High Level of Plasma Tumour Necrosis Factor-α (TNF-α) in Pregnant Mice Infected with Plasmodium berghei is Strongly Related to Low Level of Hemoglobin but not Related to Fetal Low Weight

Kadar Plasma Tumor Necrosis Factor-α (TNF-α) Tinggi pada Mencit Hamil Terinfeksi Plasmodium berghei Sangat Terkait dengan Tingkat Hemoglobin Rendah bukan pada Berat Janin Rendah

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ABSTRACT
Malaria infection in pregnancy may increase the morbidity and mortality for both mother and fetus. In pregnant women, it can lead to severe anemia, cerebral malaria, pulmonary edema, renal failure and even death, while in the fetus it can cause abortion, premature birth, low birth weight, and fetal death. Elevated levels of tumor necrosis factor-α (TNF-α) are associated with low birth weight and anemia in pregnant women. This study was conducted to measure the levels of TNF-α in plasma and hemoglobin levels as well as fetal weight to determine the relationship among them in P. berghei infected pregnant mice and normal pregnant mice. Seventeen BALB/c mice used in this study were divided into two groups, those were the study group (9 pregnant mice infected with P. berghei) and control group (8 pregnant mice not infected with P. berghei). Levels of TNF-α were measured using Enzyme Linked Immunosorbent assay (R&D Systems, catalog A00B MT). Hemoglobin levels were determined using flowcytometri, whereas fetal weights were measured with Mettler analytical balance AE 50. T-test statistical analysis showed that the levels of plasma TNF-α in study group were higher than control group (p=0,000). Hemoglobin levels in the study group were lower than control group (p=0,025). Fetal weights were also lower in fetuses of infected mice than fetuses of uninfected mice (p=0,002). Pearson correlation test showed that increasing plasma levels of TNF-α in infected P. berghei pregnant mice were related with the decreasing levels of Hb, (p=0,020; r=-0,748). However plasma levels of TNF-α were not associated with the incidence of fetal low weight (p=0,380, and p=0,365). It can be concluded that the increasing levels of TNF-α is associated with decreasing levels of hemoglobin (Hb), but not associated with fetal low weight.

Keywords: Birth weight, hemoglobin, Plasmodium berghei, Tumor Necrosis Factor-α (TNF-α)

ABSTRAK
Infeksi malaria pada masa kehamilan dapat meningkatkan morbiditas dan mortalitas ibu dan janin. Pada wanita hamil, infeksi malaria dapat menyebabkan anemia berat, malaria serebral, edema paru, gagal ginjal, dan bahkan kematian, sedangkan pada janin dapat menyebabkan aborsi, kelahiran prematur, berat badan lahir rendah, dan kematian janin. Peningkatan kadar Tumor Necrosis Factor-α (TNF-α) berhubungan dengan berat badan lahir rendah dan anemia pada ibu hamil. Penelitian ini dilakukan untuk mengukur dan menentukan hubungan kadar TNF-α dalam plasma dan tingkat hemoglobin serta berat janin pada tikus hamil terinfeksi P. berghei dan tikus hamil normal. Tujuh belas tikus BALB/c yang digunakan dalam penelitian ini dibagi menjadi dua kelompok, kelompok studi (9 tikus hamil yang terinfeksi P. berghei) dan kelompok kontrol (8 tikus hamil tidak terinfeksi P. berghei). Tingkat TNF-α diukur dengan menggunakan enzim Linked Immunosorbent assay (R&D Systems, katalog A00B MT). Kadar hemoglobin ditentukan dengan menggunakan flowcytometri, sedangkan bobot janin ditimbang dengan menggunakan timbangan analitis Mettler AE 50. Analisis statistik T-test menunjukkan bahwa tingkat plasma TNF-α dalam kelompok studi lebih tinggi daripada kelompok kontrol (p=0,000). Kadar hemoglobin dalam kelompok studi lebih rendah dari kelompok kontrol (p=0,025). Bobot janin juga lebih rendah pada janin tikus yang terinfeksi dibanding janin tikus yang tidak terinfeksi (p=0,002). Uji korelasi Pearson menunjukkan peningkatan kadar plasma TNF-α pada tikus hamil yang terinfeksi P. berghei berhubungan dengan menurunnya kadar Hb, (p=0,020; r=-0,748). Namun kadar plasma TNF-α tidak berhubungan dengan berat badan rendah janin (p=0,380 dan p=0,365). Dapat disimpulkan bahwa tingkat peningkatan TNF-α berhubungan dengan penurunan tingkat hemoglobin (Hb) tapi tidak berhubungan dengan berat badan rendah janin.

