Plasmodium berghei infection associated with adverse birth outcomes in pregnant Swiss albino mice

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ABSTRACT

Malaria in pregnancy has been seen to cause poor pregnancy and foetal outcomes. In this study, mice infected with *Plasmodium berghei* (*P. berghei*) during the second and third stages of pregnancy were examined for their pregnancy's outcome and changes in their blood's biochemical composition after delivery. Additionally, the physical and behavioural reactions of the mice's pups were also investigated. Thirty pregnant female Swiss Albino mice were randomly divided into three groups; two received intraperitoneal injections of 10^6 *P. berghei*-infected red blood cells on gestational days (GD12 and 17), while the third group was left uninfected (control). Pregnancy termination occurred in 20% of mice infected during GD12, whereas mortality before parturition occurred in 40% and 30% of mice infected during GD12 and GD17, respectively. Non-infected group's total protein and glucose concentrations were significantly higher ($p<0.05$), while cholesterol and triglyceride concentrations were significantly lower ($p<0.05$) when compared to the infected groups. The Mean birth weights (1.82 ± 0.37 g) of pups were considerably higher ($p < 0.05$) in control mice compared to pups from infected groups. Offspring born to infected mothers exhibited poor physical and behavioural responses. Mice infection by *Plasmodium berghei* during pregnancy resulted in adverse birth outcomes, altered measured biochemical parameters, poor physical and behavioural responses in their offspring and was more severe during the 2nd stage of pregnancy.

Keywords: *Plasmodium berghei,* Swiss Albino mice, Pregnancy outcome, Nigeria
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INTRODUCTION

Malaria continues to cause significant mortality and morbidity globally, despite advances in malaria knowledge. Due to the coronavirus pandemic in 2020, work to tackle malaria in sub-Sahara Africa may have been interrupted, which may have led to an increase in the number of malaria infections in the region (W.H.O, 2021; Audu *et al.,*2022). *P. falciparum* is to blame for humans' most severe type of malaria. Other

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species that produce fewer complex types of human malaria include *P. vivax, P. ovale, P. malariae*, and *P. knowlesi* (Déchamps *et al*., 2010; Audu *et al*., 2022). Malaria research requires laboratory animal models, yet none of the human malaria agents can infect mice or rats. However, *P. berghei, P. chabaudi, P. vinckei,* and *P. yoelii* are well-known species of *Plasmodium* that infect rodents. In terms of order in so-called syntenic regions and content there is a high degree of conservation between 85% of the genes from *P. falciparum, P. berghei, P. chabaudi, and P. yoelii* (Kooij *et al*., 2005; Déchamps *et al*., 2010).

Malaria in Nigeria is a severe parasitic infection that affects pregnant women and children between zero and five (Idowu *et al.*, 2006). Pregnant women are three times more likely to be affected by severe malaria (Kovacs *et al.*, 2015). Therefore, millions of pregnant women in malaria-endemic countries risk having serious malaria complications such as high maternal anaemia, miscarriage, pre-term delivery, maternal death, low birth weight, intrauterine growth restriction and neonatal death (Rogerson *et al.*, 2007; Duffy and Fried, 2011; De Beaudrap *et al.*, 2016;). Furthermore, pregnant women residing in low malaria transmission regions with lowered acquired immunity experience more malaria complications, including pulmonary oedema, renal failure, and cerebral malaria. However, the clinical features of malaria in pregnancy (MIP) vary from no symptoms to severe (Menendez, 2006). Nevertheless, the overall mortality in MIP is similar in the malaria endemic region compared to low transmission regions (Desai *et al.*, 2007).

Despite enough data on the severe outcomes of *Plasmodium* infections during pregnancy, there is a scarcity of information on the effect of malaria in different stages of gestational age. Furthermore, most studies investigating the association between malaria during pregnancy and its diverse effects do not consider gestational age. Therefore, laboratory animal investigations are pertinent and contribute to knowledge, human health and societal development (Corvino *et al.*, 2015).

The experimental model used in studying MIP in mice infected during the second and third stages of pregnancy and its effects on the vital organs of its offspring have been studied (Audu *et al.*, 2021). However, in MIP, the gestational period in pregnancy when infections are initiated can alter the pregnancy outcomes (Neres *et al.*, 2008). Furthermore, numerous post-natal diagnostic problems are encountered during follow-up treatment of both mother and offspring in malaria-endemic locations (Uneke, 2008). Therefore, it is necessary to examine the blood biochemistry of the mother mice after delivery, pregnancy outcomes, and the physical and behavioural reactions of offspring in mice infected during the second and third stages of pregnancy. In addition, it would help to generate local knowledge on the different pregnancy outcomes in malaria infection during the second and third stages of pregnancy.

