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(RESEARCH ARTICLE)

The correlation between inducible nitric oxide synthase (iNOS) expression and level of malondialdehyde (MDA) on placenta tissue of pregnant mice in malaria infection with intra uteri growth restriction

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Abstract

Malaria infection in pregnant women can cause various clinical manifestations, one of them is fetus low birth weight. Sequestrations of infected erythrocytes and macrophages on intervillous space has been revealed by previous study, however the mechanism of this sequestration in placenta insufficiency is still unknown. We suggested that local immunity on placenta tissue can induce the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that can cause the destructions of placenta tissue due to the fetus low birth weight. This research was conducted to explore the path of immunity response on malaria placenta which involved the expression of inducible nitric oxide synthase (iNOS) and the level of malondialdehyde (MDA). This experimental laboratory study was done in pregnant Balb/c strain mice that infected with *Plasmodium berghei* in 9th day of pregnancy. Eighteen mice were divided into control group (pregnant mice) and treatment group (pregnant mice infected with *Plasmodium berghei*). iNOS expression was detected by using polyclonal antibody t iNOS, and level of MDA in the placenta tissue was measured by NTB assay. Fetal birth weight was measured on 15th day of pregnancy using analytic balance. The t-test independent showed a significant difference on iNOS expression between control group and treatment group ($p<0.000$), as well as fetal birth weight. ($p<0.000$). However there was no significant difference in the level of MDA between control group and treatment group ($p = 0.101$), The Pearson correlation test showed a significant correlation between iNOS expression with fetus birth weight ($p<0.000$); $r=-0.925$). It can be concluded that local immune response in placenta that involve nitric oxide pathway is responsible to placenta insufficiency.

Keywords: iNOS; Intra uteri growth restriction; Malaria; Malondialdehyde

1. Introduction

Malaria infection occurs in many countries in the world, especially over the tropic areas. Most cases of malaria occur in countries around the African continent as well as some other countries such as India, Brazil, Afghanistan, Sri Lanka, Thailand, Indonesia, Vietnam, Cambodia, and China [1-2]. It is estimated that more than 50 million pregnancies occur in malaria endemic areas each year, and 50% of cases occur in Africa while the rest are spread worldwide [3]. Pregnant women in malaria endemic areas are at higher risk of infection and exhibit more severe clinical manifestations in both the mother and baby aspects, as pregnancy is a period in which the mother's immune status is in low condition which

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making it easier to be infected. Pregnancy with malaria will result in the birth of infants with low birth weight and severe anemia in the mother [4-5].

The main function of the placenta is not only to exchange gas products and metabolism between mother and fetus but also hormone production. The secretions of lymphokines and cytokines often fill in the intervillous placental space (maternal-fetal interface) to prevent rejection by the placenta and control the infections that enter the fetus. The placenta also has a very important barrier in preventing infection by bacteria, viruses and parasites, so that microbes do not infect the fetus [4, 6]. A massive sequestration by *Plasmodium* parasite on the placenta will also occur during pregnancy, so that there will be changes in the supply of nutrients and oxygen (O_2) from the mother to the fetus, which will inhibit the growth of the fetus inside the womb. Plasmodium infection during pregnancy also results in a local or systemic immune response and the mother's body will produce cytokines for an inflammatory response [4-5, 7-8).

Sequestration of malaria parasites results in endothelial dysfunction; this is largely determined by the presence of endothelial receptors against Parasitized Red Blood Cells (PRBCs) [4, 9-10]. The receptors on PRBCs on each network vary. In placental malaria Chondroitin-sulat A (CSA) is the largest receptor in the placenta for sequestration in addition to several other receptors, including CD36 and Hyaluronic Acid (HA) [10]. CSA and HA receptors are more responsible for the occurrence of malarial syndrome in pregnant women than CD36 [3]. The attachment of PRBCs to the endothelial also results in a local immune response by activating macrophages [9, 11-13].

