HAEMATOLOGICAL ALTERATION AND HISTOPATHOLOGY OF VITAL ORGANS OF PUPS DELIVERED BY MICE INFECTED WITH *PLASMODIUM BERGHEI* DURING THE SECOND AND THIRD STAGE OF PREGNANCY

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ABSTRACT

Pregnancy associated malaria (PAM) is a potentially life-threatening condition that affects the new-born. The aim of this study was to investigate the influence of PAM on the haematological parameters and histopathology of some vital organs of pups delivered by mice infected with malaria in the second and third stages of pregnancy. Thirty female Swiss albino mice were randomly assigned into three groups, two groups of which were intraperitoneally infected with inoculums containing 3.97 x 10^6 Plasmodium berghei infected red blood cells at gestational days (GD 12 and 17), while the third group were uninfected (control). Pregnant females were allowed to deliver and progenies were monitored for three weeks. The red blood cell (RBC), white blood cell (WBC), packed cell volume (PCV) and haemoglobin (HB) concentration was significantly lower ($p<0.05$) in pups of mice infected in the 2nd and 3rd stages of pregnancy as compared to the pups from non-infected mother. Histopathological alterations observed in the pup's organs of mice infected in the 2nd and 3rd stages of pregnancy include fatty degeneration in the liver, interstitial pneumonia and oedema in the alveolar sac of the lungs, severe lymphoid depletion of the spleen, degeneration of tubular epithelial cells of the kidney and vacuolar degeneration in the brain. More severe damages were seen in pups from mice infected in the 2nd stage of pregnancy. This study showed that pregnancy associated malaria induce anaemia and damaging effects on vital organs of progeny of mice infected in the 2nd and 3rd stages of pregnancy.

Keyword: Pregnancy associated malaria, *Plasmodium berghei,* Parasitaemia, Birth weight, Haematological profile, Organ histopathology

INTRODUCTION

Malaria still threatens the lives of about 40 % of the world's population (Sha'a *et al*., 2011). The majority of who are pregnant women and young children under 5 years of age (WHO, 2015). The World Health Organization reported that the number of malaria in sub-Sahara Africa may double in 2020 as work done in tackling the endemic disease have been interrupted by coronavirus pandemic (WHO, 2020). The reduction of *P. falciparum* prevalence in Africa has progress significantly in the past decade. However, malaria in pregnancy still remains a threat in malaria endemic region, with over 50 % of the women having *P. falciparum* detected in their blood during antenatal (Kakuru *et al*., 2016). This high prevalence of pregnancy

associated malaria in endemic region may be due to increased rate of being infected with malaria in gravid women as compared to non-gravid women. Women who are younger, malnourished, primigravidae/secundigravidae, or living with HIV lack immunity to pregnancy-associated malaria and are at the highest risk of malaria-associated adverse pregnancy outcomes (Desai *et al*., 2007).

Malaria in pregnancy is significantly associated with higher infant morbidity and mortality (PMI, 2015), including cerebral malaria (Román and Senanayake, 1992), maternal anaemia, intrauterine growth retardation (Dondorp *et al*., 2010), premature labour (Bardají *et al*., 2011) stillbirth, low birth weight (LBW) (Huynh *et al*., 2011) and untimely abortions of developing embryos and foetus (Ndam *et al*., 2015). The study showed that 11 % infant deaths in the first month after birth and up to 14 % of LBW in area of high malaria transmission in Africa were sequel to complications due to pregnancy associated malaria.

It is important to note that murine model of malaria using *P. berghei* have recapitulated features analogue to malaria during pregnancy caused by *P. falciparum* in humans (Hviid *et al*., 2010). Previous studies concerning PAM in rodents have focused on congenital malaria (Adachi *et al*., 2000) and characterization of placenta pathology (Tegoshi *et al*., 1992). Other studies of rodent malaria in pregnancy observed the disease dynamics and recrudescence (Pavia and Niederbuhl, 1991) and analysed pregnancy outcome upon drug treatment of the disease (Boareto *et al*., 2019).