MATERIALS AND METHODS

Procurement and management of animals

This study was carried out in the animal house of the Pure and Applied Zoology Department, College of Biological Sciences, Federal University of Agriculture, Abeokuta. Sixty Females and thirty male adult Swiss Albino mice weighing between 20g to 25 g were obtained from the Institute for Advance Medical Research and Training, University College Hospital (UCH), Ibadan (Nigeria), and acclimatised for seven days. Steel cages were used to house the animals. They were fed with standard animal feed produced by Ladokun feed Limited, Ibadan, Oyo state, Nigeria, containing crude protein of 21%, Fibre 6.0%, Fat 3.5%, calcium 0.8%, and phosphorus 0.8%.

Experimental design

Thirty female mice were divided randomly into three groups (A, B, and C), having ten female mice per group. The females from each group were separated from the males for a week to suppress the oestrus cycle, after which the induction of the oestrus cycle was carried out by placing two females to a male in a cage; this caused the female to be in oestrus at 3rd night of exposure (Freyre *et al.*, 2006). After confirmation of pregnancy, groups A and B were intraperitoneally injected with 10^6 parasitised red blood cells at gestational days (GD 12 and 17), while group C was uninfected (Control). Mice in each group were monitored for various pregnancy outcomes (Pre-term delivery (<18 days), Term delivery (18 – 21 days), post-term delivery (> 21 days), Termination of pregnancy and dead before parturition). After delivery, the offspring's birth weight was taken, and physical and behavioural responses were observed. The remaining 30 females and 15 males were paired and mated (2 females with 1 male) and used as foster mothers for post-natal study as *P. berghei* was lethal to infected mothers. This study was carried out in agreement with the national institute of health guide for the use and care of laboratory research animals (NIH publication 8023, revised 1978). The research was conducted with the approval of the department of pure and applied Zoology, Federal University of Agriculture Abeokuta, Nigeria.

Gestation timing and pregnancy monitoring

Noticing the presence of a vaginal plug coupled with body weight measurement was used in pregnancy gestational timing as described by (Freyre *et al.*, 2006). Two females were put together with one male for 3 days and examined for the presence of a vaginal plug every morning. The day of noticing the presence of a vaginal plug was taken as gestational day one (G1), and the progression of pregnancy was monitored by weighing the pregnant female every other day. Successful fertilisation was confirmed between

G10 and G13 when the animal had an average increase of 3-4 g in body weight. Thus, an increase in weight was taken as a pregnancy sign, while abrupt loss of weight as abortion or interruption of pregnancy (Neres *et al.*, 2008). In addition, bulging of the female mice's abdomen region and enlargement of the nipples were used to rule out weight gain that was not associated with pregnancy.

Malaria parasite

The NK65 strain of *P. berghei* used for this study was obtained from the chemotherapy Research Laboratory, Institute of Advance Medical Research and Training, University College Hospital (UCH), Ibadan, Nigeria, and sustained in mice by week to week passaging to fresh mice. Each mouse was inoculated intraperitoneally with 0.2 mL of infected blood containing about 10^6 parasitised red blood cells obtained from a donor mouse having about 79% Parasitaemia. Thin blood films were conducted by collecting blood from the tail, stained with Giemsa stain, and the percentage of parasitaemia was ascertained by counting the number of parasitised cells per the total number of red blood cells (RBC) in a minimum of 4 random fields and multiplied by 100 to express it as a percentage (Moody, 2002).

Offspring monitoring

NK65 strain of *P. berghei* infection is fatal in mice; hence, foster mothers were used to nurturing newborns for postpartum follow-up studies. Therefore, to avoid biases in weight due to differences in nutritional content from mothers, newborns from both infected and non-infected mothers were transferred to foster mothers. Newburn's weight was taken every other day (Neres *et al.*, 2008). In addition, pups were physically observed daily for the physical development of the fur, eye, teeth, and behavioural responses (Nibbling for solid food, Activeness and Weaning); this observation was carried out daily for four weeks in the morning, afternoon, and evening.

