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Potentiation of Antimalarial Activity of Chloroquine by Chlorpheniramine in Plasmodium berghei-Infected Mice

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ABSTRACT

The resistance of malaria parasite to the cheapest and most available antimalarial, chloroquine, has limited its use. In this study, chlorpheniramine was combined with chloroquine in order to enhance the susceptibility of plasmodium to chloroquine. Using Swiss albino mice, *in vivo* antimalarial curative activity was determined with chloroquine (14 mg/kg for the first two days, and 7 mg/kg on the third day) alone or in combination with chlorpheniramine, (0.057 or 0.029 mg/kg). The effect of such combination on the haematopoetic and hepatic systems were also evaluated. The parasite reduction by the combination of chloroquine and 0.057 mg/kg chlorpheniramine was significantly greater (94.5%) than chloroquine given alone (43.9%) at $P<0.05$. This combination was safe on the haematopoetic system. There were indications of deleterious effect on the liver when Chloroquine or chlorpheniramine was used alone, whereas the combination showed safety. In conclusion, chloroquine and chlorpheniramine combination showed high synergy of antimalarial activity which is dose-dependent; this combination can be a cost effective, clinically efficacious alternative to ACT's.

KEYWORDS: *Antimalarial activity*; *Chloroquine, Chlorpheniramine, Mice, Potentiation, Safety*

INTRODUCTION

Chloroquine has remained the cheapest of the antimalarial therapies [1] and was designated the drug of choice in 1946 [2]. But, resistance of the malaria parasite to this drug has limited its use. The current trend in malaria treatment is the use of combination therapy, especially as artemisinin-based combination therapies (ACTs) to curb drug resistance. But the affordability and accessibility of the ACTs to rural dwellers in sub-Saharan Africa has remained a problem even with government interventions [3]. The first case of resistance to chloroquine was reported in 1963, in Malaysia [4]. However, the first case in Africa was reported in 1978 [5, 6]. It spread to a lot of areas in less than 5 years. Chloroquine resistance is associated with a decrease in the amount of chloroquine that accumulates in the food vacuole, the site of action for chloroquine. The mechanism for this decreased accumulation is controversial. Some

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studies suggest that the decrease in drug accumulation is due to an increase in drug efflux [7], whereas other studies suggest that diminished levels of chloroquine accumulation are more important [8].
However, resistant genes responsible for chloroquine resistance, and that of other antimalarial drugs have been identified as *pfcrt* and *pfmdr* respectively. The protein *pfcrt* codes for is located in the food vacuole. There are several mutations in the *pfcrt* gene with mutations in codon 76 being the most predominant [7]. These mutations may affect the accumulation of chloroquine in the food vacuole, but the exact mechanism of chloroquine resistance remains elusive. Perhaps, resistance did not occur due to mutations to one gene at a time but to several genes because chloroquine resistance arose relatively few times and then subsequently spread [9].
There are several methods used in evaluating the antimalarial activity of a compound, including; *in vitro* and *in vivo* methods. One of the *in vivo* methods, the Rane's test evaluates antimalarial activity on already established plasmodium infection. Here, the infection must be allowed to reach high parasitemia before the treatment is administered for the number of days it is meant for.
Data on enhancement of antimalarial effect of chloroquine by chlorpheniramine in animals are few. Also, previous work by Sowunmi and Oduola [10] did not consider the effect of the combination on haematopoietic system and the liver. Chloroquine is very affordable antimalarial especially in sub-Saharan Africa. Development of new antimalarial drugs may turn out to be a costly venture that will fashion out new drugs that are not only costly, but to which resistance will develop in no time. In a view to try and maintain our existing antimalarial drug stock, we have to emphasize resistance reversal. The present study thus demonstrates the ability of chlorpheniramine to enhance the activity of chloroquine in plasmodium using the Rane's test, and to assess the effect of such combinations on the haematopoietic system and the liver. This enhancement of activity will help in curbing resistance.

MATERIALS AND METHODS

Experimental animals and parasite used

Male and female (non-pregnant) Swiss albino mice, 8weeks of age, weighing 25 ±5 g, bred in the Animal House of College of Veterinary Medicine, University of Nigeria, Nsukka (UNN) were used in this study. They were kept in the Experimental Animal House of the Department of Pharmacology, for acclimatization prior to the study, where they were given standard mice pellet diet (Ladokun Farms, Nigeria) and water ad libitum, and observed under 12 h light/dark cycles in a well-ventilated rodent cage. The animals were handled according to the guidelines approved by the Animal Ethical Committee, Nnamdi Azikiwe University, Awka, Nigeria. The animals were randomly placed into the various experimental groups, after a 7 days period of acclimatization.