This experimental model used in this study captured infecting mice during the 2nd and 3rd gestational stages of pregnancy and studying its effects on the progeny haematological parameters and some of its vital organs histology. The consequences of PAM on progeny can be affected by the stages of pregnancy in which infection was initiated (Neres *et al*., 2008).This was necessary as a lot of diagnostic challenges were encountered during postnatal follow up of offspring from malaria infected mothers during pregnancy.

MATERIALS AND METHODS

Animal Procurement and Management: The present study was conducted in the Animal House of the Pure and Applied Zoology Department, College of Biosciences, Federal University of Agriculture, Abeokuta, Nigeria. 60 female and 30 male adult Swiss Albino mice weighing 22.5 ± 2.5 g were obtained from the Institute for Advance Medical Research and Training, University College Hospital (UCH), Ibadan, Nigeria. The animals were housed in steel cages and fed with animal feed containing 21 % crude protein, 3.5 % fat, 6.0 % fibre, 0.8 % calcium and 0.8 % phosphorus produced by Ladokun Feed Limited, Ibadan, Oyo State, Nigeria.

Experimental Design: A total of 30 female mice were randomly divided into 3 groups (A, B and C) of a completely randomized design (CRD) having 10 female mice per group. Each group were divided into two replicates containing five mice per cage. The females were isolated from the males for a week to suppress the oestrus cycle, after which the oestrus cycle were induce by exposing two female to a male, this makes the female to be in oestrus the 3rd night of exposure (Freyre *et al*., 2006). After pregnancy was established, group A and B were intraperitoneally inoculated with 3.97 x 10^6/µl of *Plasmodium berghei* infected red blood cells at gestation day (GD) 12 and 17 respectively, while group C were uninfected during pregnancy which served as the control. Pregnant females (both infected and control) were allow to deliver and progeny were monitored. At week three postpartum, one pup from each replicate was randomly selected and euthanized for the study.

The remaining 30 females obtained from UCH were mated and used as foster mother for new born post-natal follow up study, since *P. berghei* was lethal to infected mother mice. Experimental animal management and experimental protocols /procedures were conducted in accordance to the guidelines for caring and using of laboratory animals (NRC, 2011). The experiment was approved by the Department of Pure and Applied Zoology,

federal University of Agriculture Abeokuta, Nigeria.

Gestation Timing and Pregnancy Monitoring: Spotting of vaginal plug coupled with measurement of body weight was used to time gestation, as described by Freyre *et al*. (2006). Two females and one male were put together in a cage for 3 days, and examination for the presence of vaginal plug was carried out every morning. The detection of vaginal plug was considered to be gestational day one (GD 1) and monitoring of pregnancy progression was carried out by weighing the pregnant female every other day. Successful fertilization was confirmed between GD 10 and 13 when the animal had an increase of 3 – 4 g in body weight. Thus, Additional weight gain was taken as an indication of pregnancy and abrupt loss of weight as sign of pregnancy loss (Neres *et al*., 2008).

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 maintained in mice by weekly passaging in to fresh mice. Each experimental mouse was inoculated intraperitoneally with 0.2 mL of infected blood containing about 3.97 x 10^6 /µl of parasitized red blood cells obtained from a donor mouse having about 79.35 % parasitaemia. Thin blood smears were made by collecting blood from the tail and stained with Giemsa stain and the percentage parasitaemia was determined by using the percentage of parasitized red blood cells (RBC) in a minimum of 4 random fields (Moody, 2002).

Offspring Monitoring: As *P. berghei* NK65 strain infection is lethal in mice; foster mothers were used in new-born post-natal follow-up studies. Hence, to avoid bias in weight due to differences in nutritional content from mother, new born from both infected and control mother were transferred to foster mothers (Neres *et al*., 2008).