The accumulation of macrophages in placenta with malaria is induced by endothelial activation in cyncitiotrophoblast placenta. The number of macrophages increases with the increasing number of parasites that induce endothelium [6, 8, 14]. The mechanism of macrophages to face the agent is by 1) expressing CD38 located on the surface of the cell membrane to induce a natural killer cell, 2) producing Nitric Oxic (NO) which serves to protect from infection in cells [4, 10, 15], and 3) producing Reactive Oxygen Species (ROS). Reactive oxygen powder will cause detectable oxidative stress using Malondialdehyde (MDA), which is a decomposition of reactive oxygen [7, 16].

Recent studies of malarial infections in pregnancy are associated only with the occurrence of adhesion that makes up rosetting and sequestration [4, 5, 7, 13, 17] which leads to inhibition of fetal growth resulting in low birth weight, but few studies have linked localized immune responses to sequestration and rosetting of the placenta.

In this study, we will examine the effect of *Plasmodium berghei* infection on pregnant mice on local immunity responses to placental malaria, particularly the presence of inducible nitric oxide synthase (iNOS) and malondialdehyde (MDA).

2. Material and methods

2.1. Ethical clearance

The use of strain Balb/c mice has fulfilled the requirement of ethical committee of experimental research from the Medical Ethics Committee of Universitas Brawijaya, Malang Indonesia number 220/EC/ KEPK-S2-JK/09/2011.

2.2. Research design

The research was conducted in Central Laboratory of Biomedicine and Parasitology Laboratory of Faculty of Medicine, University of Brawijaya, Malang, Indonesia. Procedures research on laboratory was done by comparing the results obtained from the infected mice with the controlled mice. The animal used in this study were Balb/c strain mice. The use of mice to substitute the guinea pig was based on several reasons, some of them were because mice are easy to handle, easy to maintain, and easy to breed. While strain Balb/c was selected because this strain is a good model in demonstrating the status of malaria, especially placenta malaria. This study used 2 groups of mice, the control group consisted of 9 pregnant mice and the treatment group consisted of 9 infected mice with *Plasmodium berghei*. The criteria used for the subjects were Balb/c primigravide female and male mice, adult mice between the ages of 13-16 weeks, mice weight between 20-30 grams, and has no physical defect. The variable in this study was the number of *P. berghei* infections and the duration of its infection. The degree of parasitaemia, fetal body weight, apoptosis of placental cells, and angiogenesis in Balb/c mice placenta were calculated in several steps.

2.3. Impregnating the mice

Impregnating the mice was conducted simultaneously after preparing synchronization of oestrus by exploiting biological phenomenon that is Leeboot effect (18). Pheromone effect and Whiten effect (18) by placing female mice in one cage during acclimation time (7-10 days). In this way the mice will be in an un-oestrus condition (Leeboot effect).

Female mice that have been separated will begin the cycle when exposed to odors derived from the male, such as its urine (pheromone effect). Mice were mated for 1 night at a 1: 1 ratio.

2.4. Inoculation of *Plasmodium berghei*

Inoculation of *Plasmodium berghei* [19] ANKA strain for donor mice, was conducted by intraperitoneally, then after the 4th day after inoculation, a parasitemic count was performed. Parasitaemia was calculated from a thin blood-smearing preparation taken from the tip of the tail and had been doped with Giemsa dye. The number of parasites is calculated per 1000 erythrocytes with a 1000x magnification microscope. After the parasitaemia of donor mice reached above 15%, it means that the donor mice are ready to be used as donor.

2.5. Preparation of inoculants

The amount of erythrocytes per mL of blood and parasitaemia of the donor mice which parasites were going to be transferred was calculated. To calculate the amount of erythrocytes, blood was taken from the tail end of 10 µL and diluted 103 x with PBS solution. Then the amount of erythrocytes is counted in the Naubauer count chamber. By multiplying the amount of erythrocytes per mL of blood with parasitaemia, the number of parasites per mL of blood was obtained. The next step was by diluting with M+ solution to obtain parasitic concentration 106 in 0.2 mL of blood, then the solution was transferred intraperitoneally to the treatment mice as much as 0.2 mL.