Kata Kunci: Berat badan lahir, hemoglobin, Plasmodium berghei, Tumor Necrosis Factor-α (TNF-α)

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INTRODUCTION

Pregnant women are more susceptible than non pregnant women to malaria, and this susceptibility is highest in the first pregnancy. The incidence of malaria infection and anemia in primigravidae is higher compared with multigravidae subjects (1,2). Susceptibility to pregnancy-associated malaria represents a combination of immunological and hormonal changes associated with pregnancy (3-5). Malaria infection in pregnant women are more easily occurs because of changes in immunity status during pregnancy, from Th1 to Th2 dominance (6).

The manifestation of Plasmodium falciparum infection in pregnant women can also be more severe and complicated compared to the non pregnant population, includes severe anemia, fever illness, hypoglycemia, cerebral malaria and pulmonary edema (4,7,8). Malaria infection during pregnancy also increases the risk of puerperal pyrexia, abortion, prematurity (9), anemia, intrauterine death in pregnancy woman (10), and low birth weight (11) as well as intrauterine growth retardation babies (12,13).

The strong Th1 responses during pregnancy especially increased levels of TNF have been associated with low birth weight (LBW), maternal anemia, spontaneous abortions, and premature deliveries (14). The clearest finding has been an association between raising levels of TNF-α and lowering of babies's birth weights (12,15) including low birth weight due to fetal growth restriction (16) and preterm delivery (17).

The mechanism of TNF-α in causing anemia and other TNF-α related complications in pregnant women remains unclear and the downstream consequence of high TNF-α production has been linked to fetal growth restriction is intriguing, since fetal growth restriction seems to result from chronic infection (14). This study was performed to determine the effect of Plasmodium berghei infection in pregnant mice on the levels of plasma TNF-α, hemoglobin level, the fetal birth weights and the relationship among them.

METHODS

Research Design and Samples

This experimental study was conducted using 50 adult female BALB/c mice 13-15 weeks old, 20-30 grams in weight, obtained from the Integrated Research and Testing Laboratory University of Gadjah Mada. In order to get a group of pregnant mice with the same gestation, their estrus were synchronized cycles using Leebot, Pheromone and Whitten effects and then mated simultaneously in pairs within one night (18). They were then divided into two groups and observed daily. On the ninth day post mating (estimated to be the second trimester of pregnancy), the study group was infected by Plasmodium berghei.

On the 18th day post mating (estimated to be the third trimester of pregnancy), the mice were sacrificed humanely by placing them into plastic cages containing cotton wool soaked with chloroform. Intra-cardiac puncture for blood collections were performed immediately, the gravid uterus were weighted individually and the placentas were isolated and stored in -80°C.

The measurement of levels of TNF-α, and fetal weight were conducted at the Laboratory of Parasitology and Central Biomedical Laboratory Faculty of Medicine, University of Brawijaya Malang. The hemoglobin levels were measured at the private laboratory. The level of TNF-α in plasma were measured using Quantikine ELISA (R & D Systems, catalog A00B MT). Hemoglobin levels were determined by hematoanalyzer and fetal birth weight were measured using analytical scale (Mettler AE 50). His study had received ethical clearance from the Ethics Committee of Health Researches of the Faculty of Medicine, University of Brawijaya No. 104/EC/KEPF – S2/03/2013.

Preparations and Inoculation of Plasmodium berghei

Plasmodium berghei ANKA strain were obtained from Central Biomedical Laboratory, Faculty of Medicine University of Brawijaya. Pellet of erythrocytes infected with P.berghei from the storage of liquid nitrogen tank with -135°C temperature was thawed and centrifuged at 2000 rpm (0,26832 G) with Sentrifuse Hettich Mikro 2R for 5 minutes, and washed twice in RPMI medium and diluted as needed for inoculation intra-peritoneally (ip) to donor mice. Mice in the study group were inoculated as much as 10^5 parasites in 0,2ml of blood per mice.

The degrees of parasitemia were measured by examining slides of thin blood smear taken from the tip tail of mice with 1000x magnification and counted the number of infected erythrocytes per 1000 erythrocytes by two different observers.