Collection of Blood: Blood samples were collected from pups of each group at day 21 (week three) by ocular puncture via heparinized capillary tubes into EDTA bottles. The haematological parameters such as: red blood cell (RBC) count, white blood cell (WBC) count, packed cell volume (PCV) and Haemoglobin (HB) concentration were determined using standard methods (Sood, 2006).

Sample Collection from Pups: The pups were euthanized by anaesthetizing them using gaseous chloroform in a 70 litre airtight plastic jar. A mass of cotton wool was put in the jar and 500 ml of chloroform was added in which the cotton wool was soaked just before introducing the mice (Blackshaw *et al.,* 1988). Samples of the lungs, kidney, liver, spleen and brain were collected and fixed in 10 % buffered formalin for histopathological studies.

Procedure for Histopathology: Tissue processing for histopathological examination was carried out in the Pathology Laboratory, College of Veterinary Medicine, and Federal University of Agriculture Abeokuta, Nigeria. Tissue samples from each group were retrieved from the 10 % buffered formalin and dehydrated through ascending grades of ethyl alcohol (70, 80, 90 and 100 %), cleared in xylene, infiltrated with molten paraffin wax at 58^o C and embedded. Tissue blocks were prepared from the paraffin embedded tissues. Serial sections of the tissue blocks were cut at 5 µm thickness with a rotary microtome and stained with Haematoxylin and Eosin stains for histopathological studies, according to the methods described by Avwioro (2002). Tissue sections were examined using light microscope and photomicrograph of pathological changes were taken using Microscope Digital Camera (AmScope, FMA050, China) with fixed microscope adapter.

Statistical Analysis: Data obtained were analysed using one way analysis of variance (ANOVA). Significant differences between means were separated using Duncan's New Multiple Range Test of the same statistical package. Significant difference was set at

p<0.05 and all analyses were done using SPSS Version 16. Results obtain were expressed as mean ± standard error of mean.

RESULTS AND DISCUSION

Pregnancy Outcome: Mice infected in the 2nd stage of pregnancy with parasitaemia level of not less than 43 % before delivery recorded only four successful deliveries with a total of 41 pups, but only four pups survived until 3rd week of postpartum. Mice infected in the 3rd stage of pregnancy with parasitaemia level of not less 8 % before delivery recorded only seven successful deliveries with a total of 51 pups, but only 8 pups survived until 3rd week of postpartum. Non-infected pregnant mice recorded 100 % success in delivery with a total of 68 pups, only four pups' dead's were recorded before week three postpartum (Table 1).

Table 1: Pregnancy outcome of mice infected with *Plasmodium berghei* at 2nd and 3rd stages of pregnancy

Characteristics	Non-infected Mice	Mice Infected in the Various Stages of Pregnancy	
		2nd Stage (GD 12)	3rd Stage (GD 17)
Parasitemia (Range) (%)	0	43 - 87	8 – 11
Successful deliveries (%)	100	40	70
Total Number of Pups Delivered	68	41	51
% mortality at week 3	5.88	90.24	84.31

Guyatt and Snow (2001) reported that 200,000 infant's die yearly as a result of pregnancy associated malaria, while De Beaudrap *et al.* (2016) showed a strong positive relationship between infant mortality and pregnancy associated malaria. Malaria in pregnancy is seen as a preventable cause of infant morbidity and death, as progeny can be affected by placenta malaria into childhood (Bauserman *et al.*, 2019).

Haematological Parameters of Pups: This study showed significant reduction (p<0.05) in the PCV, Hb and RBC counts in pups from mice infected with *P. berghei* in the 2nd and 3rd stages of pregnancy as compared to the pups from non-infected mice. Among the pups of mice infected in the 2nd and 3rd stages of pregnancy, the highest PCV, Hb and RBC were seen in pups from mice infected in the 3rd stage of pregnancy (Table 2).