2.6. Inoculation and measurement of parasitaemia for treatment mice

Inoculation and measurement of parasitaemia for treatment mice was performed on the 9th day of mice pregnancy of 106 parasites in 0.2 mL of blood for each mouse intraperitoneally. Blood collection for the measurement of parasitaemia was done every day by making a blood smear from the tail of the mouse and then dripped on the object glass. The droplets were smeared and then dried, then methanol until evenly distributed and allowed to dry, after which the smear was dyed with giemsa which was a mixture of giemsa and giemsa buffer with a ratio of 1: 8. The dying process was allowed to stand for 20 minutes. Then rinsed with water and dried. The degree of parasitaemia was checked by examining the blood smear with a 100x magnification microscope. The number of parasites was calculated per 1000 erythrocytes. The calculation of the percentage of parasitaemia was calculated based on the number of erythrocytes infected with malaria in 200 blood cells in one field of view.

2.7. Surgery

Surgery was performed on the 15th day of pregnancy. The mice were drugged with chloroform that had been saturated in large jars. Surgery was performed to remove the uterus and then separated between the placenta and the fetus. The fetus was weighed and recorded from the weighing, while the placenta was introduced into the formalin solution to be used in subsequent analysis (apoptosis and VEGF expression examination).

2.8. iNOS measurement

Inducible nitric oxide synthase (iNOS) in this study was observed from placental tissue as measured by immunohistochemical method. The placental tissue was stored in formaldehyde and parafinized for slicing with a thickness of 5 micros. The first day of coloring process included Depolarization (xylol, alcohol ethanol absolute, ethanol 90%, ethanol 80%, ethanol 70%) Blocks with non-specific proteins (FBS 5% and 0.25% Triton), as well as anti-body incubation Primary administration with dilution: primary anti-body: FBS 5% = 1: 100 On the second day, secondary anti-body incubation with 1: 200 sterile PBS dilution, incubation of SA-HRP with ratio 1: 500 sterile PBS DAB chromagen application with ratio of 1: 50 DAB buffer Counter staining By administering meyer hematoxilin, after washing and drying and observed under a microscope.

2.9. MDA measurement

Malondialdehide (MDA) is a decomposition of peroxidised polyunsaturated fatty acids due to cell membrane damage caused by ROIs activity. MDA can be used to detect ROIs of the impact of the infection process. In this study, MDA measurements were taken from mole placental tissue of diffuse mice, Malondialdehide was a decomposition of peroxidised polyunsaturated fatty acids due to cell membrane damage caused by ROIs activity. MDA can be used to detect ROIs of the impact of the infection process. In this study, MDA measurements were taken from placental tissue of mice that had been diffused, then homogenized, homogenized cold-cooled homogenization and supernatant taken and stored in cooling cupboard. Initial heating was done by inserting in water bath with temperature of 60 °C. TBA reagents addition with 10.5 mL pure water, 10 µL BHT reagents, 250 µL calibrators, 250 µL posphoric acid reagents, 250 µL TBA sealed and vortex reagents. It was then heated in water bath with temperature of 60 °C for 60 minutes. The results were centrifuged at 10,000 rpm for 3 min, analyzed by spectrophotometer.

3. Results and discussion

Based on mean and p-value variable measurement consists of the average of fetal weight, iNOS expression, and MDA levels using independent t-test, the results are as follows:

Table 1 Mean of iNOS expression, MDA levels and fetal weight

Variable	Mean		p-value
	Control	Treatment	
iNOS expression	1.71	54.50	<0.000
MDA level	1.55	1.79	0.101
Mean of fetal weight	0.27	0.20	<0.000

The results of the analysis showed that there was a significant difference in the mean of fetal weight of control and treatment group, as well as the expression of iNOS in the normal group and treatment group (p value < 0.000), but not the MDA level, there was no significant difference between normal group and treatment group (p value = 0.101)

3.1. iNOS expression

The results of iNOS expression calculation showed significant differences in the number of controlled mice and mice infected with *Plasmodium berghei*. There was a significant difference between the two groups. The treatment group had a wide data range and median above/greater than the maximum value of the control group (figure 1). The expression of iNOS in Plasmodium-infected placental tissue was the highest in the intervillous space, due to the local immune response of endothelium to PRBC.

