Research Article

The Oxidative Stress-Mediated Effects in Pregnant Mice with *Plasmodium berghei* Infection

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Abstract | Malaria during pregnancy can lead to various pathological conditions for pregnant women and fetuses such as fever, abortion, low birth weight, and fetal death. In this study, we examined differences in oxidative stress-mediated effects in pregnant and offspring mice in *Plasmodium berghei* infection. A Complete randomized design was used in this study, in which 25 mice were divided into five groups. Group 1 as a control group, consisted of non-pregnant and *Plasmodium*-uninfected mice, group 2 comprised pregnant and uninfected mice, group 3 to 5 were pregnant mice and were infected with 1 x 10^1iRBCs, 1 x 10^2 iRBCs, and 1 x 10^3 iRBCs respectively. On the fourth day of post-treatment, the parasite level was calculated. Malondialdehyde (MDA) levels of mother's and offspring's liver and spleen were observed by using the TBARS Assay Kit, also the superoxide dismutase activities. As supporting data the histological analysis of the mother's and offspring's liver and spleen were prepared by using the paraffin method. There were sigificant results in the parasites levels and the increase in *P. berghei* infection followed an increase of oxidative stress in mothers and offspring mouse in the treatment and control groups. The liver and spleen of mothers have been affected with *P. berghei* infection, however, there are still no effects on the offsprings, and the body weight of the offsprings from infected mothers were lower than uninfected mothers. This study revealed that *P. berghei* infection had different effects in oxidative stress-mediated in pregnant mice, but not their offspring.

Keywords | Parasitemic level, *Plasmodium berghei*, Pregnancy, Offspring, Oxidative stress

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INTRODUCTION

Malaria is a public health problem in the world, including in Indonesia. WHO reported that in 2016 there were 216 million cases of malaria worldwide, and 445.000 deaths (WHO, 2018). Malaria is an infectious disease caused by several species of *Plasmodium*, a single-celled protozoan. It is transmitted through the bite of a female *Anopheles* mosquito that harbours sporozoit of *Plasmodium*, which then infects and multiplies in red blood cells, leading to malaria that attacks all age groups of humans. In addition, malaria directly causes anemia, even can cause death, especially in high-risk groups like infants, toddlers, and pregnant women.

Malaria during pregnancy can lead to various pathological

conditions such as fever, anemia, abortion, and even death. In the fetus, this disease cause low birth weight and fetal death. On the other hand, clinical incidence and the degree of parasitemia are more severe in primigravida and young pregnant women (Quinn, 2002; WHO, 2018). Human malaria research has been modeled by using rodents such as mice as test animals and *Plasmodium berghei* as the parasite. This parasite has proven to mimic malaria in humans and other primates in most important aspects such as structure, physiology, and life cycle (De Niz and Heussler, 2018).

Plasmodium berghei infection results in oxidative stress manifested as a rise in reactive oxygen species (ROS) in the body of BALB/c mice. Oxidative stress is an indicator of pathogenesis in various diseases including malaria. During malarial infection, antigenic stimulation activates the immune system in the body causing the release of ROS. ROS compounds in the body are also generated by malaria parasites due to hemoglobin degradation (Akanbi et al., 2010; Sharma et al., 2012).

Sharma et al. (2012) suggested that pregnant mice infected with *P. berghei* have higher oxidative stress than non-pregnant infected mice. The malondialdehyde (MDA) level is significantly higher in the liver, spleen, kidney, and brain of infected pregnant mice than of uninfected pregnant mice (Sharma et al., 2012). Histopathologic observations of the organ tissues clearly show cellular and morphological changes that may be attributable to the increasing lipid peroxidation. The increased severity of malarial infections during pregnancy is assumed to be due to oxidative stress (Sharma et al., 2012). As a result of *P. berghei* infection, the MDA levels increased in the liver, however, the SOD activities in the liver decreased, relative to uninfected mice. The increasing levels of MDA indicate the reduced production of peroxynitrite and oxidative stress (Akanbi et al., 2012; Dogruman-Al et al., 2015).