Table 2: Haematological parameters of pups from mice infected with *Plasmodium berghei* at various stages of pregnancy

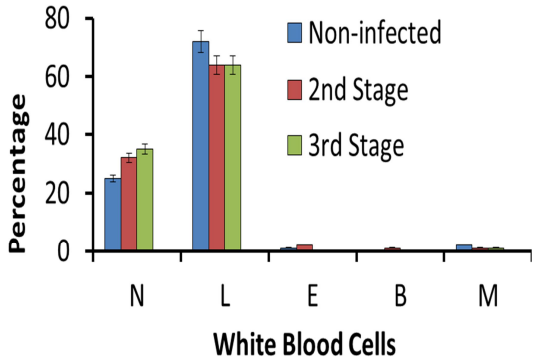
Parameter	Non-infected Mice	Mice Infected in the Various Stages of Pregnancy	
		2nd Stage (GD 12)	3rd Stage (GD 17)
PCV (%)	38.00 ± 0.58^{b}	28.00 ± 1.73^{a}	31.00 ± 2.31^{a}
HB (g/dl)	12.30 ± 0.12^{b}	9.30 ± 0.17^{a}	10.00 ± 0.58^{a}
RBC ($X10^{12/l}$)	6.70 ± 0.17^{b}	4.70 ± 0.12^{a}	5.07 ± 0.89^{a}
WBC ($X10^{9/l}$)	9.40 ± 0.58^{c}	5.80 ± 0.23^{a}	7.40 ± 0.29^{b}

^{abc}Mean (± standard error) values in the same row with different superscript are significantly different (p<0.05)

The reduction in PCV, Hb and RBC levels is indicative of anaemia. Anaemia is one of the most common complications in malaria infection especially in pregnant women in high transmission areas (Menendez *et al.*, 2000). Research has shown high maternal parasitaemia to be associated with fetal and new-born anaemia (Accrombessi *et al.*, 2015). Malaria in pregnancy is frequently associated with infant anaemia, and consequently the child development and survival are at risk (Shulman, 1999; Brabin *et al.*, 2004). In high malaria transmission area, the haemoglobin concentration is low in nearly all infants and young children (White, 2018).

Pups of control mice at week three postpartum had significantly high WBC compared to pups from mice infected in the 2nd and 3rd stage of pregnancy. Neutrophil count was higher in pups from mice infected in the 2nd and 3rdstages of pregnancy compared to those

from uninfected mice. Pups from infected mother had lower lymphocyte and monocyte count compared to those of uninfected mother. The eosinophil and basophil count were significantly higher in pups from mice infected in the 2nd stage of pregnancy (Figure 1).



Key: N = Neutrophils, L = Lymphocyte, E = Eosinophil, B = Basophil, M = Monocyte

Figure 1: Percentage of white blood cells differentials counts of pups from mice infected with *Plasmodium berghei* at various stages of pregnancy

The low lymphocytes in pups of mice infected in the 2nd and 3rd stages of pregnancy may indicate a compromised immune system (Cyril-Olutayo *et al*., 2013). This may be due to infecting of mother mice during pregnancy, as the pups from control mother mice had high lymphocytes. Therefore, the low WBC count of pups from mice infected in the 2nd and 3rd stages of pregnancy might have resulted from the low lymphocyte count instead from all the differential WBCs.

Histology of Pups Organs: The histological examination of the liver of non-infected pups showed normal histological architecture (Figure 2a), as no degeneration and necrosis of hepatocyte occurred. Vacuolar/fatty degeneration of hepatocyte in pups from mice infected with malaria in the 2nd and 3rd stages of pregnancy was observed (Figures 2b and c). This may be due to fat infiltration which according to Ganti (2007), degeneration of hepatocyte occur when the liver is too sick to metabolize glucose and other substrates or due to chronic venous congestion or nutritional inadequate levels of choline.