Blood chemistry analysis of mother mice immediately after birth

After birth, blood samples were collected from the mother mice of each group. The blood was immediately transferred into a heparinised tube to prevent coagulation. Glucose was determined after enzymatic oxidation in the presence of glucose oxidase with Randox commercial kits (United Kingdom). The total protein concentration of each animal's blood was determined using Randox kits (UK) using the biuret method. Total cholesterol was determined after enzymatic hydrolysis and oxidation using Randox kit (UK), while triglycerides were determined by enzymatic hydrolysis with lipases using Randox Kits (UK). Each parameter absorbance of the sample, standard and blank, was measured using a spectrophotometer (Surgispec SM-23D, Surgifield Medical England).

Statistical analysis

Data were analysed using one-way Analysis of Variance (ANOVA). Significant differences between means were separated using 'Duncan's New Multiple Range Test. The significant difference was set at $p < 0.05$, and all analyses and Data were analysed as mean ± standard deviation. SPSS statistical package version 16 was the software used for the statistical analysis.

RESULTS AND DISCUSSION

Outcomes of malaria in pregnant mice

Parasitaemia density before delivery was significantly ($p<0.05$) higher in mice infected during the 2nd stage of pregnancy (63.6%) compared to mice infected during the 3rd stage of pregnancy (11.51 %) (Table 1). Pre-term delivery was observed in 50% and 28.87% of mice infected in the 2nd and 3rd stages of pregnancy, respectively. Among the successful pregnant mice infected in the 2nd and 3rd stages, term delivery was found only in one mouse, while 80% of non-infected mice recorded term delivery. Post-term delivery was recorded in 20% of non-infected mice and 25% and 57% of mice infected during GD 12 and GD17, respectively (Table 1). The high pre-term and post-term delivery observed in mice infected in the 2nd and 3rd stages of pregnancy might be due to the malaria parasite infection. MIP has been documented as the leading cause of pre-term delivery and pregnancy complications, which could delay delivery (Bardají *et al.*, 2011; De Beaudrap *et al.*, 2016).

Pregnancy termination was observed in 20% of mice infected during GD12, while mortality before parturition was observed in 40% and 30% of mice infected during GD12 and GD17 of pregnancy, respectively (Table 1). This could be due to the parasitaemia load recorded before parturition in mice infected in the 2nd and 3rd stage (Table 1) compared to the uninfected groups. In humans, abortion and mortality before parturition are adverse pregnancy consequences related to malaria during pregnancy. Malaria during pregnancy is the dominant cause of miscarriages (De Beaudrap *et al.*, 2016), and mortality during pregnancy (Kovacs *et al.*, 2015) because pregnancy raises the risk and vulnerability to malaria infection (Idowu *et al.*, 2006).

The mean birth weight of pups delivered by non-infected mothers (1.82, %) was significantly higher ($p <0.05$) compared to pups of mice infected in the 2nd (1.55, %) and 3rd (1.62, %) stages of pregnancy (Table 1). Low birth weight (LBW) is one of the after-effects of malaria in pregnancy (Umbers *et al.*, 2011). Furthermore (Guyatt and Snow, 2001) showed that pregnancy duration is an essential factor in determining the size at

birth; This may account for the mean low birth weight recorded in mice infected in the 2nd and 3rd stages of pregnancy. Pre-term delivered pups registered low birth weight (Table 1), as a study by (Hack *et al.*, 1995) shows that children with low birth weight can be born at or before term and are at varying levels of social and medical risk.

Table 1. Pregnancy outcomes of mice infected with *P. berghei* during the 2nd and 3rd stages of pregnancy.

Variables (Pregnancy outcomes)	Stages of pregnancy		
	Control (N=10)	2nd Stage (N=10)	3rd Stage (N=10)
Parasite density before delivery (mean± SE)	-	63.60±4.49[a]	11.51±0.67[b]
Pre-term delivery (< 18 days) (%)	0	2(50)	2(28.57)
Term delivery (18-21 days) (%)	8(80)	1(25)	1((14.29)
Post-Term delivery (>21 days) (%)	2(20)	1(25)	4(57.14)
Pregnancy Aborted (%)	0	2(20)	0
Mortality before parturition (%)	0	4(40)	3(30)
Survived Mean litter size (mean± SE)	6.80±0.66[a]	2.40±1.01[b]	4.50±1.19[ab]
Pup birth weight (g) (mean± SE)	1.82±0.37[a]	1.55±0.22[b]	1.62±0.04[b]
Prematurely delivered pups' mean weight (mean± SE)	0	0.87±0.22	0.73±0.03
Mortality of pups at day 1 (%)	0	11(45.83)	0
Mortality of pups on day 7 (%)	2(2.94)	6(46.15)	13(28.89)