However, *P. berghei* infection and the occurrence levels of oxidative stress in the fetus and parent were not well understood. In this study, we examined differences in oxidative stress-mediated effects in pregnant and offspring mice in *Plasmodium berghei* infection.

MATERIALS AND METHODS

Ethical approval

This study protocol was approved by the Committee of Animal Research and Ethics of the Integrated Research and Testing Laboratory (LPPT) of Universitas Gadjah Mada Indonesia (Approval Letter No. 00114/04/LPPT/IX/2017).

Experimental animals

Twenty-five female Balb/C mice were used in this study, aged 8–9 weeks and weighing 30–35 g, obtained from the reared of standard condition in the Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia.

Production of *Plasmodium berghei*

Plasmodium berghei, ANKA strain, as parasites were maintained in mice by serial passage of *P. berghei*-infected red blood cells (iRBCs) provided by the Faculty of Medicine, Universitas Gadjah Mada, Indonesia.

Assessment of pregnancy

Female and male mice were put in one cage overnight, with a ratio of 3 to 1. Pregnancy was checked by using the vaginal smear method; if sperm was found, it was assumed as the 1st day of pregnancy.

Experimental design

The mice were divided into five groups. Group 1 (nonpregnant, n=5) was injected intraperitoneal with normal saline. Group 2 (pregnant, n=5) was injected with normal saline (Offspring 1). Group 3 (pregnant-infected, n=5) was injected with 1 × 101 iRBCs as a dose 1 (Offspring 2). Group 4 (pregnant-infected, n=5) was injected with 1 × 102 iRBCs as a dose 2 (Offspring 3). Group 5 (pregnant-infected,n=5) was injected with 1 × 103 iRBCs as a dose 3 (Offspring 4). Saline or iRBCs (0.2 ml of each) were injected intraperitoneal on the 17th day of pregnancy. The offspring were weighed on the day of delivery.

Parasitemia measurement

Parasitemia was assessed by using a thin blood smear method on the 3rd day after infection. Blood was taken by cutting the tip of the tail and was stained in 3% Giemsa.

Measurement of MDA level

MDA level was measured by following the procedures of the TBARS Assay Kit (BioAssay Systems, Hayward, CA, USA). Liver tissue (~20 mg) was homogenized with 200 µL of cold PBS. Tissue lysate (100 µL) was taken and 200 µL of 10%TCA was added. The mixture was then incubated and centrifuged for 5 min (14,000 rpm in an Eppendorf Centrifuge). Standards were prepared following the kit's instructions. To each of the standards and samples were added 200 µL TBA Reagent and incubated at 100 °C for 60 min. Standards and 100 µL of samples were placed in a well and the OD was read at 535 nm wavelength.

Measurement of SOD activity

SOD enzyme activities were measured according to the T-SOD Activity Assay Kit (Elabscience, USA). Tissue samples were homogenized in the ratio of 9 g tissue: 1 mL PBS. Control, Blank control, Sample, and Blank Sample wells were prepared following the kit's instructions. Substrate application solutions (200 µL) were added to

each well and mixed fully. Reactions were incubated for 20 min at 37 ˚C, and OD was read at 450 nm.

Histological slides preparation

Separated liver and spleen tissue were cut into small pieces of approximately 3 x 1 mm and fixed in Bouin solution. The samples were dehydrated and clearing was done by toluol compound. Liver tissue samples were obtained by paraffin infiltration embedded in paraffin wax. Samples were cut at 4 µm thickness using a rotary microtome and put on the object-glass. Deparaffinization and rehydration were performed using xylene and alcohol solutions, and the sections were stained using Ehrlich's hematoxylin-eosin.

Statistical analysis

Data on parasitemic level, MDA value, SOD value, and bodyweight of mother and offsprings were analyzed with a one-way analysis of variance (ANOVA) test by using SPPS 23 program at a significant level of 5% between control and treatment groups. Significant effects were then subjected to Duncan's Multiple Range Test (DMRT) at a significance level of 5%. In addition, histological structural observations of the control livers and treatment groups were analyzed by using descriptive analysis.