The parasite used was a chloroquine-sensitive strain of *Plasmodium berghei* NK 65 (Plasmodiidae), maintained in mice, from the Department of Pharmaceutics, University of Nigeria, Nsukka. The parasite was then subsequently passaged into fresh mice, which served as donor mice in this study.

Drugs used

The Chloroquine phosphate and chlopheniramine maleate tablets used in this study were generous gifts from Rabana, Nigeria. They were reconstituted in water-for-injection to provide doses of 7 and 14 mg/kg body weight of chloroquine (CQ) and 0.057 (normal dose) and 0.029 (half dose) mg/kg body weight of chloropheniramine(CP), just prior to any of the tests.

Test for in vivo antimalarial activity against chloroquine-sensitive *Plasmodium berghei*

This was carried out using the Rane's test as described by Ryley and Peter with some modifications [11]. Four days before treatment, the parasitemia and red blood cell (RBC) count of the donor mice were determined by using a Giemsa-stained thin blood smear and an improved Neubauer counting chamber, respectively. The blood was then collected by cardiac puncture and diluted with phosphate buffered saline to give a concentration of 5×10^6 parasitized RBC per ml of suspension. A 0.2 ml volume of the cell suspension was injected intraperitoneally into each experimental mouse. The mice were randomly shared into six groups of five mice each. Their parasitemia was monitored daily by determining the parasitemia from thin blood smears of the mice stained with 10% Giemsa solution and then microscopic viewing microscopically and counting parasitized RBC from ten fields of approximately 100 erythrocytes per field.
Treatments were initiated when about 20% parasitaemia was established in infected mice, which

was approximately 96 hrs post infection on day 0, instead of 72 hrs later in the normal Rane test. The negative control group was given sterile distilled water daily for 3 days. Equal volumes of the drug solution were administered orally once daily for 3 days. One group was administered CQ 14 mg/kg daily for two days and 7 mg/kg on day 3. Another group was given the same doses of CQ but in combination with 0.057 mg/kg CP for the three days. Yet, another group was given the same dose of CQ but in combination with 0.029 mg/kg CP at each time for the three days. The last group was given 0.057 mg/kg CP alone for 3 days. One group was uninfected and untreated and served as the positive control. 24 h after each treatment thin blood smears of all the test mice were stained with10% Giemsa solution used to determine the percentage parasitemia microscopically, before the treatment for that day was administered. Three (3) slides were made per mice for viewing. The mean percentage reduction in baseline parasitemia or antimalarial activity for each treatment group was calculated from the formula;

$$Activity = 100 - \left[\frac{mean\ parasitemia\ in\ treated\ group}{mean\ baseline\ parasitemia\ in\ that\ group} \times \frac{100}{1}\right]$$

Haematological Parameters

The haematological parameters determined include: Percentage Packed Cell Volume (% PCV), Haemoglobin, and Erythrocyte and Leukocyte counts and the determination were according to the methods described by Cheesbrough [12]. The haematological parameters of each mouse were determined before infection and on day 4 after treatment, i.e., a day after the last treatment was administered. For the PCV, blood sample was collected from the ocular orbit of each mouse with a heparinized capillary tube by capillary action. The tube was sealed with plasticine at one end. The sealed capillary tubes were then arranged on the haematocrit centrifuge and spun at 10,000 revolutions per minute for five minutes to separate the blood into plasma and packed cells. The %PCV was read on the haematocrit reader and recorded. The PCV of each mouse was then measured and calculated using the formula:

$$PVC = \left[\frac{Volume\ of\ erythrocytes\ in\ a\ given\ volume\ of\ blood}{Total\ blood\ volume\ examined} \times \frac{100}{1}\right]$$

The haemoglobin concentration was also determined. Here, 20µL of blood was taken and mixed with 4 ml of Drabkin solution in a test tube. The blood mixture was allowed to stand for 5 min, and then read with a colorimeter at 540 nm wavelength. The haemoglobin concentration was noted and recorded.

