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Mice Pregnancy Failure Due to Malaria: The Role of TNF-α, Anemia, and Low Birth Weight

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A b s t r a c t

Background*: Malaria infection in pregnant women or called placental malaria is characterized by the accumulation of Plasmodium-infected red blood cells in the intervillous space of the placenta. This causes adverse perinatal outcomes such as stillbirth, low birth weight, premature birth, and small neonates of gestational age while in the mother it causes anemia. Inflammatory responses such as TNF-α expression can promote complications in pregnancy. TNF-α plays an important role in the immune response in acute malaria but inhibits erythropoiesis.* ***Objective****: This study aims to determine the relationship between malaria infection and TNF-α expression with the incidence of anemia and birth weight in pregnant mice infected with Plasmodium berghei.* ***Methods****: Twenty BALB/C pregnant mice were divided into 2 groups, control group (10 pregnant mice without infection) treatment group (10 pregnant mice infected with Plasmodium berghei). TNF-α expression was observed by immunohistochemical method using anti-TNF-α Chip Grade antibody from abcam, anemia examination using Cyanmethemoglobin and all fetuses were weighed using an analytical balance. Statistical analysis using Structural Equation Modeling.* ***Results****: Malaria infection causes high expression of TNF-α in the placenta (t_{count}=2.97≥ t_{table} = 1.96), causes anemia (t_{count}=1,97≥t_{table} = 1.96) and causes low fetal weight t_{count}=2,16 ≥ t_{table} =1, 96.* ***Conclusion****: Malaria infection can cause high expression of TNF-α in the placenta causing anemia and low birth weight of the fetus.*

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INTRODUCTION

Malaria is a blood-borne disease caused by *Plasmodium spp.*[1] Pregnant women, especially mothers who have given birth for the first time, are at high risk of developing severe malaria due to *P. falciparum*. Long-term exposure to the parasite in childhood can result in the development of protective antibodies however, first-time pregnant women are at high risk.[2] Their susceptibility could be attributed to the erythrocyte membrane protein *P. falciparum-1* (PfEMP-1), a major variant surface antigen displayed on the surface of *P. falciparum*-infected erythrocytes (IEs) that functions as an adhesin.[3]

Malaria infection in pregnancy is a major cause of maternal death, maternal anemia, and adverse pregnancy outcome (spontaneous abortion, preterm delivery, growth restriction/low birth weight, stillbirth, congenital infection, neonatal mortality) in geographic areas where malaria infection occurs commonly in pregnant women.[3] Pregnant women are particularly vulnerable to *Plasmodium falciparum* infection because red cells are infected with the parasite can sequester in the placenta, and thereby cause adverse fetal effects. If anti-malarial drugs do not achieve therapeutic levels in the placenta, parasites sequestered there may be released intermittently into the peripheral blood and cause recurrent maternal infection.[4]

Plasmodium infection in pregnancy is the occurrence of placental sequestration. Infected erythrocytes bind to chondroitin sulfate A, which is present on the surface of the syncytiotrophoblast lining the intervillous space of the placenta, thereby avoiding splenic clearance. This process is mediated by the parasite antigen VAR2CSA. Placental malaria triggers a cascade of inflammatory processes leading to impaired placental development and transplacental transfer of nutrients. In areas of moderate to high transmission intensity, women with placental infection are often asymptomatic and apathetic, and the risk of placental malaria decreases with increasing gravidity.[5]

Pregnant women and children under five years of age have an increased risk of severe complications in malaria-infected areas. It is well accepted that *P. falciparum* infection in pregnant women is known to cause maternal and fetal morbidity and mortality. However, there are emerging reports worldwide of *P. vivax* infection also resulting in maternal anemia and low birth weight A.[6,7,8]

The condition of anemia is exacerbated by suppression of the bone marrow, thereby inhibiting erythropoiesis caused by an increase in TNF- α.[9] Tumor necrosis factor-α (TNF-α) inhibits all stages of erythropoiesis by inhibiting erythroid growth and differentiation through high expression of TRAIL and CD95L in erythroblast development.[10]

METHOD

Research Design

This study is a pure experimental study carried out in the laboratory in vivo by comparing the results obtained in pregnant infected mice with controls. The experimental animals used in this study were mice of the BALB/c strain. The use of mice as experimental animals is based on several reasons, including mice are experimental animals that are easy to handle, easy to maintain, and easy to breed. Meanwhile, the selection of the BALB/c line was because this strain is a good model in demonstrating the status of malaria, especially placental malaria. This study used 2 groups of mice, namely: the control group and the other group as the treatment group (the group of mice infected with *P. berghei*).