RESULTS AND DISCUSSION

Level of parasitemia

The level of parasitemia varied in treatment groups infected with *P. berghei* at different doses. The percentage of parasitemia in groups 3, 4, and 5 was 5.93%, 10.27%, and 21.13%, respectively (Figure 1). These results indicated that the higher dose of amount parasite injection followed by the higher parasitemic levels.

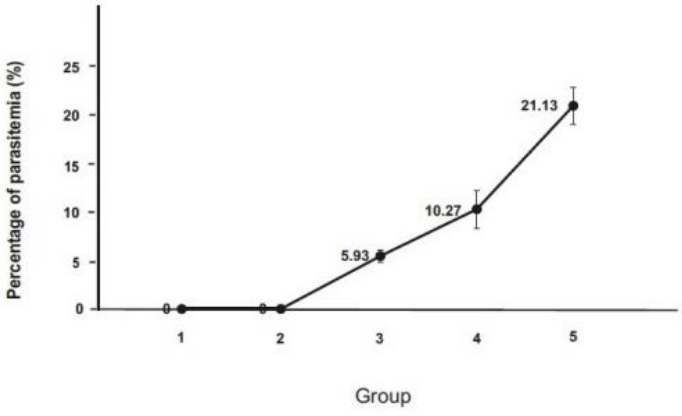

Figure 1: Level of parasitemia in mother. Group 1: non-pregnant; Group 2: pregnant (Offspring 1); Group 3: pregnant-infected was injected with dose 1 (Offspring 2); Group 4: pregnant-infected was injected with dose 2 (Offspring 3); Group 5: pregnant-infected was injected with dose 3 (Offspring 4).

MDA levels in liver and spleen

Figure 2A showed that the MDA level in the maternal liver differs between the control and the treatment groups infected with *P. berghei*. MDA levels in the treatment groups (3, 4, and 5) were significantly ($p<0.05$) higher than those in the control groups (1 and 2). MDA levels in the spleen (Figure 2B) of groups 4 and 5 were higher than those in the control groups (1 and 2), while the MDA level in group 3 did not differ from those in the control groups (1 and 2).

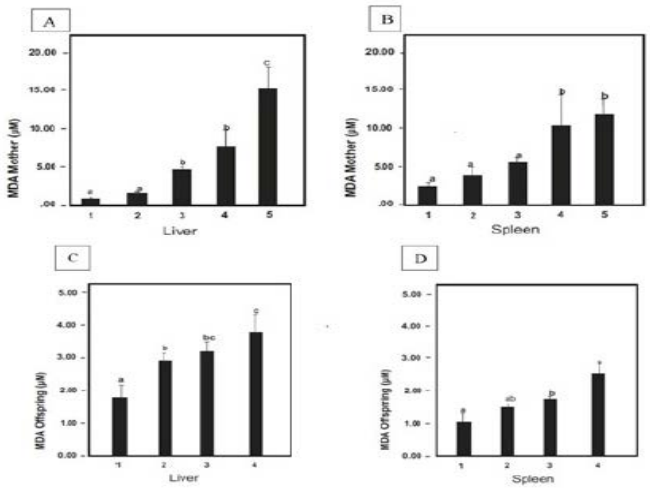

Figure 2: MDA levels of liver and spleen in mothers (A, B) and offspring (C, D). The MDA level in the maternal liver differs between the control group and the treatment groups infected with *P. berghei*. Group 1: non-pregnant; Group 2: pregnant (Offspring 1); Group 3: pregnant-infected was injected with dose 1 (Offspring 2); Group 4: pregnant-infected was injected with dose 2 (Offspring 3); Group 5: pregnant-infected was injected with dose 3 (Offspring 4). Different letters above the bars indicate a significant difference ($p<0.05$).

The offspring's liver of treatment groups showed higher MDA levels than in the control groups (Figure 2C). MDA levels in the offspring's spleen (Figure 2D) in treatment groups 4 and 5 were significantly different ($p<0.05$) with the control group (group 2), while that in group 3 was no different from the control group. Infection with high doses of *P. berghei* followed by increasing MDA levels in Balb/C mice offspring's liver and spleen.

