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Plasmodium berghei: efficacy and safety of combinations of chloroquine and promethazine in chloroquine resistant infections in Gravid mice.

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Summary

Efficacy and safety of combinations of Chloroquine (CQ) and doses of Promethazine (PR) against CQ resistant *Plasmodium berghei* infections in gravid mice was evaluated. Parasites were cleared faster in mice treated with CQ combined with doses of PR ranging from 20mg/kg to 50mg/kg (3.4 ± 0.5 to 2.7± 0.7) compared with CQ alone (4.7 ± 0.8) (P<0.5). Parturition resulting in live pups in animals treated with CQ and 20mg/kg and 30mg/kg of PR (81%) was significantly higher than in animals treated with CQ alone (44%) or saline (13%). Mean birth weight of pups delivered by infected gravid animals treated with CQ and 30mg/kg or 40mg/kg of PR (1.51± 0.16 or 1.56 ± 0.16) was significantly higher than animals treated with CQ alone (1.33±0.13) (P=0.00004, 0.0014 respectively). No gross malformations were observed in pups delivered by infected or non-infected animals treated with the combinations of chloroquine and Promethazine.

Keywords: *Plasmodium berghei, malaria, resistance, chloroquine, promethazine and Gravid mice*

Résumé

L'éfficacité et la protection de la combinaison chloroquine (CQ) et different doses de promethazine(PR) contre les infections de la chloroquine résistant *plasmodium berghei* étaient évaluées. Les parasites étaient elliminés plus rapidement aux groupes des souris traités avec la combinaison de la chloroquine avec des doses de promethazine variant de 20mg/kg a 50mg/kg (3.4 ± 0.5 to 2.7 ± 0.7) comparé avec la chloroquine uniquement (4.7 ± 0.8) (P<0.5). La parturition des souris traitées a la CQ et 20mg/kg et 30mg/kg de PR (81%) était significativement plus elevé qu'aux groupes traités avec la CQ seulement (44%) ou a la solution salée.(13%). Le poids moyen des souris a la combinaison (CQ+PR) (1.5 ± 0.16 ou 1.56±0.16 kg) était significativement plus elevé que celui des animaux traités a la CQ uniquement (1.33 ± 0.13) (P=0.004, 0.014 respectivement).Aucune malformations n'etaient observées aux petites souris delivrées par les méres infectées ou non-infectées et traités avec les combinations de chloroquine et promethazine.

Introduction

Malaria infection is estimated to kill between 1.5 and 2.7 million people each year, mostly children less than 5 years in sub-Saharan region of Africa [1]. Similarly, pregnant women are at increased risk of malaria infection and its disease consequences than their non-pregnant counterparts [2,3]. The uteroplacental vascular space provides a relatively protected site for parasite sequestration and development [4,5]. This parasite replication in the placenta space results in altered transplacental nutrient transport which compromise fetal growth resulting in low birth weight babies. Infant mortality among for low birth weight babies is 4 times more than for normal birth weight babies [6,7]. In addition, malaria infection in pregnancy may result in abortion, intrauterine fetal death, premature delivery and even maternal death [8].

Amelioration of the deleterious effects on the foetus and the mother requires adequate chemotherapy. Chloroquine still remains the mainstay for treatment and prevention of malaria in pregnancy in some malaria endemic regions [9] and has also been shown to be safe for this purpose in pregnancy [10]. Unfortunately, the efficacy of chloroquine has been compromised by the emergence and spread of drug resistant strains of *P. falciparum*. Similarly the efficacy of most currently available antimalarial drugs has been compromised by the emergence of drug resistance [11, 12, 13, 14,15,16].

In addition the process of developing new antimalarial drug is long and very expensive. The low toxicity of chloroquine at therapeutic dose and cheapness poses a great challenge to any new drugs. Thus there is need to for development, evaluation and implementation of alternative strategies apart from development of new drugs. The ability of some non-antimalarial drugs to reverse chloroquine resistance in Plasmodium represents a useful alternative strategy to restore and prolong the clinical utility of chloroqune. The reversal of chloroquine resistance *in vitro* or in vivo by drugs such as verapamil, desipramine, chlorpromazine, chlorpheniramine, promethazine has been well established [17, 18, 19,20].

