitemia count

Daily blood samples collected from mice tails were placed on a glass side to prepare a smear which was subsequently stained with 3% Giemsa. To every smear, 3 ml Giemsa solution was added and allowed to stain the slides for 45 min and subsequently rinsed off with distilled water and viewed under the electron microscope to estimate the parasitaemia count. The entire smear was first screened at low magnification to detect suitable fields with even distribution of white blood cells after which they were viewed at high magnification. *P. berghei* parasites were counted per 200 leukocytes, which were used to estimate the parasite density per microliter of blood.

Parasitemia density (parasites/μl blood) =

$$\frac{\text{Number of parasites}}{200 \text{ leukocytes}} \times 8000$$

Chemicals

DHA, chloroquine-phosphate (CQ) and ascorbic acids were purchased from Sigma Aldrich Chemical Co., USA. All other chemicals and reagents used were of analytical grade.

Experimental design

Animals were assigned randomly into six groups of four animals each in line with the recommendations of the Babcock University Health, Research Ethics Committee. Study design was as follows:

Group I: served as normal group orally administered with 0.9% NaCl which were "non-parasitized and non-treated" (nPnT) mice.

Group II: served as control group orally administered with 0.9% NaCl which were "parasitized non-treated" (PnT) mice.

Group III: parasitized mice were orally administered with 5 mg/kg DHA in 0.9% NaCl.

Group IV: parasitized mice were orally administered with 5 mg/kg AA in 0.9% NaCl.

Group V: parasitized mice were co-administered 5 mg/kg DHA in 0.9% NaCl + 5 mg/kg AA in 0.9% NaCl.

Group VI: served as standard group: mice were administered with 25 mg/kg CQ in 0.9% NaCl.

Freshly prepared AA, DHA and CQ solutions were administered to mice via oral administration for 7 d. Mice were fasted overnight at the end of treatment which was followed by anesthesia of mice with petroleum ether and subsequently the mice were sacrificed and blood samples collected for biochemical analysis.

Collection of blood

Blood samples were obtained via cardiac puncture at the mice heart left ventricle using 2 ml sterile needle and immediately transferred into clean and sterile lithium heparin bottles to prevent clotting. The blood samples were immediately centrifuged at 3000 rpm for 10 min to separate plasma from the blood cells. The obtained plasma was used to determine alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities using Reitman and Frankel [14] spectrophotometric methods in accordance with Randox diagnostic kit assays.

Statistical analysis

Statistical analysis was performed with the aid of SPSS for Windows (Version 17.0) while chart were designed using Microsoft Excel version 2013. Mean difference was calculated using One Way Analysis of Variance (ANOVA). Data with $P<0.05$, 0.01 and 0.001 were considered significant. All results were reported as mean ± SEM of triplicate readings

RESULTS

Data in Fig. 1 showed that the plasma ALT (92 ± 1.40 U/L) and AST (103 ± 2.28 U/L) activities were significantly (P<0.01) elevated in PnT mice compared with ALT (21 ± 6.18 U/L) and AST (61 ± 16.59 U/L) in nPnT animal. DHA treated parasitized mice had significantly (P<0.05) reduced plasma ALT (29 ± 6.50 U/L) and AST (77 ± 9.89 U/L) activities compared with PnT mice. Chloroquine treated mice had significantly (P<0.01) decreased plasma ALT (18 ± 1.41 U/L) and AST (60 ± 2.12 U/L) activities compared with PnT mice. Furthermore, parasitized mice treated with AA had significantly (P<0.05) reduced plasma ALT (67 ± 2.82 U/L) and AST (91 ± 2.12 U/L) activities when compared with PnT mice. However, parasitized mice co-treated with DHA+AA had significantly (P<0.05) elevated plasma ALT (68 ± 4.50 U/L) and AST (89 ± 8.18 U/L) activities compared with DHA treated parasitized mice.
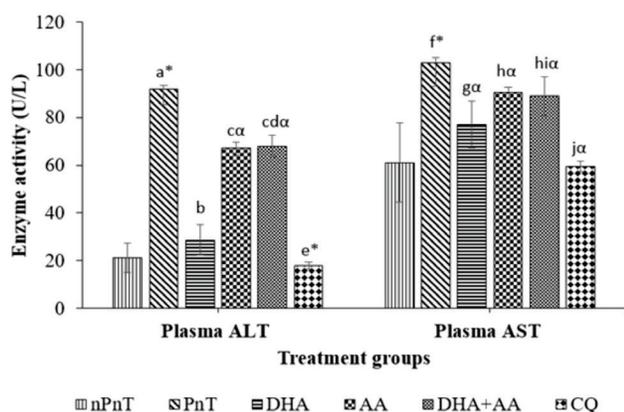