White blood cell counts were carried out using a haematocytometer. Here, 20 µL of blood was collected from each mouse in an EDTA bottle and mixed with 380 µL of Gower solution. The mixture was placed on a haematocytometer slide and viewed under a microscope with magnification of x40. The White Blood Cell (WBC) count was recorded.

The red blood cell counts were done using a haematocytometer. Here, 20µL of blood was collected from each mouse into an EDTA bottle mixed with 4ml of RBC diluting fluid in a test tube. The blood mixture was allowed for 10min, placed on a haematocytometer slide and viewed under a microscope with magnification of x40. The Red Blood Cell (RBC) count was recorded.

Biochemical Parameters

Here, blood was collected at the end of the experiment by cardiac puncture, after the animals had been anaesthesized, and dispensed into plain bottles. It was allowed to clot and centrifuged at 3500 rpm for 10 min and the clear sera aspirated off and stored at –4°C. The sera were later thawed a troom temperature and assayed for the level of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using the method of Reitman and Frankel [13].

Histopathology of the liver

Assessment of histopathological changes in the liver was carried out at the end of the experiment. Tissue sections of the liver from experimental animals were fixed in 10% formol saline at room temperature for 72 h and dehydrated in ascending grades of ethanol. Thereafter, the tissues were cleared in chloroform overnight, infiltrated and embedded in molten paraffin wax. The blocks were later trimmed and sectioned at 5-6 microns. The sections were deparaffinized in xylene, taken to water and subsequently stained with Haematoxylin and Eosin (H and E) for light microscopy.

Statistical Analysis

The results were expressed as mean± standard deviation (SD). Parameters in the groups were compared by one-way (ANOVA) using the computer Software Statistical Package for Social Sciences (SPSS) Version 16. All data was analyzed at a 95% confidence interval and values were considered statistically significant at $P<0.05$.

RESULTS & DISCUSSION

Antimalarial Results

Figure 1 shows the parasitemia counts in both the treated and untreated groups beginning from the first day of treatment, taken as day 0. The parasitemia of the untreated infected group increased as days went on and this is in agreement with previous studies [14, 15] where parasitemia will increase if there is no treatment until death. The parasitemia of the other groups decreased significantly ($P<0.05$) after each treatment including when chlorpheniramine was used alone for treatment. However, chloroquine alone produced a much more decrease in parasitemia level than chlorpheniramine alone. Also, the combination of chloroquine and 0.057 mg/kg chlorpheniramine produceda significantly lower parasitemia than chloroquine alone.

The results of mean percentage reduction in baseline parasitemia, i.e., the antimalarial activities of chloroquine alone, chlorpheniramine alone and the various combinations of the drugs are shown in Table 1. Table 1 indicates that the antimalarial activity on day 3 of chloroquine in combination with 0.05 mg/kg chlorpheniramine was significantly greater at $P<0.05$, than chloroquine alone. This finding is similar to a study carried out by Sowunmi and Oduola [10], who reported that a chloroquine and chlorpheniramine combination produced a significantly higher cure rate than chloroquine alone in African children with acute symptomatic uncomplicated falciparum malaria.

The enhanced antimalarial activity of chloroquine by chlorpheniramine shows synergy as reported in an in vitro study where chloroquine was combined with chlorpheniramine. Chlorpheniramine was found to have reversed the resistance of P. falciparum to chloroquine [16].

However, on the same day 3, chloroquine in combination with 0.029 mg/kg chlorpheniramine showed no significant difference ($P<0.05$) in percentage of baseline parasitaemia when compared with chloroquine alone.

Chlorpheniramine alone also demonstrated antiplasmodial activity. This corroborates with the work of Sunan and Phattanapog [16], who assayed chlorpheniramine for in vitro antimalarial activity against multidrug-resistant P. falciparum K1 strain and chloroquine-resistant P. falciparum T9/94 clone, by measuring the 3H-hypoxanthine incorporation and discovered that chlorphenirame inhibited P. falciparum K1 and T9/94 growth with IC50 values of 136.0 ± 40.2 µM and102.0 ± 22.6 µM respectively. Chlorpheniramine as an antihistamine prevents the degranulation of mast cells by membrane stabilization. The observed antimalarial activity may be due to membrane stabilization and prevention of efflux of haem which is toxic to the plasmodia.

Haematology

Haematological indices have been reported to be a reliable parameter for the assessment of the health status of animals [17-19]. Also, the severity of haematopoietic changes depends on the specie and physiological state of the host and acuteness or chronicity of the infection [20, 21].