Examination Levels of Plasma TNF-α

The levels of TNF-α in plasma were determined by ELISA (Enzyme Linked Immuno Assay). Fifty µL of assay diluent RD1-38 were added to each well that had been coated with the primary antibody. Standard, control, and samples was added at 50µL per well, mixed by shaking the plate for 1 minute then covered with adhesive strip and incubated for 2 hours at room temperature. Each samples were aspirated and then washed 4-5 times, by filling each well with wash buffer (400µL) and spraying with dispenser. After the last wash, the remaining wash buffer were removed by inverting the plate on a clean paper towel. Furthermore 100µL of secondary antibody which had been conjugated with biotin was added to each well. The plate was covered with a new adhesive strip and incubated for 2 hours at room temperature. The washing process was repeated 3 times. After that, 100µL substrate solution was added to each well and incubated for 30 min at room temperature and protected from light. Finally, 100µL stop solution was added to each well. Within 30 minutes the plate was read under ELISA reader at a wavelength of 450nm.

RESULTS

Among those of 50 female mice used in this research, when the operation was performed on the 18th days post mating, there were only 17 successfully pregnant mice, those were nine mice from the study group and eight mice from the control group.

The degree of parasitemia on 18th days post mating in pregnant mice that inoculated successfully with P.berghei were examined by two observers. Data show that the average parasitemia degree is 41,93±21,55 with non-homogeneous distribution.

The Comparison between Level of Plasma TNF-α, Hemoglobin and Fetal Weights in Study Group and Control
The comparison between measured variables is shown on Table 2. There are significant differences between the levels of TNF-α plasma in the study group and control group \((p=0.000)\). The level of plasma TNF-α in infected pregnant mice is significantly higher than non-infected pregnant mice \((p=0.000)\). There is a significant difference on the level of hemoglobin \((p=0.025)\) and fetal body weights between the study group and the control group \((p=0.001)\). The level of TNF – α plasma as well as fetal body weight was higher compared to control group. In contact the control group has higher hemoglobin level compared to the study group.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Study Group</th>
<th>Control Group</th>
<th>(t)-Test</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levels of TNF-α plasma (pg/ml)</td>
<td>137.17±51.08</td>
<td>39.05±18.31</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Levels of hemoglobin (gr/dl)</td>
<td>10.69±1.23</td>
<td>13.01±1.36</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>Fetal weights (gr)</td>
<td>0.63±0.11</td>
<td>0.94±0.19</td>
<td>0.001</td>
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</tbody>
</table>

The relationship between the degree of parasitemia with levels of plasma TNF-α is not significant \((p=0.257; r=-0.423)\) as well as the relationship between the degree of parasitemia with hemoglobin levels \((Hb)\) \((p=0.086; r=0.602)\). Relationship analysis between levels of TNF-α in plasma and hemoglobin with fetal weights using 5% confidence, interval \((α)\) shows that there are no relationships between levels of plasma TNF-α with fetal body weight \((p=0.380; r=0.334)\), but there is a significant relationship between levels of plasma TNF-α with hemoglobin levels \((Hb)\) \((p=0.020; r=-0.748)\). There is also no significant relationship between the fetal weights and the level of hemoglobin \((p=0.774; r=0.112)\).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>(r)</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parasitemia degree vs TNF-α</td>
<td>-0.423</td>
<td>0.257</td>
</tr>
<tr>
<td>Parasitemia degree vs Hb</td>
<td>0.602</td>
<td>0.086</td>
</tr>
<tr>
<td>TNF-α vs fetal body weight</td>
<td>0.334</td>
<td>0.380</td>
</tr>
<tr>
<td>TNF-α Hb</td>
<td>-0.748</td>
<td>0.020</td>
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**DISCUSSION**

**Increasing Levels of Plasma Tumor Necrosis Factor Alpha (TNF-α) in the Study Group**

The levels of plasma TNF-α of pregnant mice infected with *Plasmodium berghei* demonstrated a higher value in the treatment group than control group (Table 2; Fig. 1). Statistical analysis using T test, showed that *Plasmodium berghei* infection could cause elevation of TNF-α levels in plasma \((p=0.000)\). In normal condition, increased level of TNF-α occurs in any human infections. The TNF-α is a pyrogenic and multifunctional cytokine, produced by macrophages, T, B and mast cells. This molecule is not only involved in immuno-protection against infection, but also plays a role in inflammation, autoimmune and pathophysiology of many diseases (19).

During malaria infection, IFN-γ produced by activated lymphocytes activates macrophages to produce TNF-α and induce phagocytosis (4). TNF-α is pro-inflammatory cytokine that has been most closely investigated in malaria and usually act as homeostatic agents, but can cause pathology if produced excessively (20).

