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Interactions between Retinol and Some Established Antimalarials in *Plasmodium yoelii nigeriensis* Infection in Mice

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Abstract: The aim of present study was to investigate the effect of retinol on the efficacy of chloroquine and dihydroartemisinin against *Plasmodium yoelii nigeriensis* infected mice. Sixty Swiss albino mice of either sex and average weight of 18-25 g were inoculated with *Plasmodium yoelii* and divided into 5 treatment groups: retinol alone, chloroquine alone, chloroquine and retinol, dihydroartemisinin alone, dihydroartemisinin and retinol and control group (retinol vehicle). Treatment was started on the fifth day (post inoculation) and continued for 5 consecutive days. The level of Malondialdehyde (MDA) in the treatment group was also measured to determine the extent of lipid peroxidation. The result of the study showed that retinol increased the antiplasmodial effect of chloroquine while antagonizing that of dihydroartemisinin. The lipid peroxidation assay also showed that retinol reduced the extent of oxidative stress when combined with dihydroartemisinin while not having any significant effect on lipid peroxidation when combined with chloroquine. The co-administration of retinol may enhance the activity of chloroquine but reduce the antimalarial potency of artemisinin.

Key words: Retinol, dihydroartemisinin, malaria, lipid peroxidation, *Plasmodium yoelii nigeriensis*, mice

INTRODUCTION

Vitamin A refers to a group of fat-soluble substances that are structurally related to and possess the biological activity of the parent substance of the group called retinoids Vitamin A is essential for normal immune function (Semba, 1994) and several studies suggest that it could play a role in potentiating resistance to malaria. (Serghides and Kain 2002; Shankar *et al*., 2000). In an *in vitro* study by Hamzah *et al*. (2004), direct antiplasmodial effect was confirmed across all stages of parasite development but at actual media retinol concentration that were above the range in normal human serum.

We have also shown *in vivo*, using malaria models in rodents that retinol possesses some degree of antiplasmodial activity and that toxic doses led to total parasite clearance (Oreagba and Ashorobi, 2006). Retinol supplementation in conventionally treated patients with malaria may, therefore hasten parasite clearance.

The World Health Organization (WHO) recommends the use of Artemisinin Combination Therapies (ACTs), as first line treatment of uncomplicated malaria (FMOH, 2005). When used in combination with other anti-malarial drugs, artemisinin is nearly 95% effective in curing malaria and the parasite is highly unlikely to become drug resistant (Meshnick *et al*., 1996). However, the companion drug must have a long half life to prevent recrudescence (McIntosh and Olliaro, 2000) Combination regimens have the advantage of a shorter duration of treatment, thus increasing the likelihood that patients will complete the entire course and be cured.

The major disadvantage of the existing ACTs is that of relatively high cost thus making it difficult for poor malaria endemic countries to have easy access to them (Mutabingwa, 2005). Dihydroartemisinin, a short acting artemisinin derivative, formulated for human use, is the main active metabolite of artesunate, artemether and arteether (Meshnick *et al*., 1996). Vitamin A, a fat soluble, long acting and relatively inexpensive retinoid (McEvoy, 1988), in combination with artemisinin, may thus prove to be very effective in malaria control.

An *in vitro* study on the activity of vitamin A in combination with artemisinin showed that retinol therapy may accelerate parasite clearance in quinine-treated patients but that the reverse may apply when retinol is combined with chloroquine or artemisinin (Skinner-Adams *et al*., 1999). A synergistic effect between retinol and artemisinin was however observed in a recent *in vitro* study (Thriemer *et al*., 2005) although this has not been verified in animal models.

This study therefore aims to evaluate the efficacy of retinol in combination with dihydroartemisinin against *Plasmodium yoelii* infection in mice and to compare this with its effect when combined with chloroquine.

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MATERIALS AND METHODS

This study was conducted between June and October 2006 at the Chemotherapy Research Laboratory, Department of Pharmacology and College of Medicine University of Lagos Lagos Nigeria.