Sample Size

20 mice needed for this study. Based on the theory that to get mice of the same gestational age through marriage in pairs for one night the pregnancy rate of success

(pregnancy rate) is 40-70%. The mice needed in this study were 50 pairs.

Research Plate

The research was conducted at the Parasitology Laboratory of the Faculty of Medicine and Health Sciences, Universitas Islam Maulana Malik Ibrahim, Malang in January - November 2020.

Mice Pregnancy

Pregnancy of 25 female mice and 25 male mice in total is 50, then carried out after preparation for estrus synchronization through three stages, namely Lee-Boot effect, Pheromones effect and Whiten effect.

Inoculation of *Plasmodium berghei* strain ANKA for Pregnant Mice

Inoculation of experimental animals was carried out on the ninth day after mating of mice (organogenesis). Inoculation was done by inoculation of *Plasmodium berghei* strain ANKA (first pass) intraperitoneally at $1x10^6$/ml. Parasitemia of donor mice was calculated from a thin blood smear that was taken from the tip of the tail and then stained with Giemsa dye. The number of parasites was counted per 1000 erythrocytes with a 1000x magnification microscope. To count the number of erythrocytes, 10 L of blood was taken from the tail end of the donor's blood and diluted 10^3 with PBS solution. Then the number of erythrocytes was counted in the Neubauer counting chamber. so that the number of erythrocytes/ml of blood is known by the formula ($N\times5\times10^4\times$dilution), where N is the number of erythrocytes. Furthermore, the number of donor mice parasites was calculated by multiplying the number of erythrocytes/ml of blood by the percentage of parasitaemia. The number of parasites to be given is 1×10^6/ml of blood, so the dilution to get the number of parasites is the number of parasites/1×10^6 ml.

Inoculation in experimental animals was carried out by injecting diluted donor mouse parasitemia into the peritoneal cavity of experimental animals. The implementation procedure is as follows: holding the mouse at the nape of the neck, turn the mouse so that the abdomen is visible, with the head lower than the body. Clean the area to be injected with 70% ethanol. A sterile needle should be inserted into the lower right or left quadrant of the mouse abdomen, inserting the needle at an angle of 30 degrees. Aspiration ensures correct puncture, and inject material.

Giemsa Blood Smear and Staining

Ten microliters of blood from the tails of mice were then dripped onto an object glass. The droplets are smeared and then dried, then given absolute methanol until evenly distributed and waited for it to dry, after that the smears are painted with Giemsa which is a mixture of Giemsa slumber with Giemsa buffer in a ratio of 1:9 for 30 minutes. Then rinsed with water and then dried. The degree of parasitemia was seen by examining the blood smear with a 1000x magnification microscope. For the calculation of the percentage of parasitemia is calculated based on the number of erythrocytes infected with malaria in 1000 erythrocytes. Blood sampling for measuring the degree of parasitemia was carried out every day to determine the increase in parasitemia. Starting from the tenth day of gestation until the 18^{th} day, the mice were dissected.

Anemia Examination

This was done by measuring the hemoglobin level of mice using the Cyanmethemoglobin method. Blood thinning was carried out using Drabkins solution. The absorbance measurement of the solution was carried out with a spectrophotometer at 540 nm. First of all, the blood in the vial is sucked with a hemoglobin pipette about 0.5 ml then the blood is put in a test tube containing 5 ml of Drabkins solution. For mixing and oxygenation, the pipette is blown vigorously against the bottom of the tube. Then the blood solution was transferred to a

spectrophotometer cuvette and read at 540 nm. Drabkins solution was used as a blank. Normal mice Hb levels are around 13-16 g/100 ml. So, if <13 g/100 ml is considered as anemia.