**Decrease Hemoglobin (Hb) Level in the Study Group**

A decrease in Hb level in pregnant mice infected with *Plasmodium berghei* evidenced by low level of Hb in treatment group (Figure 2A). Statistical analysis using independent T test, showed that *Plasmodium berghei* infection can cause a decrease in Hb levels of study group compared to the control group \((p=0.025)\). Previous studies in humans showed that decreasing hemoglobin levels could lead to anemia. Malaria was associated with lower mean hemoglobin on antenatal visit and delivery (21,22). Other studies in human showed that the mean of haematological values were significantly lower among parasitized pregnant women compared to non-parasitized pregnant and non pregnant subjects. A positive correlation was observed between the level of parasitaemia and anemia (21,23). Microcytic and hypochromic anemia were significantly higher in pregnant and parasitized subjects (2).

In human malaria infection, many uninfected red cells were destroyed, in the spleen and quite possibly in the liver. This destruction had been identified as the major contributor to malarial anemia (24). During malaria infection the obvious loss of infected erythrocytes is through recognition by macrophages (25). The activity and the number of macrophages were also increased during human malarial infection, and may therefore contribute to the increased removal of uninfected cells (26).

In murine malaria infections, high parasitemias with intravascular hemolysis of infected erythrocytes were frequently observed. Due to the high level of parasitemia, it may appear that the clearance of uninfected RBCs for the development of anemia is less significant in mice than in the majority of human malaria infections. In the mouse model, both lethal and non-lethal malaria infections induce ineffective erythropoiesis, with alterations in erythropoietic progenitor and precursor populations, as well as in the sites of erythropoiesis, erythropoietic suppression and dyserythropoiesis. Each of these mechanisms had been implicated in both human and mouse malarial anemia (25).

Lamikanra et al mentioned that the most decreasing Hb levels in relation to blood parasitemia occurred in malaria-infected primigravid and reduced in accordance with the increase in parity. Mean parasite densities were significantly higher in pregnant women compared with non-pregnant women for both *P. falciparum* and *P. vivax* infection. Pregnant women with *falciparum* or *vivax* malaria were significantly more anemic than non infected pregnant women or infected non pregnant women (27). Despite the effect of malaria on maternal Hb, which was expectedly lower, neonatal Hb levels were not decreasing in the cord blood of malaria infected compared to uninfected women. In addition, cord Hb values were not correlated to maternal Hb concentration in either group or...
The Relationship between Increased Levels of TNF-α with Decreasing Levels of Hemoglobin

Increased levels of TNF-α of plasma and placental tissue in pregnant mice infected with *Plasmodium berghei* relate with the decrease in hemoglobin levels. Analysis using the Pearson correlation test (Figure 3) shows a significant correlation between elevated levels of plasma TNF-α with the decrease of hemoglobin (p=0.020; r=−0.748). Malaria infection may increase the destruction of red blood cells and suppression of erythropoiesis. These effects may be mediated in part by an increase in pro-inflammatory cytokines such as TNF-α, which is related to the anemia of malaria-infected pregnant women (12,14).

During the acute phase of both human and mouse infections there is a strong inflammatory response, which results in increases in TNF-α and IFN-γ (29,30). Interferon-γ were over-expressed in the bone marrow of Fanconi anemia patients and TNF-α suppresses erythropoiesis in vitro (31,32).

The Relationship between TNF-α Level and Fetal Weight

There is a significant difference of fetal weight between treatment group and control group, the data range and median of treatment group were less than the control group. In previous study, the average weight of neonates from infected mothers was less than that of neonates from non-infected mothers. This difference in birth weight was statistically significant for both *P. falciparum* and *P. vivax* infection (27). However the decrease of fetal weight in treatment group of this study was not significantly associated with increase level of TNF-α in plasma. Previous research suggested that increased levels of TNF-α associated with spontaneous abortion, high levels of TNF-α is associated with impaired fetal growth and preterm birth, due to chronic infection, levels of TNF-α have been associated with LBW and anemia (14,33).

The Relationship between Hemoglobin Levels with Fetal Weight

Malaria increases the risk of preterm delivery and stillbirth through fever and contribution to severe anemia rather than through parasitemia (10,34). Abrams showed a similar result with this study. Maternal and cord hemoglobin levels and malaria status had no effect on birth outcome although increasing cord ferritin was associated with significantly decreasing birth weight and gestational length (28).

In *Plasmodium vivax*, infection can increase the weight of hematological abnormalities such as anemia and thrombocytopenia, as well as miscarriage and premature birth. There was a report of the clinical features and pregnancy outcomes in 12 cases of *P. vivax* infection in pregnant women complicated in some by either miscarriages or premature deliveries, some of them developed significant degrees of anemia and thrombocytopenia during the malaria episode (35). It can be concluded that the increased levels of TNF-α is associated with decreased levels of hemoglobin (Hb), but not associated with fetal low weight.

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