SOD levels in liver and spleen

Figure 3A and B, showed that the SOD activities in the mother's liver and spleen of treatment groups were significantly lower ($p<0.05$) than those in the control groups.

Figure 3C and D, showed that SOD activities in both the liver and spleen of offsprings in all of the treatment groups whose mothers were infected with *P. berghei* were not

significantly different ($p<0.05$) from that in the uninfected control group.

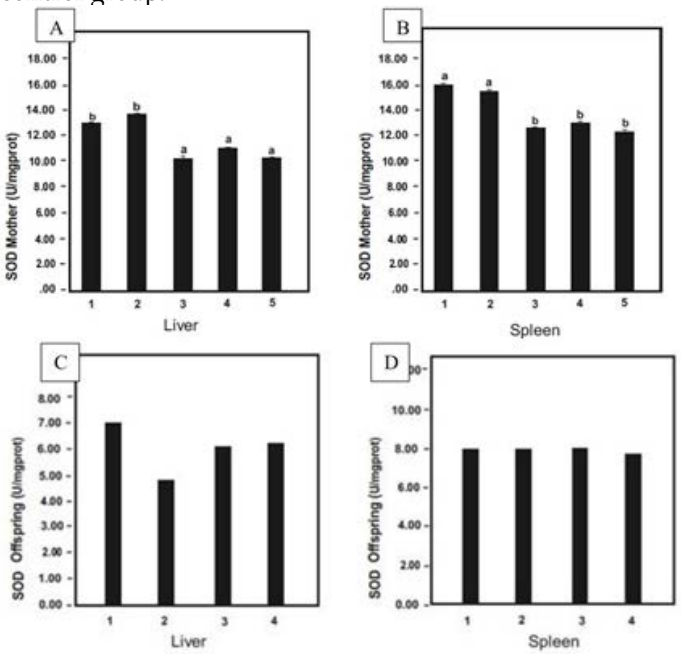

Figure 3: SOD levels of liver and spleen in mothers (A, B) and offspring (C, D). The SOD level in the maternal liver differs between the control group and the treatment groups infected with *P. berghei*. There are no significant differences on SOD Activity in offspring. Group 1: non-pregnant; Group 2: pregnant (Offspring 1); Group 3: pregnant-infected was injected with dose 1 (Offspring 2); Group 4: pregnant-infected was injected with dose 2 (Offspring 3); Group 5: pregnant-infected was injected with dose 3 (Offspring 4). Different letters above the bars indicate a significant difference ($p<0.05$).

Histological structure of liver and spleen

Histomorphometry measurement of maternal livers was shown in Table 1. It revealed a different value in the diameter of the central vein and sinusoids between the treatment and control groups. In groups 3, 4, and 5 of treatment groups showed significantly different ($p<0.05$) in the long and short diameter of the central vein and sinusoid with the control groups. In contrast, the histomorphometric measurement of the offspring's liver indicated that there were no significant differences between treatment and control groups in the long and short diameter of central vein and sinusoids. The value area of the white pulp of groups 4 and 5 was significantly different ($p<0.05$) from that of the control groups (1 and 2). The white pulp area of group 3 was not different from the positive control group (group 2) but was significantly larger than the negative control group (group 1).

Figure 4A-E, Illustrate the pathological changes of the maternal liver, in which there were differences in some cell structures between the control and treatment groups. The treatment groups showed pathological effects on the liver including necrosis, deposition of malaria pigment (hemozoin), leukocyte infiltration, and Kupffer cell hyperplasia.

Figure 4F-J, indicate the histology of the maternal spleen. The infected spleen of treatment groups (group 3, 4, and 5) showed the red pulp contained hemozoin, while in the spleen of control groups (group 1 and 2) no hemozoin was detected.