Anaemia is usually assessed by evaluating packed cell volume, haemoglobin concentration and RBC. Haematological indices were considered in this study because the most pronounced changes related to malaria involve the blood and the blood-forming system. Anaemia is a fairly common problem encountered in malaria.

The haemoglobin and packed cell volume before treatment shows no significant difference ($P>0.05$) in all the groups of the animals, data not shown. This indicates that all the animals were of comparative physiological state before treatment.

Table 2: Effect of treatment on haematological parameters

Treatment	Haematological Parameters (%)			
	PCV	Hg	RBC	WBC
CQ	77.8 ± 4.2*	81.1 ± 5.6*	74.1 ± 6.3*	124.7 ± 19.1
CQ + 0.057 mg/kg CP	72.8 ± 3.2*	78.5 ± 3.5*	73.9 ± 10.0*	114.4 ± 9.4
CQ + 0.029 mg/kg CP	70.6 ± 3.1*	75.5 ± 2.1*	76.9 ± 7.5*	120.0 ± 21.5
0.057 mg/kg CP	82.5 ± 9.0*	73.2 ± 7.8*	89.4 ± 5.7*	122.6 ± 21.6
Infected untreated (Negative control)	45.5 ± 6.2	47.7 ± 4.1	41.8 ± 8.4	160.0 ± 10.7
Uninfected (Positive control)	100.0 ± 3.2**	100.0 ± 8.7*	100.0 ± 6.4*	100.0 ± 6.9*

*-significantly difference from negative control; **-significantly different from negative control and 0.057 mg/kg CP

Table 2 shows that percentage red blood cell was significantly higher in the positive control group (uninfected and untreated) compared to the negative control and the group given only chlorpheniramine. There was a significant decrease in red blood cell count in the negative control compared with positive control. The observed decreases may be due to multiple causes including haemolysis of infected red cells. The haemolysis may be due to non-immune destruction of parasitized red cells in case of high parasitaemia or immune mediated destruction of parasitized as well as non-parasitized red cells because the changes in the red cell antigen structure brought about by the parasitic invasion stimulate the production of antibodies against the red cell. There was a significant increase in percentage red blood cell of test groups compared to the negative control.

Furthermore, it was noted that groups I, II and III did not show significant ($P<0.05$) difference in percentage red blood cells compared to the positive control. This means that combination of chloroquine with chlorpheniramine did not cause further destruction of red blood cells.

Table 2 shows that the percentage haemoglobin for negative control group were significantly ($P<0.05$) lower compared to the positive control group. This is consistent with the anaemia seen in malaria [22]. Also, its haemoglobin level is significantly ($P<0.05$) lower compared to the test groups. This is because the administration of treatment to the animals killed the parasite and prevented further digestion of haemoglobin. But, there was no significant differences between the groups at $P<0.05$.

The percentage packed cell volume (PCV) of negative control decreased significantly ($P<0.05$) compared to all the other groups (Table 2). Packed Cell Volume is volume percentage (%) of red blood cells. Expectedly, the drop in PCV corresponds with the period of progressive increase in parasitaemia, which is not seen in the test group. Also, there was no significant ($P<0.05$) difference in PCV between groups I, II, III and IV. This shows that the combination had no effect on packed cell volume

Table 2 also shows that positive control group had significantly lower percentage of white blood cell compared to the negative control. Increase in WBC has been demonstrated to be linked to severe malaria [23]. The observed increase may result from stimulation of the immune system of the animals to fight the malaria parasites.

Biochemical Parameters

Table 3 shows the effect of the plasmodium infection and above treatment on liver enzymes: alanine aminotransferase and aspartate aminotransferase.

There was a significant ($P<0.05$) increase in the activities of ALT in the blood of the animals in all the other groups, except for group IV, compared to the positive control.