[ab]Mean (±Standard Error) of the same row having similar superscript are not significantly different at p >0.05

Cross-sectional data from five locations in sub-Saharan Africa on birth weight and survival showed high mortality in LBW babies compared to normal-weight babies (Guyatt and Snow, 2001). In addition, LBW has been seen to increase the risks of infant mortality (Han *et al.*, 2011). Also, in this study, mice infected in the second stage of pregnancy with the least pup birth weight recorded 11 and 6 mortalities of pups at day 1 and 7, respectively. In contrast, pups from mice infected at the 3rd stage of pregnancy had pups' mortality of 0 and 13 on days 1 and 7, respectively (Table 1). This observation calls for close monitoring of offspring from a mother infected at the last stage of pregnancy, even when the offspring are doing well on the day of delivery.

Blood biochemical profiles of mother mice immediately after delivery

The Mean glucose levels of non-infected mother mice (100 mg/dl) were significantly higher ($p<0.05$) compared to the mother mice infected in GD12 (57 mg/dl) and GD17 (55 mg/dl) of pregnancy (Table 2). The low glucose level in mice infected in the various stages of pregnancy could be due to high glucose intake by the parasite and impairment

of hepatic gluconeogenesis (Sengupta *et al.,* 2020). The liver is severely damaged during malaria infection, and the parasites require more sugar for their growth and multiplication, which may cause the complete exhaustion of liver glycogen. It is also clearly indicated that sugar during pregnancy might be diverted from circulation to the developing foetus (needed for its growth), resulting in decreased sugar levels (Nayyar *et al.*, 2007). Therefore, this depletion of glucose in the mother mice could lead to general weakness and lack of energy to maintain pregnancy, deliver offspring successfully and keep a healthy level of motherly care after delivery.

Similarly, mean total protein levels of non-infected mother mice (4.5 g/dl) were significantly higher ($p<0.05$) compared to the mother mice infected in GD12(3.0 g/dl) and GD17 (3.5 g/dl) of pregnancy (Table 2). The observed low total protein in infected groups could be either due to an increase in plasma volume during pregnancy or the mobilisation of protein for the protein synthesis necessary for the defence mechanism against parasitic infection (Nayyar *et al.*, 2007). Also, during pregnancy, especially the 3rd trimester, protein intake by the maternal organs and foetal tissues increases. Therefore, the burden of both pregnancy and malaria could have led to a decrease in the level of total protein; this might result in the wasting and shrinking of muscle tissues, improper function of different body organs and weakening of the mother mice's immune system (Khan *et al.*, 2018).The cholesterol and triglyceride level of non-infected mother mice (48 mg/dl) (64 mg/dl) was significantly lower ($p<0.05$) compared to mice infected in the 2nd (68 mg/dl) (72 mg/dl) and 3rd stages (75 mg/dl) (83 mg/dl) of pregnancy (Table 2). Malaria is found to increase total cholesterol and triglyceride levels. Nayyar *et al.* (2007) and Sriwiphat *et al.* (2015) documented that lipid metabolism occurs in the liver, which is affected by malaria more severely than any other organ as it is the first organ where the parasite multiplies. The increased lipolysis induced by a threshold of parasitaemia promotes cholesterol and triglyceride synthesis (Sriwiphat *et al.*, 2015). Pregnancy also changes the mother's lipid metabolism (Prairie *et al.*, 2012). Hence, This rise in lipids in infected mother mice might result in pregnancy and postpartum heart failure and death in women infected with malaria during pregnancy, as research indicates that high cholesterol is an independent risk factor for coronary heart disease and death (Prairie *et al.*, 2012). The damage caused by high lipid levels may account for the various deaths observed during parturition in this study.

Many physiological adaptations are required for a successful pregnancy outcome. These adaptations involve metabolism changes in most organ systems, resulting in changes in the biochemical composition of the blood (Corvino *et al.*, 2015). Studies have shown that both pathological and biochemical alterations caused by malaria during pregnancy might account for the maternal foetal compromised relationship (Nayyar *et al.*, 2007). The alteration in the biochemical constituent after the birth of mice infected in the 2nd

and 3rd stage may account for the poor parental care, termination of pregnancy, premature delivery of pups and low birth weight observed in this study.

Table 2. Blood biochemical profiles of mice infected during the second and third stages of pregnancy.