Fig 1. Effects of orally administered dihydroartemisinin (DHA), ascorbic acid (AA) and chloroquine (CQ) on plasma alanine aminotransferases (ALT) and aspartate aminotransferases (AST) activities in *plasmodium berghei*-infected mice in 7 days. Different symbols indicates significantly different at *P<0.01 and $^{\alpha}$P<0.05 when compared with PnT.
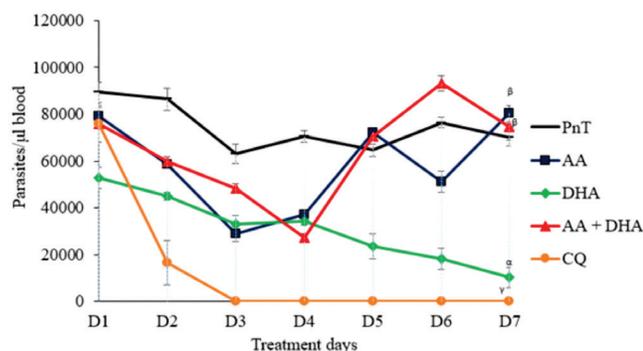

Fig 2. Effects of oral administered dihydroartemisinin (DHA), ascorbic acid (AA) and chloroquine (CQ) on parasitaemia count of *Plasmodium berghei*-infected mice in 7 days. D1-D7-indicated days 1 to 7. Different symbols indicates significantly different at $^{\gamma}$P<0.001; $^{\alpha\gamma}$P<0.01 and $^{\alpha\gamma}$P<0.05 when compared with PnT.

Data in Fig. 2 showed that there were no significant (P>0.05) difference in the blood parasite count when AA (80 ± 34.00 ×10⁻³ parasites/µl) and AA+DHA (75 ± 0.00 ×10⁻³ parasites/µl) treated parasitized mice compared with PnT (70 ± 3.70 ×10⁻³ parasites/µl) mice after 7 d. However, DHA (10 ± 43.50 ×10⁻³ parasites/µl) treated mice had significantly reduced parasite count/µl blood at P<0.05 and 0.01 when compared with PnT after 7 d. CQ (0 ± 0.00 ×10⁻³ parasites/µl) treated mice had significantly reduced parasites count/µl blood at P<0.05, 0.01 and 0.001 compared with PnT after 7d. In addition, CQ treated mice exhibited a complete clearance of parasite/µl blood from the 3rd to 7th d.

DISCUSSION

This present study showed that untreated mice infected with *P. berghei* had elevated plasma ALT and AST activities compared with normal uninfected mice and infected mice treated with anti-malarial DHA and CQ. Previous study had reported that elevated plasma ALT and AST activities could serve as an indicator of hepatotoxicity [15]. This might be due to non-clearance of parasites in mice associated with increased plasma ALT and AST activities and leakage of these enzymes from injured hepatocytes by autoimmune defense system and/or by abnormal cell activation induced by the plasmodium parasites [16].

In addition, reduction in plasma ALT and AST activities in DHA and CQ treated *P. berghei* infected-mice might be due to the anti-plasmodial activity of the test drugs. Previous study had shown that DHA anti-plasmodial mechanism action could be through CYP3A4/5 biotransformation of its endoperoxide bridge into a reactive epoxide which disrupt the parasites extracellular and intracellular membranes resulting into clearance of parasite from the host [17] while CQ anti-plasmodial mechanism of action might be through the inhibitions of heme polymerase activity within the parasite's food vacuole with a resultant parasite death [18].

Furthermore, the reduction in plasma ALT and AST activities in parasitized mice treated with AA compared with PnT mice, might be due to the protective role of AA as an antioxidant against the deleterious effects of free radicals generated by the activity of the plasmodium parasites on hepatocytes. AA is a known water soluble free radical scavenger that protect biological tissues against the deleterious effects of reactive species [19]. This seems to support the hypothesis which suggest that high administration of AA might suppress the rate of progression of malaria parasite in infected mice [20].

However, parasitized mice co-treated with DHA+AA had elevated plasma ALT and AST activities compared with DHA treated parasitized mice. It was hypothesized that AA might have neutralized or mopped up the free radicals generated by DHA meant to be toxic to the plasmodium parasite.

Further study showed that the mean parasitemia count from *P. berghei*-infected mice co-treated with AA+DHA which was similar to those in PnT and AA treated *P. berghei*-infected mice when compared with mean parasitemia count from DHA and CQ treated *P. berghei*-infected mice. This seems to support the previous observation that AA could interfere with the antiplasmodial activity of DHA *in vivo*. More so, this observation was in agreement with previous work performed by Meshnick et al. [11] where it was reported that antioxidant vitamins such as α-tocopherol and ascorbate interfered with the antimalarial activity of artesunate and artemether. Therefore, this study showed that the oral administration of AA could antagonize the anti-plasmodial activity of DHA against *P. berghei*-infected mice. It further indicated that the administration AA and co-treatment with AA+DHA could elicit liver injury in individuals infected with plasmodium parasite

ACKNOWLEDGEMENTS

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