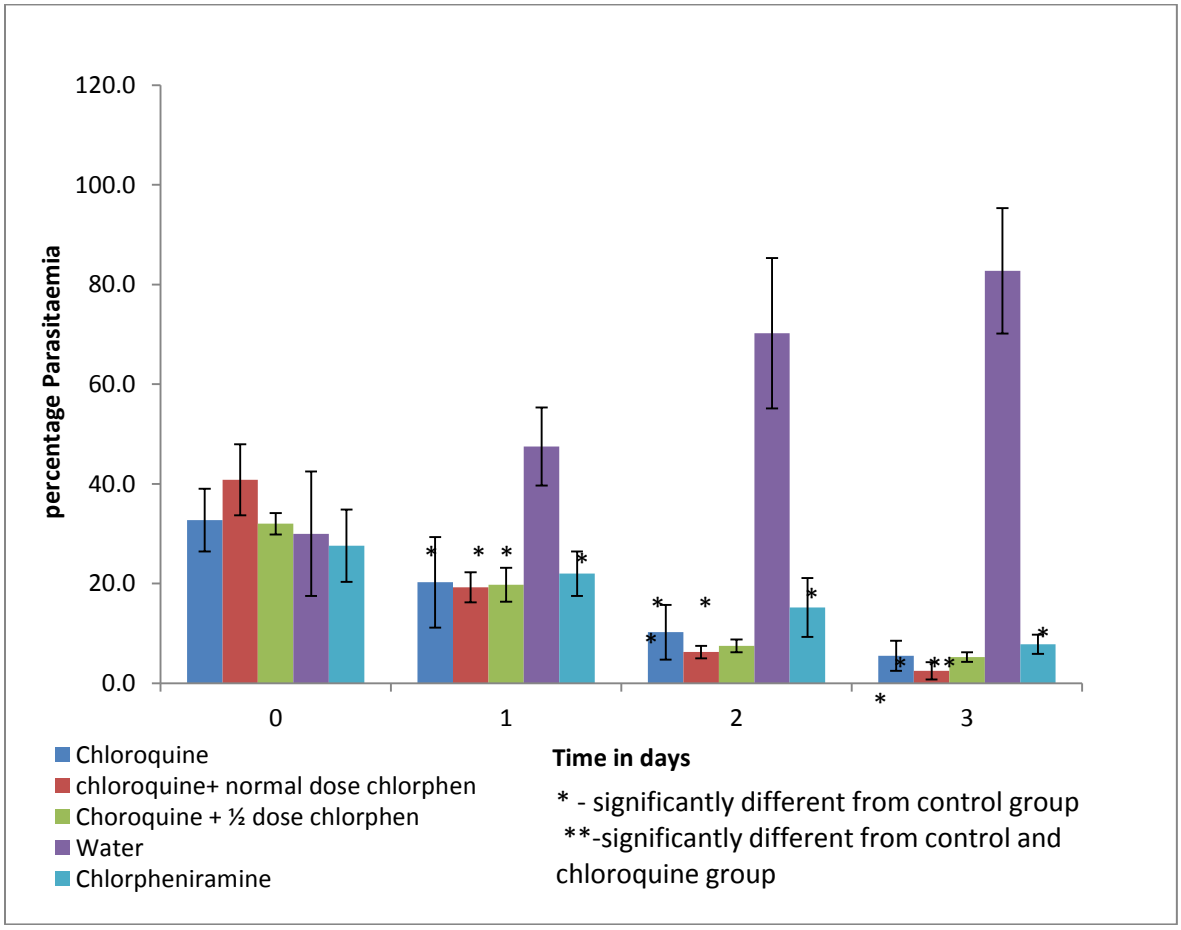

Figure 1: Effect of Treatment on Parasitemia of infected mice

Table 3: Effect of treatment on biochemical parameters

Treatment	Liver enzymes	
	% alanine aminotransferase	% aspartate aminotransferase
CQ	129.6 ± 6.9**	115.4 ± 13.6
CQ + 0.057 mg/kg CP	119.2 ± 5.6***	115.7 ± 20.1
CQ + 0.029 mg/kg CP	125.0 ± 11.3**	114.4 ± 29.2
0.057 mg/kg CP	131.0 ± 2.8**	127.9 ± 13.3
Infected untreated (Negative control)	217.9 ± 7.0 *	167.3 ± 30.7
Uninfected (Positive control)	100.0 ± 13.0	100.0 ± 8.6

*-significantly different from positive control
**-significantly different from positive control and negative control
***significantly different from negative control

This observed increase in ALT may be as a result of liver injury and altered hepatocyte integrity caused by the *Plasmodium* infection with the consequent release of the enzymes into the blood stream. Several studies have reported that liver injuries could occur as a complication of severe malaria [24, 25]. The increase in AST activities may be related to liver inflammation and is an indication of abnormal function of the liver. Both ALT and AST are normally present in the blood, but their blood level increases when there is organ damage, and liver cells are rich in these enzymes [26].