Laboratory animals: All the mice employed in the course of this project were obtained from the Laboratory Animal Centre, College of Medicine, University of Lagos, Idi Araba, Lagos. Mice collected were of average weight between 18 and 25 g and only healthy mice were used. They were kept in a well ventilated environment, housed in standard cages and acclimatized for a period of ten days. They were maintained on standard pellets (Pfizer Livestock Feeds, Lagos Nigeria) and water *ad libitum.* The mice were divided into 5 treatment groups (A-E) of 10 mice per group and 1 control group (F).

Parasite: The NQ67 strain of *Plasmodium yoelii nigeriensis* used in this study was obtained from Dr. O.G. Ademowo's Research Laboratory, College of Medicine University of Ibadan Nigeria and maintained in mice by weekly passage. Each mouse was inoculated on day 0 intraperitoneally with 0.2 mL of infected blood containing about 1×10^7 *P. yoelii* parasitized red blood cells obtained from a donor mouse having about 60% parasitaemia.

Thin blood films were made by collecting blood from the tail and stained evenly to form a monolayer with the edge of another slide placed by the blood droplet at angle 45 degrees. The blood was allowed to dry and then fixed with methanol and left to dry before staining with Giemsa stain for 30 min, afterwards, the slide was rinsed and dried.

The percentage parasitaemia was determined by counting the number of parasitized erythrocytes out of 1000 erythrocytes in 10 random microscopic fields.

Drug administration: Retinol acetate (Sigma, USA) was dissolved in a vehicle consisting of Acetone-Tween 20-Water (0.25:5:4.75v v^{-1} v^{-1}) as described by Collins *et al.* (1992). Administration of drugs was through the oral route and with the aid of a stainless metallic feeding canular. All drugs were administered after regular morning feeding. Dihydroartemisinin and chloroquine (Emzor Pharmaceuticals Ltd.) were used as study drugs.

Procedure: Group A received chloroquine (5 mg kg^{-1} day^{-1}) alone, group B received chloroquine (5 mg kg^{-1} day^{-1}) and retinol (100 mg kg^{-1}). Group C received dihydroartemisinin (2 mg kg^{-1}) alone, group D received dihydroartemisiinin (2 mg kg^{-1}) and retinol (100 mg kg^{-1}), group E received retinol (100 mg kg^{-1}) and group F served as the control group (retinol vehicle). For groups B and D, retinol was administered prior to the second drug.

The chemotherapeutic efficacy of the different combinations on established *Plasmodium yoelii nigeriensis* infection was determined using the modified method similar to that by Ryley and Peters (1970). The average suppression of parasitaemia in each group was calculated in comparison to control on day 9. The percentage parasitaemia was determined subsequently on alternate days throughout the study period.

Assay of lipid peroxidation: Pooled blood from the animals was assayed for lipid peroxidation by determining their Malondialdehyde (MDA) levels according to the protocol outlined by Gutteridge and Wilkins (1982).The data obtained was quantified using a molar extinction.

Coefficient of 1.56×10 M cm^{-1} and expressed as MDA μM mL^{-1} (Buege and Aust, 1978).

Statistical analysis: All data generated are reported as means±SEM and differences between group data were determined by student t-test. One-way analysis of variance (ANOVA) and F-test computations were further employed to determine the significance of the variations between and within treatment groups. All p-values less than 0.05 were considered to be statistically significant.

RESULTS

Figure 1 shows the effect of retinol, chloroquine, chloroquine/retinol, dihydroartemisiinin, dihydroartemisinin/chloroquine and control on established malaria infection. Retinol demonstrated mild anti-malarial activity *in vivo* in monotherapy. Dihydroartemisinin/retinol combination caused a delay in parasite clearance compared with dihydroartemisinin alone as a result of reduced chemosuppression (76.47% versus 82.35%) on day 4 of curative treatment (Table 1) Also, peak percentage parasitaemia was higher in the dihydroartemisinin/retinol group compared with the dihydroartemisinin only group. However chloroquine/retinol and chloroquine reduced parasitemia level by 72.06 and 59.12%, respectively. The chemosuppressive effect of chloroquine/retinol combination was significantly higher ($p<0.05$) than that of chloroquine alone (Table 1). Also, peak percentage parasitaemia was lower in the chloroquine/retinol group compared with the chloroquine only group. In the control group, there was a sustained increase in parasitaemia until the animals eventually died.