Chemical constituent of the blood	Control	Mice infected in the 2nd and 3rd stages of pregnancy	
		2nd Stage	3rd stage
Total protein (g/dl)	4.50±0.29[a]	3.00±0.12[b]	3.50±0.23[b]
Glucose (mg/dl)	100.00±0.58[a]	57.00±0.58[c]	55.00±1.15[c]
Cholesterol (mg/dl)	48.00±1.73[c]	68.00±1.73[b]	75.00±0.58[a]
Triglyceride (mg/dl)	64.00±2.31[c]	72.00±0.58[b]	83.00±1.15[a]

[abc]Mean (± Standard Error) values in the same row with different superscripts are significantly different ($p < 0.05$)

Figure 1. Four weeks old pups (A) Showing pink colouration of the epidermis from mice infected in GD17 of pregnancy (B) showing normal appearance from non-infected mice.

Physical appearance and behavioural responses in Pups

At birth, there were no observable differences in the physical appearances and behavioural responses of pups from non-infected mother mice and mice infected during the second and third stages of pregnancy (Table 3). However, on day 10, it was observed that pups from mice infected in the various stages of pregnancy showed slow development of fur and ear flaps were erected. Still, movement activity was low compared to the non-infected mothers' offspring. At week three, pups from mothers infected in the 2nd and 3rd stages of pregnancy were inactive, and their eyes were always closed. Only the offspring of mice infected at GD17, and Non-infected groups showed evidence of weaning at week three (Table 3). Pink colouration of the epidermis was observed in four-week-old pups of mice infected in the 3rd stage of pregnancy (Figure 1). LBW could be a risk factor for poor fur growth, low activities at day ten, eyes not primarily open, and little nibbling of food of offspring of mice infected in the various

stages of pregnancy. LBW increases the risks of infant-impaired development (Han *et al.*, 2011). LBW is a risk factor for poor neurosensory, cognitive, and behavioural development and limited school performance and academic achievement (Mccormick *et al.*, 1992). However, the most vulnerable group appears to be those infants born prematurely, who need educational services and exceptional support as they would likely experience academic failure in school more than infant born with an average birth weight (Hack *et al.*, 1995). De Beaudrap *et al.*(2016) stated that pregnancy-associated malaria in the endemic malaria region decreases the ability of progeny to develop its full potential.

Table 3. Physical appearances and behavioural responses in pups from mice not infected during pregnancy and mice infected during the second and third stages of pregnancy.

Days	**Non-infected**	**Pups of mice infected in the 2nd and 3rd stages of pregnancy *Gestation Day (GD5,12, and 17)**	
		2nd Stage	**3rd stage**
DAY 1	Pups were very red and helpless. Their ears and eyes were shut.	Pups were very red and helpless. Their ears and eyes were shut.	Pups were very red and helpless. Their ears and eyes were shut.
DAY10	Fur growth was completed, ear flaps have opened and erected, and pup activity is high.	Fur growth was not completed, ear flaps have opened and erected, and low activity.	Fur growth was not completed, ear flaps have opened and erected, and low activity.
DAY,12,13 AND 14	The eyes were mostly open, teeth begin to erupt, and they were observed to nibble for solid food	The eyes were not primarily open, and teeth begin to erupt and little nibble for solid food.	The eyes were not primarily open, and teeth begin to erupt and little nibbling for food.
WEEK 3	They were very active, and there was an observation of pup weaning	They were inactive, and their eye was always closed.	They were inactive, and there was an observation of pup weaning.

CONCLUSION

This study has shown that malaria infection during the 2nd and 3rd stages of pregnancy in mice resulted in poor pregnancy outcomes such as pregnancy termination, pre-term delivery, and mother mortality before parturition, and blood biochemical alteration with poor pups' growth and development. Furthermore, the severity of pregnancy outcome was more significant in mice infected during the 2nd stage than those infected during the

3rd stage. Therefore, further robust related studies are required to generate data and knowledge on different *Plasmodium* species' effects on pregnancy outcomes.

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DECLARATIONS

Ethical statement

Approval for this research was granted by the department of pure and applied Zoology, College of Biosciences, Federal University of Agriculture, Abeokuta, Nigeria, with reference number PG14/0766. Experiments were performed following the guidelines for the care and use of laboratory Animals of the National Institutes of health.

Conflict of interest

The authors declared they have no conflict of interest.

Contribution of each author

Audu David was responsible for the study design, experiment, data collection, and analysis. Adewunmi B. Idowu provided supervision and technical guidance during the investigation, and Olufunmilayo A. Idowu supervised the malaria portion of the work.

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