Furthermore, the ALT values for all the groups were significantly lower when compared to the negative control group. However, chloroquine in combination with the higher dose chlorpheniramine (0.057 mg/kg) showed no significant difference in ALT compared to the positive control group. This shows that chloroquine in combination with 0.057 mg/kg dose chlorpheniramine ameliorated the liver damage caused by the parasitaemia. Furthermore, the AST activities for the entire test groups were not significantly higher than the positive control. This showed that the administration of treatment ameliorated the liver damages as a result of the malaria parasitaemia.

Histopathological effects on the Liver

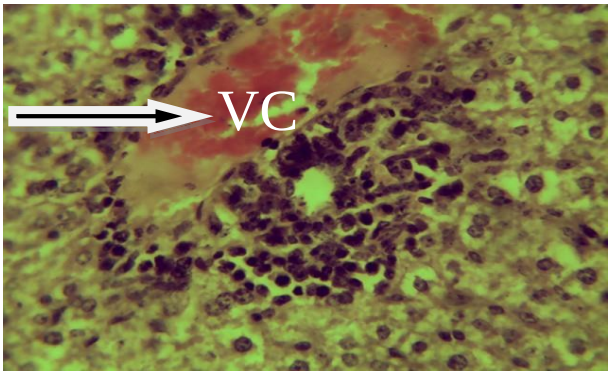

Figure 2: Liver section from group I animal (treated with chloroquine alone) showing periportal mononuclear cell infiltration (arrow) and vascular congestion (vc). H and E x 40.

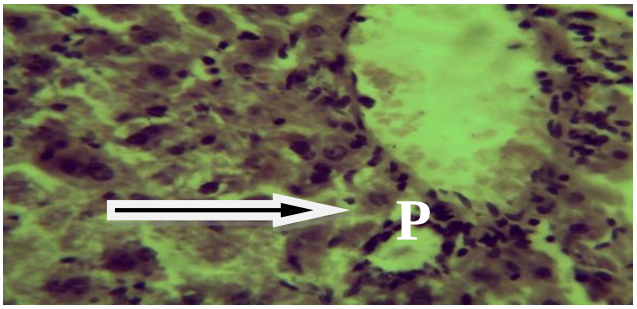

Figure 3: Liver section from group II animal (treated with chloroquine and 0.057 mg/kg (normal dose) chlorpheniramine showing the portal area (P) with no remarkable histological change.H and E x 40.

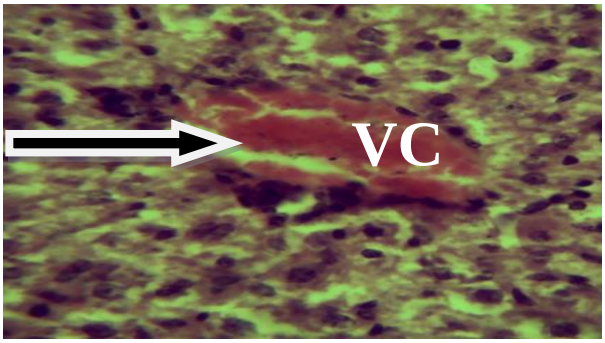

Figure 4: Liver section from group III animal (treated with chloroquine in combination with 0.029 mg/kg (half the dose) of chlorpheniramine) with no remarkable histological change except vascular congestion (vc) .H and E x 40.

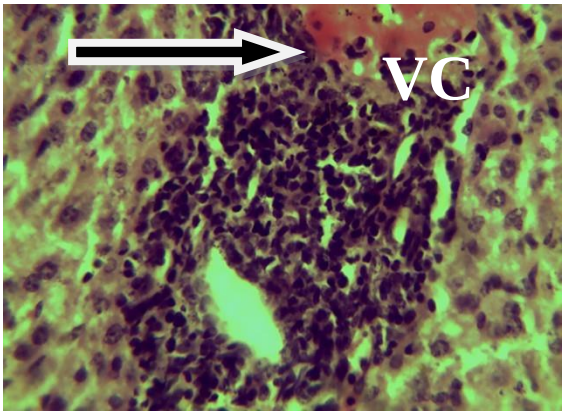

Figure 5: Liver section from group IV animal (infected and untreated) showing severe periportal mononuclear cell infiltration mainly lymphocytes(arrow) and vascular congestion (vc).H and E x 40.