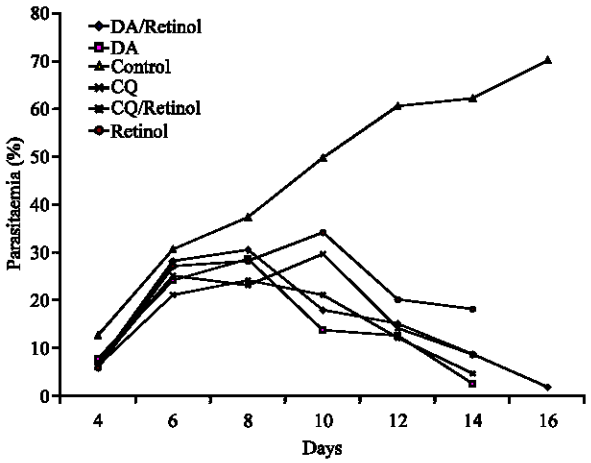

Fig. 1: Effect of retinol on the efficacy of CQ and DA during *P. yoelii* infection in mice. DA/Retinol-Dihydroartemisinin (2 mg kg^{-1}) + Retinol 100 mg kg^{-1}. DA: Dihydroartemisinin (2 mg kg^{-1}), CQ: Chloroquine 5 mg kg^{-1}, CQ/Retinol: Chloroquine 5 mg kg^{-1} + Retinol 100 mg kg^{-1} and Control: Inert Vehicle used to dissolve Retinol

Table 1: Mean parasitaemia levels of *P. yoelii* infection after four days of treatment with retinol and study antimalarials

Drugs	Dose (mg kg^{-1} day^{-1})	Average parasitaemia* (%)	Average suppression (%)
Retinol	100	42.0±0.04	38.24
Retinol/Chloroquine	100/5.0	19.0±0.06	72.06
Chloroquine	5.0	27.8±0.02	59.12
Retinol/DA	100/2.0	16.0±0.03	76.47
DA	2.0	12.0±0.01	82.35
Control** (Retinol vehicle)		68.0±0.02	-
One-way ANOVA	F	7.45	
	p<0.05		

Data expressed as mean±SEM for 10 animals per group df 3, 36; *p<0.05 when compared to control; **Equal volume (0.1 mL) of drug and control vehicle was administered; DA: Dihydroartemisinin (2 mg kg^{-1})

Table 2: Lipid peroxidant Levels in *P. yoelii* infected mice treated with retinol and study antimalarials

Treatment group	MDA (μM mL^{-1})
Dihydroartemisinin + Retinol	1.34±0.16*
Dihydroartemisinin (DA)	2.50±0.05
Chloroquine (CQ)	2.60±0.23
Chloroquine + retinol	2.76±0.40†
Control	5.40±0.45

Data expressed as mean±SEM for 10 animals per group; *p<0.05 compared to DA; †p>0.05 compared to CQ; MDA: Malondialdehyde

Two animals died on day 11, 4 died on day 13 while the remaining 4 died on day 17. No death was recorded in the treatment groups. The lipid peroxidation assay showed that retinol, when combined with dihydroartemisinin reduced MDA levels from 2.50 for the dihydroartemisinin only group to 1.34 for the dihydroartemisinin/retinol group (Table 2) indicating reduced extent of lipid peroxidation. There was however a slight increase in the extent of lipid peroxidation when retinol was combined with chloroquine. The MDA levels were 2.60 and 2.76, respectively for the chloroquine only group and the chloroquine/retinol group.