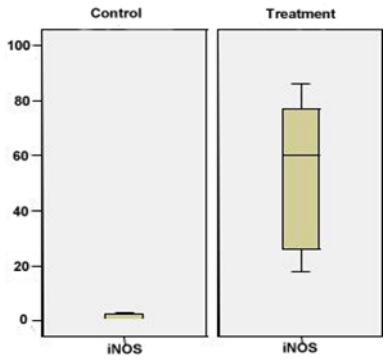

Figure 1 Expression of iNOS in the control and treatment groups

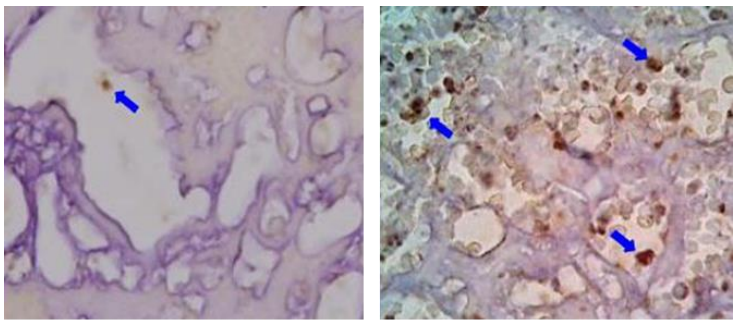

Figure 2 Expression of iNOS in mice placenta. In uninfected placenta tissue (A) there was minimum expression of iNOS. Other side in infected placenta tissue (B) there were many expressions of iNOS

3.2. MDA levels

The results of MDA levels did not show significant differences between the control group and the treatment group. The result of data analysis by using independent t-test obtained a p value of 0.101. Figure 3 shows that data distribution between the control group and the treatment group was normal, there was a significant difference between the two groups, the normal group had a narrow data range and the median was above/greater than the maximum value of the treatment group.

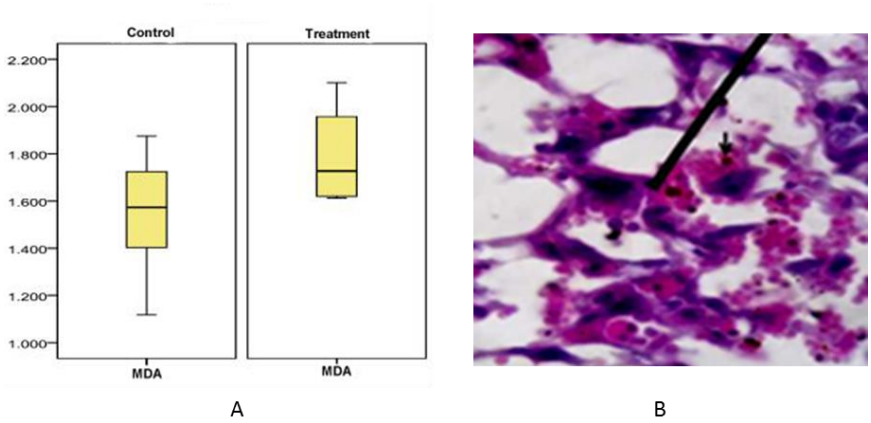

Figure 3 (A) The average fetal weight of the fetus, and (B) sequestration of infected erythrocytes. The black arrows showed the sequestration of infected erythrocytes in the placental intervillous area (HE staining, 1000x)

3.3. Correlation between the average of fetal weight and iNOS expression and MDA

The analysis of the relationship between two variables showed that there was a significant relationship between iNOS expression with the average of fetal weight (p value < 0.000), but there was no significant relationship between MDA levels with the average of fetal weight (p-value = 0.207). The visualization of data correlation between iNOS and average fetal weight by using linear regression graph can be seen on figure 4 (A). It illustrates the negative relationship where the higher the iNOS expression and lower the mean fetal weight, with p-value of 0.000 and R square of 0.856. The association of MDA with the average mean of fetal weight did not indicate a significant correlation, p value of 0.207 and r value of -0.375 meaning that there is no significant correlation between placental MDA level and the mean of fetal weight (Figure 4 B).