Examination of TNF-α in Liver

TNF-α examination was carried out using the immunohistochemical method at the Anatomical Pathology Laboratory of FKUB. For immunohistochemical staining on slides, an anti-TNF-α Chip Grade antibody kit from Abcam.

Data Analysis

The data were analysed by using Structural Equation Modeling (SEM) method with true tool of Smart Partial Least Square (PLS) software. The purpose of this analysis was to build and test the statistical model in the form of causal model.

Research Ethics

The Health Research Ethics Committee (KEPK) of the Faculty of Medicine and Health Sciences, State Islamic University of Maulana Malik Ibrahim Malang, has provided ethical clearance in this study with the number of Ethics Statement Letter No. 028/EC/KEPK-FKIK/2020. In vivo, experimental animals are treated as well as possible so as not to hurt during treatment. Neck dislocation method that is suitable for euthanasia techniques in experimental animals.

RESULT

Pregnancy of mice is carried out simultaneously after estrus synchronization by utilizing biological phenomena, namely the Lee-Boot effect, pheromone effect and whiten effect. From a total of 40 mice that were mated, on the eighteenth day after mating there were Twenty pregnant mice were eligible in this study, namely ten mice from the infected/treatment group and ten mice from the uninfected/control group.

Inoculation of *Plasmodium berghei* strain ANKA for pregnant mice was carried out on the ninth day after mating of mice (organogenesis). Inoculation was carried out intraperitoneally as much as $1x10^6$/ml (10^6 parasites in 0.2 ml of injected blood) on the ninth post-breeding day. The measurement of the degree of parasitemia was carried out on the eighteenth day before the mice were dissected.

The calculation of the degree of parasitemia was carried out for 9 days before surgery for mice by counting the number of infected erythrocytes among 1000 erythrocytes from a thin smear of mouse blood using a microscope with 1000 times magnification. The average degree of parasitemia can be seen in Figure 1.

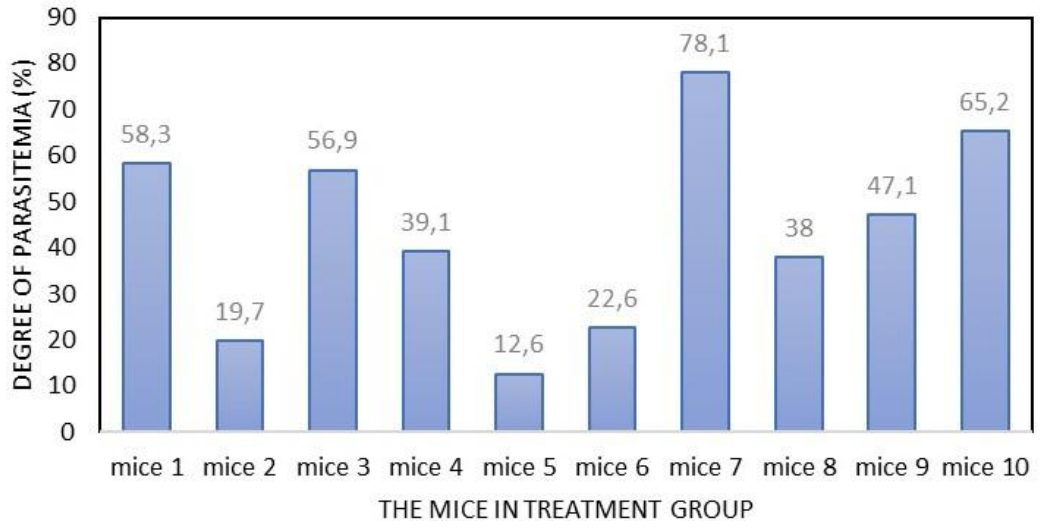

Figure 1. Average Parasitemia Degrees in Mice

Based on the bar chart in Figure 1, it can be concluded that the highest parasite density was found in the treatment group (78.1%) and the lowest parasite density was found in the treatment group 5 (12.6%).