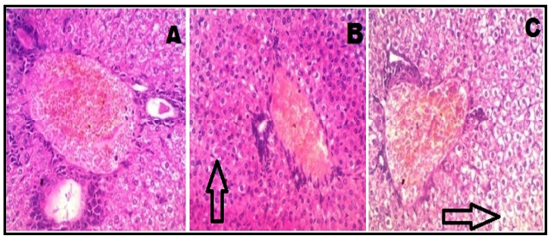


Figure 2: Section of the liver of pups of mice infected at the 2nd and 3rd stages of pregnancy and control pups. (A) Liver of control pups showing normal appearance. (B) Liver of pup of mice infected in the 2nd stage of pregnancy: showing mild vacuolar/ fatty degeneration of hepatocytes (black arrow); (C) liver of pup of mice infected in the 3rd stage of pregnancy: vacuolar/fatty degeneration of hepatocytes (black arrow) (x400; H&E)

More so, acute and severe anaemia and hypoxia, regardless of it cause, can lead to degeneration and even necrosis of hepatocytes, as this histological defect was not seen in pups of mice not infected during pregnancy.

The histological examination of the lungs of non-infected pups showed normal appearance (Figure 3a), while the pup lungs of mice infected during the 2nd and 3rd stages of pregnancy showed thickened interstitial and oedema in the alveolar sac (Figures 3b and c).

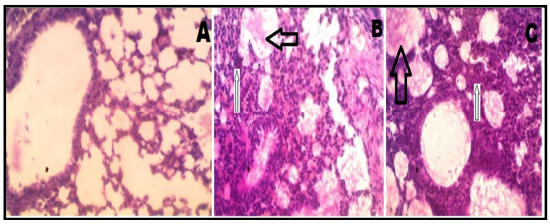


Figure 3: Section of the lung of pup from mice infected at the 2st and 3nd stages of pregnancy and control pups. (A) Lung of control pup: showing normal appearance. (B) Lungs of pup of mice infected in 2nd stage: showing moderately thickened interstitial (white arrow) and oedema in the alveolar sac (Black arrow). (C) Lung of pup from mice infected in the 3rd stage of pregnancy showing severe oedema in the alveolar sac (black arrow) and thickened interstitial (white arrow) (x400; H&E)

The thickened interstitial may be caused by increased vascular permeability, which allows fluid to leak into the lymphatic and interstitial spaces (Haschek *et al.,* 2013). These alterations

in the lungs tissues of pups may lead to respiratory insufficiency and failure (Leslie, 2004) coupled with hypoxia already caused by anaemia can greatly reduce the circulatory flow of required oxygen in the pups.

Histological examination of the spleen of pups from non-infected pregnant mice showed no alterations in the spleen architecture as there were no depletion of lymphoid tissues and no hyperplasia of mononuclear cells (Figure 4a). On the other hand, histological examination of the spleen of pups of mice infected in the 2nd stage of pregnancy showed depletion of lymphoid tissues (Figure 4b), while the histology of the pup spleen of mice infected in the 3rd stage of pregnancy had marked hyperplasia of mononuclear cells (Figure 4c).

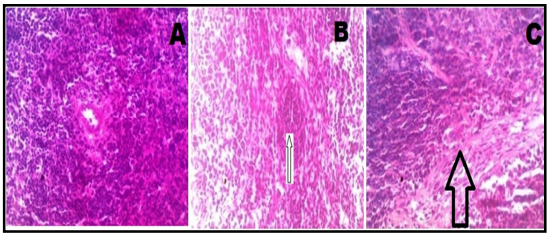


Figure 4: Section of the spleen of infected pups of mice infected at the 2nd and 3rd stages of pregnancy and spleen of non-infected pups. (A) spleen of pup of non-infected mice: showing normal appearance. (B) Spleen of pup of mice infected in the 2nd stage of pregnancy showing moderate depletion of lymphoid tissues (white arrow). (C) Spleen of infected pup of mice infected in the 3rd stage of pregnancy showing marked hyperplasia by mononuclear cells (black arrow) (x400; H&E)

This observation in the spleen may prevent breakdown of worn-out red blood cells in the pups, as the spleen is the primary site for extra medullary haematopoiesis and removal of worn-out red blood cells (Suttie, 2006).