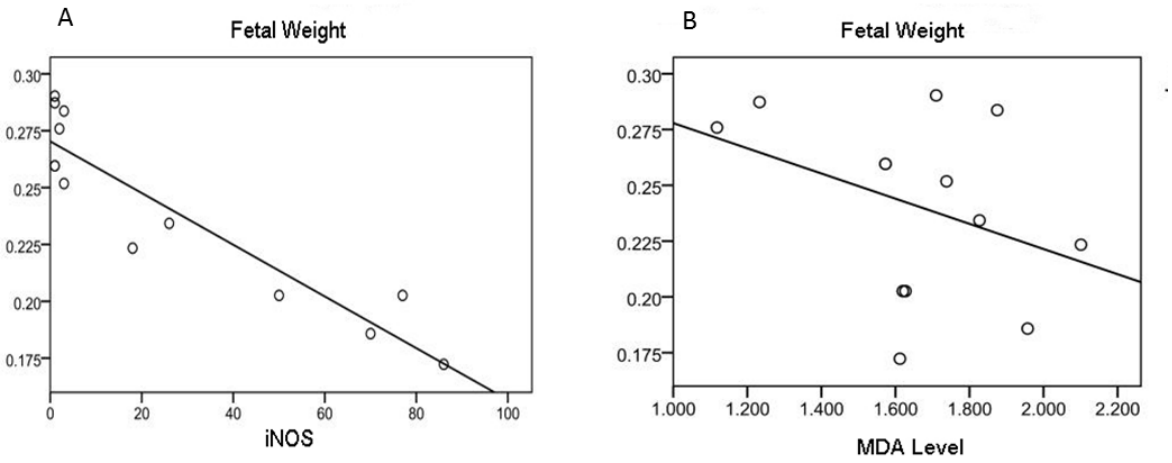

Figure 4 The linearity of the correlation between (A) iNOS expression and the average of fetal weight, (b) MDA levels and mean fetal weight.

The correlation between iNOS expression, MDA levels and Intra uteri growth restriction Based on the correlation test using Pearson analysis and linear regression graph, it can be concluded that high expression of iNOS affecting the occurrence of IUGR in placental malaria. Expression of iNOS results in placental insufficiency so that the fetus growth is

inhibited. Expression of iNOS represents the presence of reactive nitrite (RNI) as a local immune response mechanism in malaria-infected placental tissue. The reactive presentation of oxygen in the placental tissue did not show an increase in the presence of infection, it is indicated by the absence of a significant increase in MDA levels as the parameters of the presence of ROI in the placenta tissues.

The results of this analysis answer some questions that have been known as the cause of IUGR due to placental insufficiency caused by ROI, but more specific causes of insufficiency is more dominant due to RNI in production by lymphocytes and macrophages. Activation of RNI as a local immune response to the presence of Plasmodium in erythrocytes accumulated in the intervilous space. Increased RNI in intervilous addition to eliminate PRBC also resulted in damage to surrounding cells, such cells are mostly endothelial at intervilous. Endothelial damage and sequestration may lead to more severe placental insufficiency.

4. Conclusion

Insufficiency in placental malaria is caused by several factors including sequestration of intervillous space and free radical activity. Free radicals that play a role in placental malaria are more by RNI than ROI, this is due to macrophage induced by malaria antigens and inflammatory mediators (TNFα, INFTA, and IL-1), and high L-Arginine placental tissue compared to other tissues, and not so with ROI. The increasing levels of cytotoxic RNI that eliminates PRBC also destruct surrounding placental tissue. Placental insufficiency leads to a decrease in the supply of nutrients and oxygen from mother to fetus resulting in delayed fetal growth (IUGR) and the fetal weight is not suitable with the age of pregnancy. Local immune responses to placental malaria result in the increased of iNOS to produce RNI resulting in placental tissue destruction. Destruction and sequestration of the placenta affect the placental insufficiency resulting in IUGR.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare that there is no conflict of interest.