Clinical application with a potential candidate reversing compound chlorpheniramine has been demonstrated [19]. Promethazine (an H_1 receptor antagonist) is commonly used in West African countries as an adjunct in the treatment of malaria infection with chloroquine. Promethazine is found safe and well tolerated when administered in therapeutic dose [21]. Ability of promethazine to reverse chloroquine resistance in *P. falciparum* has been documented *in vitro* and in a monkey model [20]. This observation increased the number of potential candidates available to prolong clinical utility of chloroquine for treating malaria infections. In addition, development of new drug requires important parameters including its efficacy and safety in pregnancy. Studies to determine these parameters are not feasible in pregnant women for ethical reasons. Thus tyo studies were conducted to determine the effects of treatment and or infection on outcome of gestation in the animals.

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Materials and mthods

Experimental animals

Nulliparous (9-11 weeks old) Swiss albino mice were used in the studies. Mice were kept in cages at room temperature, fed with standard mouse cubes (Ladokun Feeds Nigeria Limited) and provided with access to clean drinking water ad libitum. The animals were divided into mating groups consisting of two female and a male. The presence of vaginal plug indicated the first gestation day following copulation in the animals.

Parasites/maternal infection

Chloroquine resistant ANKA clone of *Plasmodium berghei* was obtained from Dr. D. Kyle of the Division of Experimental Therapeutics, Walter Reed Army Institute for Research, Washington DC. The parasites were maintained in mice by serial passage in the animal house of the Malaria Research Laboratories, Institute for Advanced Medical Research and Training, College of Medicine, University of Ibadan. Parasitized red blood cells used for inoculation were obtained by cardiac puncture from an infected donor mouse. The blood was diluted to desired parasite density in buffered 0.9% NaCl solution (Kendall McGaw Laboratories Inc. IRVINE. CA. U.S.A). Each mouse was inoculated with $0.5x10^6$ parasitised red blood cells suspension in buffered 0.9% NaCl (0.2ml). Gravid mice were infected 12 or 14 days following successful copulation. The day of inoculation was defined as day zero (D0) and subsequent days D1, D2, e.t.c.

Treatment of infections

One hundred and twenty-eight gravid mice were randomly allocated into eight groups of sixteen animals each. Six out of the eight groups were treated with either chloroquine alone or a combination of chloroquine with selected doses of promethazine. The remaining two groups were infected gravid mice and non-infected gravid mice treated with buffered 0.9% NaCl and they served as control. Each animal was treated with chloroquine (10 mg/kg) daily for three days. Animals treated with the combination, received chloroquine (10 mg/kg) with 10mg/kg, 20mg/kg, 30mg/kg, 40mg/kg or 50mg/kg promethazine every 12 hours for seven days. The drugs were administered with an oral cannula.

Determination of responses of infection to treatment

Thin blood films were prepared daily starting 3 days post inoculation from each mouse using blood obtained from a tail snip. The thin blood films were prepared every day till day 9 and thereafter every other day till day 60 in all surviving animals. Each thin blood film was air dried, fixed with methanol and stained with Giemsa stain. Giemsa stained thin blood films were examined under a high power microscope objective (x100) to enumerate parasite density. Parasite density was determined, by counting the number of parasitized erythrocytes among at least 1000 red blood cells.

Parasite clearance time (PCT) following treatment was determined in each animal. The time it took for parasite to reappear in the animals during the 60 days of follow up was defined (RT). Number of gravid animals with successful parturition were determined in each group. Number of animals surviving till day 60 after inoculation in each group was recorded. Live pups delivered by each animal were weighed within 24 hours of parturition. The birth weight of pups from animals in each group was compared to determine the effects of malaria infection and response of infection to treatment on the birth weight of pups in the animal model.

Evaluation of effects of the drug regimen on gestation in gravid animals

Thirty-five non-infected gravid mice were randomly allocated into seven groups of five animals each. The effect of combinations of chloroquine (10mg/kg over 3 days) and selected dosages of promethazine on the development during gestation in gravid mice was studied. Six groups of animals were treated with either chloroquine alone or combination of chloroquine with 10mg/kg, 20mg/kg, 30mg/kg, 40mg/kg or 50mg/kg promethazine, while the seventh group was treated with saline as it served as control. Outcome of gestation in each animal were monitored. Abortion in the animals and successful parturition were recorded. Each live pup was weighed, examined and observed for four weeks.