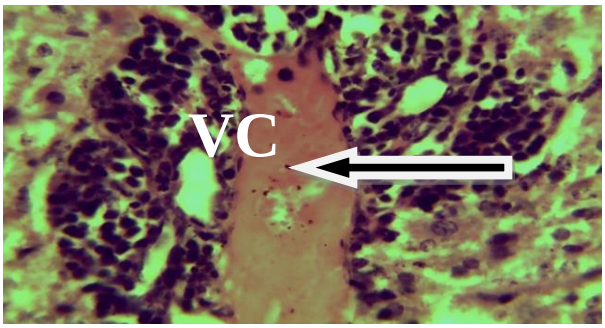

Figure 6: Liver section from group V animal (treated with 0.057mg/kg chlorpheniramine alone) showing periportal mononuclear cell infiltration (arrow) and vascular congestion (vc) .H and E x 40.

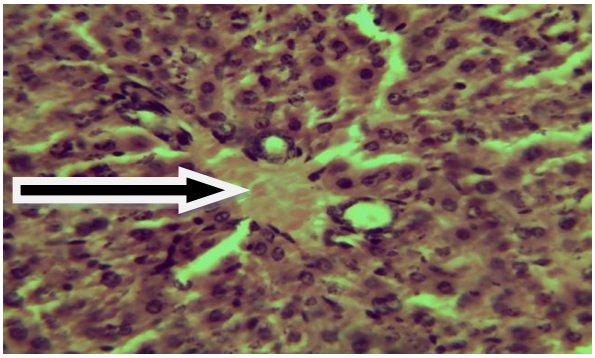

Figure 7: Liver section from group VI animal (uninfected untreated). No observable histological event is seen. H and E x 40.

Figures 2 to 7 show histological changes in the liver after treatment. Histopathology of the liver of group I, IV and V treated with chloroquine, positive control and chlorpheniramine respectively showed mononuclear cell infiltration and vascular congestion (Figure 2, 5 and 6). Chloroquine in combination with 0.057 mg/kg dose of chlorpheniramine showed vascular congestion with no remarkable histological change (figure 4). Mononuclear cell infiltration is observed in inflammatory reactions and in this case, the major cells were lymphocytes and plasma cells. Meanwhile, vascular congestion means increase blood flow to vessels within an area. The group that were not infected showed no observable histological change (figure 7) while group II treated with chloroquine in combination with 0.057 mg/kgdose chlorpheniramine showed no remarkable histological change (figure 3).

The clinical implication of vascular congestion is increase in the size of the liver while the implication of mononuclear cell infiltration is increase in white blood cells in the blood.

CONCLUSION

Chloroquine remains the regimen of choice in areas where affordability and accessibility to artemisinin combination therapy (ACT) is a constraint to adequate control of malaria. Restoration of the efficacy of chloroquine is of paramount importance as drug resistance has rendered it clinically ineffective. In this study, chlorpheniramine showed dose-dependent enhancement of the antiplasmodial activity of chloroquine and it also has antimalarial properties. Since chlorpheniramine is a safe therapeutic drug, the clinical use of chlorpheniramine as a cheap and highly effective combination with chloroquine holds great promise against malaria treatment. The haematological indices of such combination were similar to the results obtained with uninfected animals, indicating complete recovery of the infected mice and that such combination will likely not have an adverse effect on the haematopoetic system. Furthermore, histopathology revealed that indeed such combination protects the liver from the harmful effects of malaria infection and it will likely not cause any hepatic adverse effect.The combination of chlorpheniramine and chloroquine will likely overcome the resistance of the malaria parasite to chloroquine, thus restoring its clinical efficacy.

List of Abbreviations

ACT- artemisinine combination therapy
CQ- chloroquine
CP-Chlorpheniramine
PCV-Packed cell volume
RBC- Red blood cells
WBC-White blood cells
Hg- Haemoglobin
AST-aspartate aminotransferase
ALT-alanine aminotransferase

Competing interests

The author(s) declare that they have no competing interests.

Authors' Contributions

SCE carried out the antimalaria study, haematopoetic study, liver biochemistry study and histopathology of the liver. LOA performed the statistical analysis and wrote the first draft of the manuscript. UEO edited the draft. UEO, MUA and COE conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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