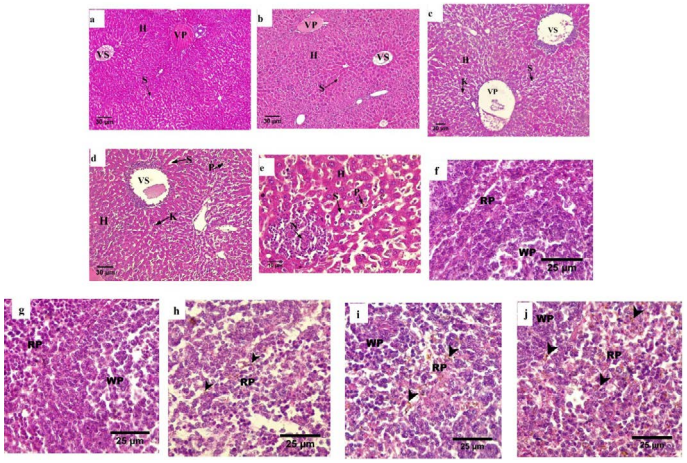

Figure 4: Hematoxylin–eosin-stained histological sections of the maternal liver (a-e) and spleen (f-j). For the liver, (a,b) normal hepatocyte devoid intracellular gaps; (c,d, e) infiltration of leukocyte and deposition of malarial pigment (H and E stained, 100x). (a) non pregnant, (b) pregnant, (c,d,e) pregnant-infected. For the spleen (f, g) normal texture of splenocytes; (h, i, j) expanded of red pulp along with the deposition of malarial pigments (H and E stained, 400x). (f) non-pregnant, (g) pregnant, (h, i, j) pregnant-infected. VS: central vein; VP: portal vein; S: sinusoid; N: necrosis; I: leukocyte infiltration; K: Kupffer cells; P: malaria pigmentation; WP: White pulp; RP: red pulp; Arrowheads: Hemozoin.

There were significant differences ($p<0.05$) in bodyweight between offspring in the maternally of treatment groups and the control groups (Figure 5). The mean of their bodyweight in group 2 was 2.184 ± 0.202 g, group 3 was 2.066 ± 0.186 g, and group 4 was 2.169 ± 0.336 g, whereas group 1 was 2.681 ± 0.036 g.

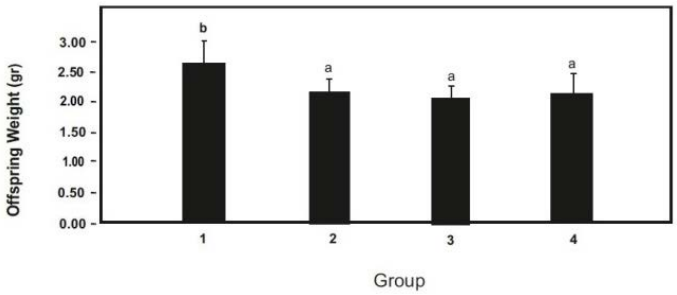

Figure 5: Offspring weight. [ab] Different letters indicate a significant difference ($p<0.05$).

Table 1: Histomorphometry of liver and spleen in mothers and offsprings.

Group	Mother liver (Mean (µm)±SD)				Offspring liver (Mean (µm)±SD)				Mother spleen (Mean (µm²)±SD)
	Long diameter of central vein	Short diameter of central vein	Long diameter of sinusoids	Short diameter of sinusoids	Long diameter of central vein	Short diameter of central vein	Long diameter of sinusoids	Short diameter of sinusoids	White pulp of lien
1	16.537 ± 1.432^{a}	8.116 ± 1.350^{a}	14.995 ± 0.488^{a}	2.619 ± 0.401^{a}	-	-	-	-	21.308.7±12.497.5^{a}
2	19.268 ± 0.092^{a}	8.270 ± 0.597^{a}	16.216 ± 1.144^{a}	2.979 ± 0.464^{a}	13.683 ± 0.564	4.570 ± 0.252	10.239 ± 1.222	2.364 ± 0.294	22.556.7±7.108.4^{ab}
3	38.331 ± 17.069^{b}	18.402 ± 7.933^{b}	20.080 ± 1.475^{b}	4.697 ± 0.404^{b}	11.980 ± 1.027	3.993 ± 0.079	11.129 ± 0.357	2.617 ± 0.158	32.027.4±12.651.3^{bc}
4	38.861 ± 8.811^{b}	16.225 ± 1.894^{b}	21.098± 1.259^{b}	4.805 ± 0.374^{b}	12.660 ± 0.422	4.050 ± 0.540	10.261 ± 0.583	2.645 ± 0.142	33.835.4±11.669.8^{c}
5	39.947 ± 5.057^{b}	18.882 ± 3.319^{b}	22.044 ± 0.995^{b}	4.995 ± 0.822^{b}	13.659 ± 1.324	3.983 ± 0.281	10.836 ± 1.470	2.497 ± 0.151	36.302.7±16.359.3^{c}