Statistical analysis

The mean birth weight, parasite clearance time, recrudescence time, survival rate, the proportion of abortion and parturition in the animals were compared using Student -t-test. P-value < 0.05 was considered significant

Results

Responses of infections to treatment

The response of infections in gravid mice to treatment with chloroquine or chloroquine combined with selected dosages of promethazine is shown in Table 1.

Table 1: Response of infection with $0.5x10^6$ chloroquine resistant *Plasmodium berghei* (ANKA) to treatment with chloroquine (30mg/kg) or chloroquine in combination with selected doses of promethazine in gravid mice

Treatment Chloroquine (CQ) 30mg/kg Promethazine (PR)	Response * Parasite clearance time ± SD (days)	* Recrudescence time ± SD (days)
CQ	4.7 ± 0.8	7.0± 0.8
CQ + (10mg/kg) PR	4.6 ± 0.5	8.8 ± 1.0
CQ + (20mg/kg) PR	3.4 ± 0.5	9.0 ± 2.1
CQ + (30mg/kg) PR	3.3 ± 0.5	10.0 ± 1.6
CQ + (40mg/kg) PR	2.9 ± 0.6	11.2 ± 1.4
CQ + (50mg/kg) PR	2.7 ± 0.7	10.8 ± 1.6

** 16 animals were allocated to each group*
Chloroquine or promethazine was prepared as suspension and administered in 0.2ml saline at desired concentration.

Infection was cleared by 4.7 ± 0.8 days following treatment with chloroquine alone but recrudesced at 7 ± 0.8 days after treatment. Treatment with combination of chloroquine with 10mg/kg, 20mg/kg, 30mg/kg, 40mg/kg or 50mg/kg promethazine cleared parasites within 2-4 days. Infection in the animals recrudesced 8 to 11 days after treatment (Table 1).

PCT in animals treated with chloroquine alone and chloroquine combined with 10mg/kg promethazine ($P>0.05$) were not significantly different. Treatment with combination of chloroquine with selected doses of promethazine resulted in a significantly different PCT when compared with animals treated with chloroquine alone ($P<0.05$)

Effect of infection and treatment with chloroquine and promethazine combinations on parturition in mice infected with P. berghei

Effects of treatment with chloroquine or chloroquine in combination with selected dosages of promethazine on the outcome of gestation in infected gravid mice are shown in Table 2. Two of 16 (13%) gravid animals infected with *P. berghei* and treated with saline delivered live pups. All the 16 (100%) non-infected gravid mice delivered live pups. Seven of the 16 (44%) infected gravid animals treated with chloroquine alone delivered successfully. Eleven of 16 (69%) infected gravid animals treated with combination of chloroquine with 10mg/kg promethazine delivered live pups. Also, 13 animals out of each 16 (81%, 81%) infected animals treated with chloroquine combined with 20mg/kg or 30mg/kg promethazine delivered live pups. Ten of 16 (63%) and 9 of 16 (56%) infected animals treated with combination of chloroquine with 40mg/kg or 50mg/kg promethazine respectively delivered successfully (Table 2).

Table 2: Effects of treatment with chloroquine or chloroquine in combination with selected dosages of promethazine on the outcome of pregnancy in mice infected with chloroquine resistant *Plasmodium. berghei* (ANKA)

Treatment Chloroquine (CQ) 30mg/kg and promethazine (PR) †	Response *Delivery rate (%)	No. of pups	Birth weight of pups (g)
Non infected [a]	100.00	29	1.63 ± 0.09
Saline [b]	12.50	8	1.09 ± 0.08
CQ	43.75	17	1.33 ± 0.13
CQ + (10mg/kg) P	68.75	10	1.42 ± 0.05
CQ + (20mg/kg) PR	81.25	12	1.44 ± 0.07
CQ + (30mg/kg) PR	81.25	13	1.51 ± 0.16
CQ + (40mg/kg) PR	62.50	19	1.56 ± 0.16
CQ + (50mg/kg) PR	56.25	6	1.36 ± 0.13

† Chloroquine or promethazine was prepared as suspension and administered in 0.2ml saline at desired concentration

** 16 animals are allocated to each group*

[a] None infected gravid animals

[b] Infected gravid mice treated with saline alone.