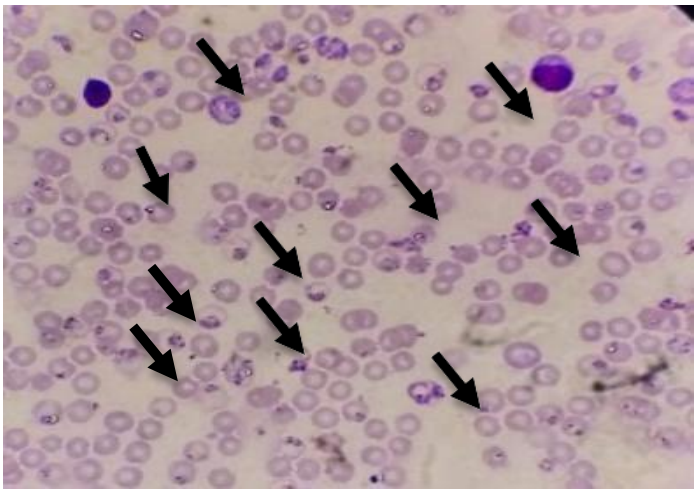

Figure 2. Erythrocytes Infected with *Plasmodium berghei*. The arrows indicate erythrocytes infected with *Plasmodium berghei* with various stages, including old ring stage, old schizont, and mature schizont.

TNF-α expression in placental tissue between the control group and the treatment group was carried out by the immunohistochemical method. Figure 3 shows the differences in TNF-α expression in placental tissue between the control group and the treatment group in the box-plot diagram as follows.

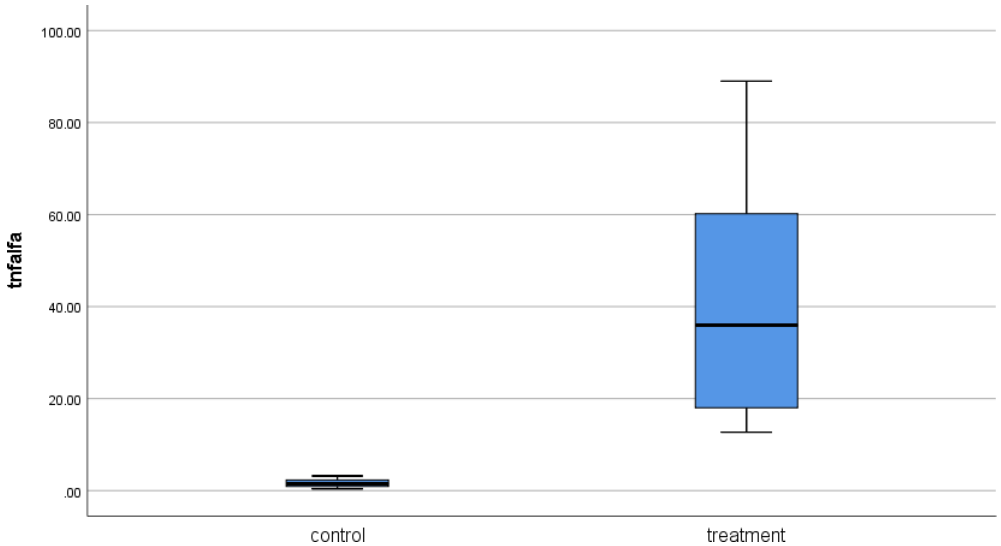

Figure 3. Placenta TNF-α Expression in the Control Group and the Treatment Group. TNF-α expression in the control group (1.66 ± .29822) was higher than in the treatment group (40.78 ± 8.14).

Examination of Hb levels between the control group and the treatment group was carried out using the Cyanmethemoglobin method. Figure 4 shows the differences in the examination of Hb levels between the control group and the treatment group in the box-plot diagram as follows:

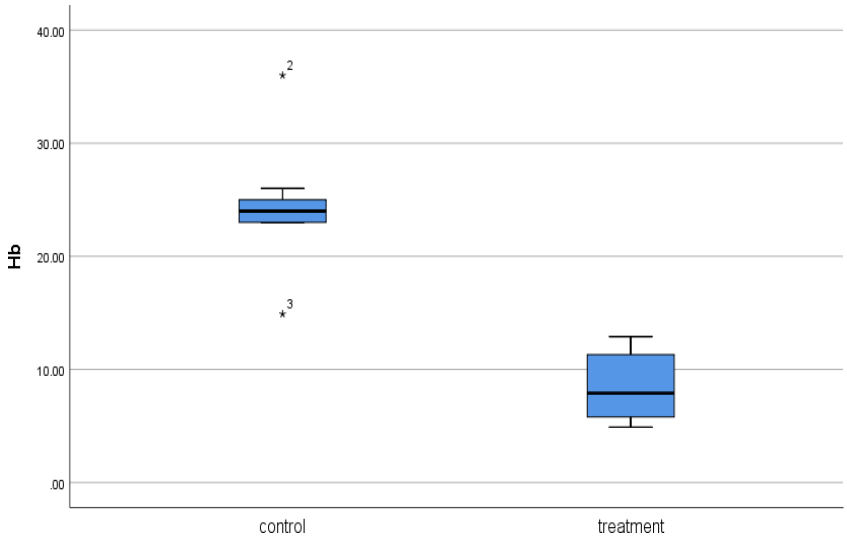

Figure 4. Hemoglobin Levels in the Control and Treatment Groups. Hemoglobin levels in the control group (24.49 ± .1.606) were higher than those in the treatment group (8.35 ± .958).

Fetal weight on the scales with analytical scales. The average fetal weight between the control group and the treatment group. Figure 5 shows the body weight of mice between the control group and the treatment group in the box-plot diagram as follows.

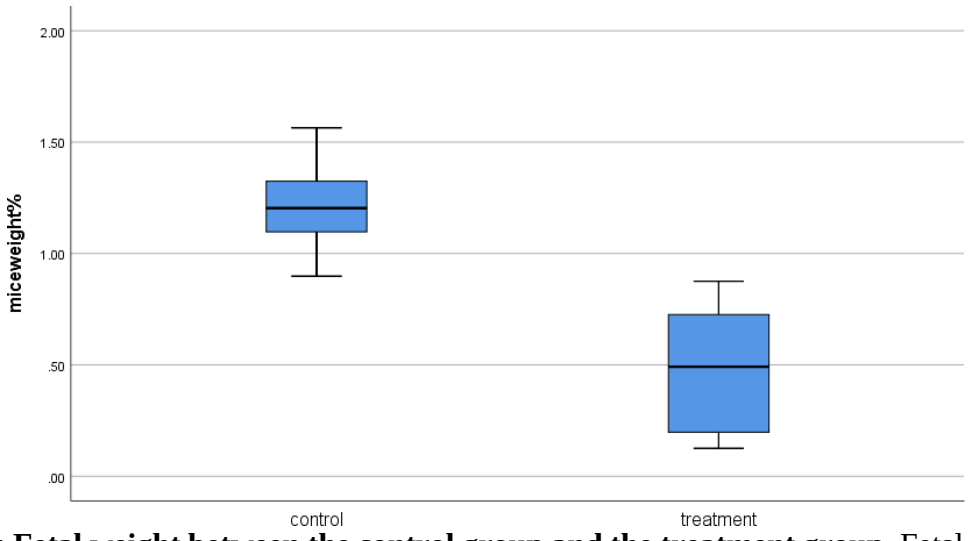

Figure 5: Fetal weight between the control group and the treatment group. Fetal weight in the control group (1.227 ± .0633) was higher than that in the treatment group (.4942 ± .08677).

This structural model was generated from statistical analysis using non-parametric structural equation modeling (SEM) using smart partial least squares (PLS). The study uses a confidence value of p = 0.05, so it is said to be meaningful if the calculated T value is 1.96. The structural model of the relationship between Plasmodium berghei infection, TNF alpha levels and fetal weight is as follows:

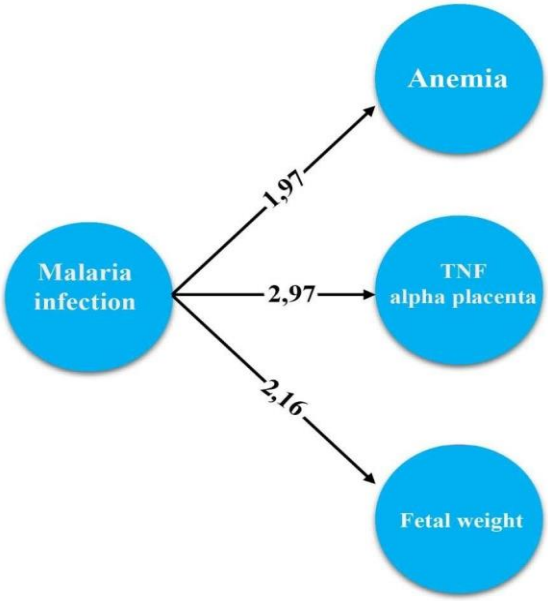

Figure 6. **Structural Equation Modeling (SEM) to Determine the Significance of the Relationship between *Plasmodium berghei* Infection, TNF Alpha Placental Tissue, Anemia and Fetal Body Weight**. Malaria infection can cause anemia (t_{table}=1.96≥t_{count}=1.97); caused high expression of placental TNF alpha (t_{table}=1.96≥ t_{count} =2.97); causes low birth weight (t_{table}= 1.96≥t_{count}=2.16).

DISCUSSION

Various studies have reported the main side effects of malaria in pregnancy, one of which is maternal anemia and low birth weight infants.[11,12] Pregnant women are susceptible to infection, although previously acquired immunity may be due to their lower immune response and reduced ability to clear malaria effectively. During pregnancy, the parasite experiences a lot of cytoadherence in the placenta rather than in the periphery because the placenta contains a lot of nutrients and oxygen.[13] In this study, malaria infection caused anemia (t_{table}=1.96≥t_{count}=1.97).

Malaria-infected pregnant women develop antibodies that inhibit the binding of infected erythrocytes to Chondroitin Sulfate A (CSA) these antibodies associated with protection against malaria infection in the placenta. Primigravida mothers have a much higher susceptibility to malaria infection than multigravida mothers. This is because antibodies obtained after multiple pregnancies reduce the number of infected erythrocytes sequestered in the placenta, thereby reducing the severity of subsequent pregnancies.[14]

It is important to note that anemia is particularly problematic as hemoglobin levels fall due to a greater expansion of plasma volume compared to an increase in red blood volume, in the case of the second trimester. It has been shown that anemia increases when there is an increase in the number of malaria cases,[15] and the incidence of anemia during pregnancy is exacerbated in settings of high malaria transmission. The detrimental effects of *P. falciparum* infection in pregnancy are most pronounced in women in their first pregnancy.[16] In addition to malaria parasite-induced hemolysis during infection, iron deficiency exacerbated by nutritional deficiency can lead to iron deficiency.[17]

Pregnant women, especially primigravida, are particularly susceptible to malaria infection, which results in maternal anemia and low birth weight infants. Placental TNF-alpha concentrations were significantly higher in the presence of maternal anemia (Fried *et al.*, 2008). In this study, it was found that malaria infection

caused high TNF-α expression (t_{table}=1.96≥t_{count}=2.97).

Pregnancy is an event of immunologic tolerance, whereby a woman accepts the implantation of the fetal allograft in her uterus (Robertson, *et al.*, 1994). Murine studies suggest a bias toward type 2 responses and away from type 1 responses during a successful pregnancy.[18] In the mouse model of pregnancy, elevated concentrations of type 1 cytokines and TNF-α cause stillbirth, abortion, and maternal anemia.[19,20] In humans, TNF-α is often elevated during severe malaria syndromes other than maternal malaria, and increased concentrations of these inflammatory mediators are associated with mortality.[21]

There are several adverse outcomes associated with malaria, affecting both mother and new born, including stillbirth, premature birth, maternal and infant mortality, congenital malaria, maternal anemia and low birth weight (LBW, i.e., birth weight < 2.5 kg).[22] Low birth weight is the most common occurrence of malaria infection in pregnant women. In 2019, it was estimated that there were 33 million pregnancies in 33 countries with moderate to high malaria transmission in the World Health Organization (WHO) Africa region. Thirty-five percent of these pregnancies were infected with malaria, and an estimated malaria infection during pregnancy was 822,000 babies with low birth weight.[1]

CONCLUSION

Malaria infection in pregnancy has a negative impact. We conclude that malaria infection can cause anemia so that TNF-alpha expression has an impact on low birth weight.

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