The kidney of pups of mice uninfected showed normal kidney tissue architecture (Figure 5a). Microscopically, the kidney of pup from mice infected at 2nd stage of pregnancy showed moderate congestion of blood vessels (Figure 5b), whilethe kidney of pup from mice infected at 3rd stage of pregnancy showed moderate degeneration and necrosis of glomerular epithelial cells (Figure 5c).

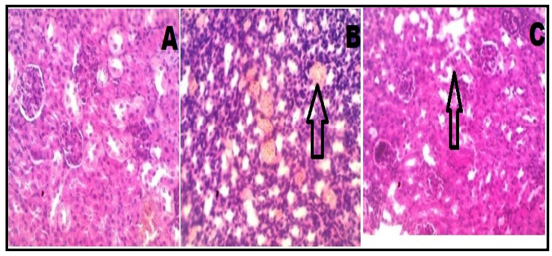


Figure 5: Section of the kidney of pups from non-infected mice and pups of mice infected at the 2nd and 3rd stages of pregnancy. (A) Kidney of pup of non-infected mice: showing normal appearance. (B) Kidney of pup of mice infected in the 2nd stage of pregnancy showing moderate congestion of blood vessels (black arrow). (C) Kidney of pup of mice infected in the 3rd stage of pregnancy: showing moderate degeneration and necrosis glomerular epithelial cells (black arrow) (x400; H&E)

Glomerular visceral epithelial cells are highly specialized and differentiated cells that play a key role in the glomerular perm-selectivity maintenance (Bijian and Cybulsky, 2005) injury of these cells may lead to glomerular scarring and dysfunction of the kidney (Schwartz and Bidani, 1993).

Histological examination of the brain tissue of pups from non-infected pregnant mice showed no distortions in the brain tissue architecture as there was no vacuolation observed (Figure 6a).

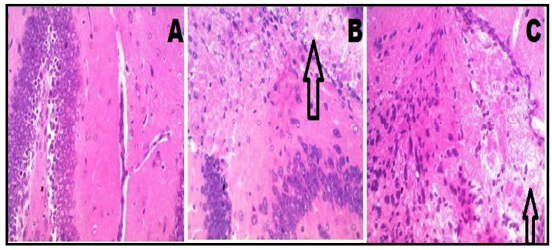


Figure 6: Section of the brain of pup of non-infected pup and mice infected in the 2nd and 3rd stages of pregnancy. (A) Brain of pup of non-infected mice: showing normal appearance. (B) Pup brain of mice infected in the 2nd stage of pregnancy showing mild vacuolation (black arrow); (C) Pup brain of mice in the 3rd stage of pregnancy showing mild vacuolation (black arrow) (x400; H&E)

The mild and moderate vacuolation observed in the brain of pups of mice infected in the 2nd and 3rd stages of pregnancy (Figures 6b and c) may

be due to the anaemic nature of the pups. Goyal *et al.* (2016) reported that risk factors such as anaemia can retard the brain development, and this may result in cognitive disorders.

Conclusion: Results obtained from this study showed that malaria infection during the 2nd and 3rd stages of pregnancy caused anaemia, fatty degeneration of hepatocytes of the liver, interstitial pneumonia and oedema of the lungs, severe lymphoid depletion of the spleen, degeneration of tubular epithelial cells of the kidney and vacuolar degeneration of the brain tissues of their progenies. Pups from mice infected in the 2nd stage of pregnancy had more severe damages in blood cells and vital organs. These alterations may compromise the condition of the organs and blood of the offspring, making it vulnerable to another infection. Therefore, early diagnosis and treatment of malaria during 2nd and 3rd stages pregnancy is very essential in order to avoid complications.

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