Parturition resulting in live pups in animals treated with chloroquine in combination with 20mg/kg or 30mg/kg promethazine was significantly higher ($P = 0.02$) than in animals treated with chloroquine alone. There was no significant difference in parturition rate resulting in live pups in animals treated with chloroquine and those treated with chloroquine combined with 10mg/kg, 40mg/kg, 50mg/kg promethazine ($P = 0.15, 0.5, 0.5$ respectively).

Effect of infection and treatment with chloroquine and promethazine combination on the birth weight of pups in mice infected with P. berghei.

Mean birth weight of pups delivered by uninfected and infected animals treated with chloroquine alone or chloroquine combined with selected dosages of promethazine are shown in Table 2. The mean birth weight of pups delivered by animals treated with chloroquine alone was 1.33 ± 0.13g. Mean birth weight of pups delivered by animals treated with combination of chloroquine with 10mg/kg, 20mg/kg, 30 mg/kg, 40 mg/kg or 50mg/kg promethazine were 1.42 ± 0.05g, 1.44± 0.07g, 1.51 ± 0.16g, 1.56 ± 0.16g or 1.36 ± 0.13g respectively.

Mean birth weight of pups delivered by gravid animals treated with chloroquine alone or chloroquine in combination with 10mg/kg, 20mg/kg or 50mg/kg were not significantly different ($P=0.12$, $P=0.10$, $P=0.64$ respectively). However, the mean birth weight of pups delivered by animals treated with chloroquine alone and chloroquine in combination with 30 mg/kg or 40 mg/kg were significantly higher than pups delivered by animals treated with chloroquine alone ($P=0.00004, 0.0014$ respectively).

Table 3: Effects of treatment with chloroquine or chloroquine in combination with selected dosages of promethazine on the survival of gravid mice infected with chloroquine resistant *Plasmodium. berghei* (ANKA)

Treatment Chloroquine (CQ) 30mg/kg and promethazine (PR) †	Response No of mice dead/ days died	No alive on day 60 / Total no. examined	Percent survival (%)
Non infected [a]	0	16/16	100
Saline [b]	1/3, 1/4, 5/5, 5/6, 4/7,	0/16	0
CQ	2/10, 3/14, 1/19, 1/23, 1/42	8/16	50
CQ + PR (10mg/kg)	1/7, 1/10, 2/14, 2/42	10/16	62.5
CQ + PR (20mg/kg)	3/13, 1/14	12/16	75.0
CQ + PR (30mg/kg)	1/12, 1/15	14/16	87.5
CQ + PR (40mg/kg)	1/6	15/16	93.8
CQ + PR (50mg/kg)	1/6	15/16	93.8

† Chloroquine or promethazine was prepared as suspension and administered in 0.2ml saline at desired concentration.

[a] Saline - gravid mice treated with normal saline

[b] Non infected - non infected gravid animals

Mean birth weight of pups delivered by non-infected animals (1.63 ± 0.09g), was significantly different from mean birth weight of animals treated with chloroquine alone or chloroquine combined with 10mg/kg 20mg/kg or 50mg/kg promethazine (P = 0.00, 0.04, 0.00 respectively). However, mean birth weight of pups delivered by non-infected animals, animals treated with chloroquine combined with 30mg/kg or 40mg/kg promethazine were not significantly different ($P=0.13, 0.19$ respectively).

Studies on effects of chloroquine and promethazine on gestation in mice.

All non-infected gravid mice treated with either saline, chloroquine alone or chloroquine combined with 10mg/kg, 30mg/kg, 40mg/kg or 50mg/kg of promethazine delivered live pups. One animal treated with chloroquine combined with 40mg/kg promethazine died two days after parturition. Four of 5 mice treated with chloroquine combined with 20mg/kg promethazine delivered live pups, while the remaining mouse aborted the foetus. There was no gross malformation in the pups delivered by all animals treated with the drug regimen. All the pups developed normally and compared with pups delivered by animals treated with saline for the period of 60 days observed.