Statement of ethical approval

The use of strain Balb/c mice has fulfilled the requirement of ethical committee of experimental research from the Medical Ethics Committee of University of Brawijaya, Malang Indonesia number 220/EC/ KEPK-S2-JK/09/2011.

References

[1] Kakkilaya's BS. Life Cycle of Malaria. https://www.malariasite.com/, Last accessed on 05/01/2016

[2] Tuteja R. (2007). Malaria–an overview. The FEBS journal, 274(18), 4670-4679.

[3] Kakkilaya's BS. (2009). Malaria and pregnance are mutually aggravatting. https://www.malariasite.com/, Last accessed on 22/7/2017.

[4] Ndam NT and Deloron P. (2007). Molecular aspects of *Plasmodium falciparum* infection during pregnancy. BioMed Research International, 2007.

[5] Neres R, Marinho CR, Gonçalves LA, Catarino MB and Penha-Gonçalves C. (2008). Pregnancy outcome and placenta pathology in *Plasmodium berghei* ANKA infected mice reproduce the pathogenesis of severe malaria in pregnant women. PloS one, 3(2), e1608.

[6] Wahlgren M and Perlmann P. (2003). Malaria: molecular and clinical aspects. CRC Press.

[7] Olayaki LA, Ajao SM, Jimoh GA, Aremu IT and Soladoye AO. (2008). Effect of vitamin C on malondialdehyde (MDA) in pregnant Nigerian women. J Basic Appl Sci, 4(2), 105-108.

[8] Ribera JM, Hausmann-Muela S, D'Alessandro U and Grietens KP. (2007). Malaria in pregnancy: what can the social sciences contribute? PLoS Medicine, 4(4), e92.

[9] Langhorne J, Quin SJ and Sanni LA. (2002). Mouse models of blood-stage malaria infections: immune responses and cytokines involved in protection and pathology. In Malaria immunology, 80, 204-228.

[10] Fried MI and Duffy PE. (1998). Maternal malaria and parasite adhesion. Journal of Molecular Medicine, 76(3-4), 162-171.

[11] Abbas AK, Lichtman AH and Pillai S. (2014). Cellular and molecular immunology E-book. Fifth editions, Elsevier.

[12] Biggs BA and Brown GV. (2001). Principle and Practice of Clinical Parasitology. John Wiley & Sons Ltd, England.

[13] Rogerson SJ, Hviid L, Duffy PE, Leke RF and Taylor DW. (2007). Malaria in pregnancy: pathogenesis and immunity. The Lancet infectious diseases, 7(2), 105-117.

[14] Suguitan Jr AL, Cadigan TJ, Nguyen TA, Zhou A, Leke RJ, Metenou S, Thuita L, Megnekou R, Fogako J, Leke RG and Taylor DW (2003). Malaria-associated cytokine changes in the placenta of women with pre-term deliveries in Yaounde, Cameroon. The American journal of tropical medicine and hygiene, 69(6), 574-581.

[15] O'Gorman MR and Donnenberg AD. (2008). Handbook of human immunology. CRC press.

[16] Hunt NH, Kopp M and Stocker R. (1992). Free radicals and antioxidants in malaria. Biochemistry and clinical applications. Basel, Birkhäuser Verlag.

[17] Scherf A, Pouvelle B, Buffet PA and Gysin J. (2001). (2001). Molecular mechanisms of Plasmodium falciparum placental adhesion. Cellular microbiology, 3(3), 125-131.

[18] Sardjono TW. (2005). Pengaruh Infeksi Toxoplasma Pada Hasil Kehamilan Melalui Interferon Gama Ifn-Γ Caspase-3 Dan Apoptosis Sel-Sel Plasenta. Penelitian Eksperimental Laboratoris Pada Mencit Balb/C Bunting Yang Diinokulasi Dengan Takhizoit Toxoplasma Gondii Galur Rh. Doctoral dissertation, Airlangga University Library, Surabaya.

[19] Supargiono. (1993). Production, proliferation and functional activities of mononuclear phagocytes during *P. vincke* Petteri infection in mice. Doctoral dissertation, University of London 1993.

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