Group 1: non-pregnant; Group 2: pregnant (Offspring 1); Group 3: pregnant-infected dose 2 (Offspring 3); Group 5: pregnant-infected was injected with dose 3 (Offspring 4). SD: standard deviation.abc Different letters indicate a significant difference ($p<0.05$). was injected with dose 1 (Offspring 2); Group 4: pregnant-infected was injected with

Plasmodium is a source of malaria and can attack pregnant women. *Plasmodium* infects red blood cells and uses hemoglobin as the main source of amino acids.

Degradation of hemoglobin in the *P. berghei* food vacuole produces toxic free heme, superoxide, and hydrogen peroxide (H_2O_2), which can increase lipid peroxidation and oxidative stress in host cells (Bilgin et al., 2012). A large amount of extracellular Hb (hemoglobin) causes an increase in the rate of autoxidation, formation of superoxide, and H_2O_2 as the ROS. The reaction of H_2O_2 and extracellular Hb also leads to the release of Fe from the heme. Furthermore, ROS can damage the RBC membrane and follow to damage other cells and tissues, finally, oxidative stress conditions will be increased in the organism (Chiabrando et al., 2014; Rifkind et al., 2015). The host body has a defense mechanism to deal with free heme, namely its degradation by heme oxygenase-1 (HO-1). In severe malaria, free heme accumulates to a high level in plasma it may cause the defense system can work (Ferreira et al., 2008).

In pregnant mothers, malaria infection leads to an increase in ROS production as a response to infection and free radical production (Percário et al., 2012). The defense mechanism of *P. berghei* is by taking host SOD concentrations in large amounts to increase the amount of endogenous SOD. Inadequate levels of SOD can bring about an overabundance of superoxide radicals (Dogruman-Al et al., 2015). Besides *P. berghei* infection, the increasing of SOD activities may due to the inhibition or inactivation of O_2^- by the parent cells. Thus, the pathology of malaria goes hand in hand with excessive ROS production or decreased SOD activity (Siddiqi and Pandey, 1999).

Plasmodium can increase MDA production. The MDA levels of the mother were significantly higher in mice infected with *P. berghei* than in uninfected mice, which indicated considerable lipid peroxidation due to inadequate antioxidant capacity in the parent. As said that the increasing MDA levels indicated the increasing membrane lipid peroxidation in malaria patients (Dogruman-Al et al., 2015; Scaccabarozzi et al., 2018).

On the other hand, malaria infections can cause a decrease in SOD activities (Raza et al., 2015), which marks oxidative stress. These findings were in line with the previous researcher, that malaria caused the decreasing of SOD activities (Akanbi et al., 2012; Dogruman-Al et al., 2015; Sharma et al., 2012). SOD enzymes are constitutively produced in the body, but in the case of an imbalance between oxidative compounds and antioxidants that enter the body, there is a decrease in the ability of SOD to act as an efficient antioxidant (Murray et al., 2014).

In short, this study found that *P. berghei* infection had no direct effect on the condition of the offspring of infected mothers. Neres et al. (2008) added that placental pathology due to malaria parasites cause the uterus to have an inadequate supply of hemoglobin/iron/oxygen and nutrition to the fetus (Neres et al., 2008).

In this study, it was seen that plasmodium can cause pathology in the liver including necrosis, malaria pigment deposition (hemozoin), leukocyte infiltration, and Kupffer cell hyperplasia. Dilatation of sinusoid in the liver tissue can be observed by the necrosis, deposition of malaria pigment (hemozoin), leukocyte infiltration, and Kupffer cell hyperplasia (Monfared and Salati, 2013). The appearance of hepatocyte necrosis is highly dependent on sporozoite infection *Plasmodium*. This is the same as previous research which states that *Plasmodium* can cause hepatocyte necrosis (Asmilia et al., 2020).