Discussion

Spontaneous abortion, reduced birth weight of foetus and potential effects on post natal development of the child associated with *Plasmodium falciparum* infection underscore a need for adequate and careful management of malaria infection during pregnancy. Chloroquine has been the mainstay for treatment and prevention of malaria in pregnancy in malaria endemic regions [7]. It is one of the only few antimalarial drugs found safe for treatment of malaria in pregnant women in areas where the parasites are still sensitive to the drug [10]. Unfortunately, the widespread of *Plasmodium falciparum* strains resistant to chloroquine in sub-Saharan Africa constitute a public health problem and necessitate consideration of other drugs with limited safety for use during pregnancy. These underscore the need for current effort focused on developing strategies to prolong chloroquine utility by circumventing chloroquine resistance through combination with resistant reversing compounds.

Chloroquine remains a valued blood schizontocide, it produces rapid resolution of malaria symptoms and clearance of infection associated with sensitive parasites. In this study the beneficial effects of chloroquine in pregnancy was restored against chloroquine resistant parasites by co-administration with promethazine. Reversal of chloroquine resistant *P. berghei* infection in pregnancy in the study however appeared to be dose dependent. Parasite clearance time was reduced with increasing doses of promethazine combined with modified dose of chloroquine. Animals treated with combination of chloroquine and the highest dose of promethazine (50mg/kg) studied had the shortest parasite clearance time compared to animals treated with lower doses of the combinations or chloroquine alone. Although, an initial clearance of parasitaemia was observed in all animals treated with the combinations, recrudescence also occurred indicating a measure of resistance of the *P. berghei* ANKA clone to treatment. The length of reduction of infection to subpatent level was also dependent on the dosage of promethazine. Infections in animals treated with chloroquine and the highest dose of promethazine (50mg/kg) remain subpatent for longer period compared with those treated with lower dosages of promethazine.

Malaria infection has been shown to severely compromise the maternal - fetal relationship in animal models [22,23]. Parasite load and replication in the placenta have also been shown to alter transplacental nutrient transport, thereby seriously compromising fetal growth [6,7]. In this study rapid reduction in parasite load and eventual clearance in animals treated with combination of chloroquine with selected doses of promethazine resulted in higher birth weight pups. Pups delivered by the few infected untreated mice had the lowest birth weight followed by pups delivered by animals treated with chloroquine alone. The mean birth weight of pups delivered by mice treated with chloroquine in combination with 30mg/kg or 40mg/kg of promethazine were significantly higher than those treated with chloroquine alone. More importantly, there was no significant difference between the birth weight of pups delivered by non-infected control animals and those delivered by infected animals treated with the combination of chloroquine and the two doses of promethazine. The proportion of animals that delivered live pups increased as the concentration of promethazine combined with chloroquine in the combinations was increased. This beneficial effect was observed in animals treated with combination of chloroquine with dosages of promethazine ranging from 10-30mg/kg. However, combination of chloroquine with higher dosages of promethazine (40mg/kg or 50mg/kg) resulted in a decline of the beneficial effects of the combinations.

Survival of infected mice was significantly prolonged when retreated after recrudescence with the combination of chloroquine and promethazine with animals surviving to 60 days. None of the infected untreated pregnant animals survived for 60 days while a significant number of animals treated with the combination survived. Treatment of animals with a combination of 40mg/kg or 50mg/kg promethazine and chloroquine produced the most significant survival rate of 93.75% compared with only 50% in animals treated with chloroquine alone. However, optimal beneficial effects of treatment with chloroquine and promethazine was obtained when chloroquine was combined with (20mg/kg or 30mg/kg) promethazine

The results from this study showed the potential value of combination therapy in the management of chloroquine resistant *Plasmodium berghei* infection in gravid mice. The increased birth weight of pups and survival rate in animals treated with these combination support the need for prompt and effective antimalarial drug intervention in pregnancy in order to reduce malaria associated low birth weight. Further pharmacokinetics and toxicological studies are however important prerequisites to clinical application of the reversal phenomenom.

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