DISCUSSION

This study is a follow up on our earlier findings (Oreagba and Ashorobi, 2006) on the efficacy of retinol in rodent malaria models. Since drug combinations are being advocated to slow down the emergence of drug resistance in the malaria parasite, retinol was tested as a potential complement to the antimalarial activity of dihydroartemisinin and chloroquine. Probable mechanisms for these interactions were also investigated.

The mild anti-malarial activity demonstrated by retinol monotherapy in this study has already been confirmed in previous *in vivo* (Oreagba and Ashorobi, 2006) and *in vitro* (Hamzah *et al*., 2004) studies.

Present findings reveal that retinol reduced the efficacy of dihydroartemisinin, thus suggesting some form of pharmacological antagonism. Previous *in vitro* studies have shown conflicting activities of retinol on the efficacy of artemisinin derivatives. One study (Skinner Adams *et al*., 1999) which aligns with ours, suggest that retinol supplementation could reduce the efficacy of artemisinin derivatives including dihydroartemisinin by virtue of its antagonistic activity when the combinations were assessed against *P. falciparum* clone 3D7 for their effect on hypoxanthine incorporation. The antioxidant property of retinol was suggested as a possible mechanism for its antagonistic interaction. Antioxidant vitamins (such as α-tocopherol and ascorbate) and antioxidant enzymes (such as catalase and reduced glutathione) have been shown to block antimalarial activity of artemisinin derivatives (Meshnick *et al*., 1989). Conversely, α-tocopherol deficiency enhances the antimalarial action of artemisinin against *P. yoelii* in mice (Levander *et al*., 1989) and inhibitors of endogenous antioxidants promote artemisinin action (Kamchonwongpaisan *et al*., 1992).

Thriemer *et al*. (2005) demonstrated a synergistic interaction between retinol and artemisinin. They used the WHO *in vitro* micro technique for the assessment of the response of *P. falciparum* to antimalarial drugs (WHO, 1990) which measures drug dependent inhibition of schizont maturation. Lower drug concentrations in the combination were required to inhibit schizont maturation indicating synergism between the two drugs. It was hypothesized that retinol may slow down the transformation of artemisinin to a free radical in a reaction

catalyzed by iron (Meshnick, 1994) and thus extend its active half life. This may eventually increase overall parasite clearance.

Another probable mechanism for the synergistic interaction between retinol and artemisinin arise from the observation that the *in vitro* antimalarial activities of artemisinin and artesunate are enhanced by high oxygen tension and by the addition of other free-radical-generating compounds, such as Doxorubicin, Miconazole (Krungkrai and Yuthavong, 1987), Castecin and Artemitin (Elford *et al*., 1987).

Retinol, like ascorbate and other antioxidant vitamins have been shown to act as a pro-oxidant in some situations (Marva *et al*., 1992) thereby complementing the oxidative stress generated by artemisinin. This might also be responsible for the enhanced antimalarial activity of chloroquine/retinol compared to chloroquine monotherapy as observed in this study. Chloroquine is a weakly basic compound that has been shown to accumulate in the food vacuole. In this location it is thought to interact with toxic haem (ferri/ferroprotoporphyrin IX-FP IX) which is released upon haemoglobin digestion and prevent its detoxification. The build-up of FP IX enhances the toxicity of the ROS thereby killing the parasites (Loria *et al*., 1999).

Therefore the pro-oxidant effect of retinol further enhances the antiplasmodial action of chloroquine This mechanism is consistent with our observation that the chloroquine/retinol combination produced higher lipid peroxidant levels compared to the chloroquine only group although this was not statistically significant.

This study has shown that, *in vivo*, retinol impairs the antiplasmodial activity of dihydroartemisinin against *P. yoelii* infection, probably by protecting the malaria target cells against lipid peroxidation. However it enhanced the efficacy of chloroquine. This finding may have important implications for malaria control especially in developing countries. Studies to further highlight the mechanisms involved would be beneficial.

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