Generally, the histological structure of the infected liver with malaria parasites shows that sinusoidal cells dilate and contain a large amount of hemozoin (Kuntz and Kuntz, 2006). Hemozoin is a waste product arising from the digestion of red blood cells by the malaria parasite and is observed as black or brown granules under a conventional light microscope (Pham et al., 2021).

Kupffer cells are specifically located in the periportal sinusoid in the liver and structurally hyperplasia in Kupffer cells occurs because of *P. Berghei* infection. According to Nobes et al. (2002) the higher level of parasitemia may cause the greater activities of Kupffer cells phagocytic capacities. Increased Kupffer cells may become a defense response to clean up remaining infected erythrocytes and malaria parasites (Nobes et al., 2002).

Mouse spleens infected with malaria undergo expansion of white pulp, loss of structure in the germinal center, activation of T and B cells, and loss of marginal zones in the white pulp so that the boundaries of white and red pulp become blurred (Carvalho et al., 2007; Kumar and Bagai, 2014). B cells and antibodies play an important role in the development of immunity against malaria infection. The germinal center area of the follicle is reduced in the spleen infected with malaria, which is an indication of the B cells differentiation into plasma and memory cells (Perez et al., 2017; Urban et al., 2005).

Likewise, the histologic structure of the liver and spleen in offspring showed no deposition of malaria pigment or other pathologies. The absence of abnormalities in the offspring's spleen may be caused by the formation of marginal zone structure and the white pulp of the mouse's spleen begins at the developmental stage after birth. Meanwhile, the formation of red pulp begins during the

development of the embryonic spleen (Tan and Watanabe, 2018). Our results agree with those obtained by Neres et al. (2008) who reported that parasites and hemozoin were not found in the circulation of offspring and that positive parasitemia was never recorded in newborns of infected mothers (Neres et al., 2008). The lack of evidence for congenital infection, despite the large numbers of infected erythrocytes found in the maternal blood of the placenta, demonstrates the effectiveness of the placental trophoblast layer in preventing parasites from accessing fetal blood (Albieri et al., 2005). These findings suggested that the absence of an effect of *P. berghei* infection in the mother on the offspring's histomorphometry and histopathologic was due to malaria parasites being unable to cross the placenta.

The present results highlighted that the offspring's weight in treatment groups in which the infected mothers were lower than the control groups, in which the uninfected mothers. This concurs with a report by Neres *et al*. that *P. berghei* infection in pregnant mice caused the fetus to be underweight, and some offspring died in the womb (Neres et al., 2008). The effect of maternal malaria on fetal status is believed to be caused by placental insufficiency (Avery et al., 2012).

Based on that results, this study revealed that infection of *P. berghei* significantly increases the oxidative stress effects in mothers, and it has pathological changes effects on the maternal liver and spleen. On the other hand, in offspring infection of *P. berghei* have no oxidative stress effects and pathological changes effects on the liver or spleen, but the bodyweight of the offsprings from infected mothers was lower than from uninfected mothers.

CONCLUSIONS AND RECOMMENDATIONS

Based on the results, this study revealed that infection of *P. berghei* significantly increases the oxidative stress effects in mothers, and it has pathological changes effects on the maternal liver and spleen. On the other hand, in offspring infection of *P. berghei* have no oxidative stress effects and pathological changes effects on the liver or spleen, but the bodyweight of the offsprings from infected mothers was lower than from uninfected mothers.

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NOVELTY STATEMENT

There has been a lot of research on malaria in pregnancy, but no one has seen its effect on offspring. Therefore, in this study, we want to see how the incidence of oxidative stress in mothers and offsprings caused by Plasmodium berghei infection. *P. berghei* can cause abnormalities in the liver and spleen in the mother, but not their offspring.

AUTHOR'S CONTRIBUTION

HTSS, SS, UNF, and SHP contributed equally to the experimentation. HTSS, RRUNWA, SI, and RFY wrote and edited the article. HTSS, SS, and UNF equally designed the experiment. All authors read and approved the final manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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