7. comparison of the effaect of sambiloto as 201

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ORIGINAL ARTICLE:

Comparison of the effect of sambiloto (AS 201-01) tablet and dihydroartemisinin-piperaquine on macrophage MIF expression in mice placenta infected with *Plasmodium berghei*

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ABSTRACT

Objective: To compare the administration of sambiloto tablets (AS201-01) and dihydroartemisinin-piperaquine phosphate tablets in pregnant mice infected by *P. berghei* on the expression of MIF in the placenta.
Materials and Methods: Experimental laboratory research, 24 pregnant mice were divided into 4 groups with randomization, ie. non-infected, placebo, sambiloto (AS201-01) and DHP groups. On day 9 *P. berghei* was infected, on day 11 the treatment was given, day 15 the surgery was performed, placental samples were taken, immunohistochemical staining was given, and MIF expression was assessed.
Results: The expression of MIF in the group not infected with *P. berghei* had the lowest mean, while the highest mean was found in the placebo group. Uninfected groups were not significantly different from sambiloto (AS201-01) tablet group. Sambiloto tablet group (AS201-01) had lower MIF expression than DHP group, but it was not significantly different. Sambiloto tablet group (AS201-01) showed lower MIF expression than placebo. MIF expression in DHP group was lower than that in placebo group. From the lowest, the MIF expressions were as follows: group not infected with *P. berghei*, group receiving sambiloto (AS201-01) tablet, DHP group and placebo group.
Conclusion: MIF expression in the placenta of pregnant mice infected with *P. berghei* and receiving sambiloto (AS20-01) tablets was not different from those receiving DHP tablets.

Keywords: Placental malaria; MIF; sambiloto; DHP

ABSTRAK

Tujuan: Membandingkan pemberian tablet sambiloto (AS201-01) dan tablet dihydroartemisinin– piperaquine phosphate pada mencit bunting yang dinfeksii *P. berghei* terhadap ekspresi MIF pada plasenta.
Bahan dan Metode: Penelitian eksperimental laboratorium, 24 mencit bunting dibagi dalam 4 kelompok dengan randomisasi, yaitu kelompok tidak diinfeksi, plasebo, sambiloto (AS201-01) dan DHP. Pada hari 9 *P. berghei* diinfeksikan, hari 11 perlakuan diberikan, hari 15 pembedahan dilakukan, diambil sampel plasenta, pewarnaan imunohistokimia diberikan, dan ekspresi MIF dihitung.
Hasil: Ekspresi MIF pada kelompok yang tidak diinfeksi *P. berghei* memiliki rerata terendah dan rerata tertinggi dimiliki kelompok plasebo. Kelompok tidak diinfeksi tidak berbeda bermakna dengan kelompok tablet sambiloto (AS201-01). Kelompok tablet sambiloto (AS201-01) memiliki ekspresi MIF lebih rendah dibandingkan kelompok DHP, namun tidak berbeda bermakna. Kelompok tablet sambiloto (AS201-01) menunjukkan ekspresi MIF lebih rendah daripada plasebo. Ekspresi MIF pada kelompok DHP lebih rendah dibandingkan kelompok plasebo. Mulai dari yang terendah ekspresi MIF adalah sebagai berikut: kelompok tidak diinfeksi *P. berghei*, kelompok yang diberi tablet sambiloto (AS201-01), DHP dan plasebo.
Simpulan: Ekspresi MIF pada plasenta mencit bunting yang diinfeksi *P. berghei* dan menerima tablet sambiloto (AS20-01) tidak berbeda dari yang menerima tablet DHP.

Kata kunci: Malaria plasenta; MIF; sambiloto; DHP

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INTRODUCTION

Twenty-five million pregnant women are currently at risk of malaria, and according to the World Health Organization malaria accounts for more than 10,000 maternal deaths and 200,000 neonatal deaths per year. By 2015, there were an estimated 214 million cases of malaria, of which over 400,000 cases were the cause of death. In Indonesia alone there were 417,819 cases of positive malaria in 2012, and there are still 16.5 million people living in high risk areas of malaria.[1] Pregnant women are more easily infected with malaria than the general population. In addition, pregnant women are also susceptible to recurrent infections with severe complications resulting in death. This is caused by weakness and decreased immunity in pregnant women.[2] Primigravids are generally most susceptible to malaria, such as anemia, fever, hypoglycemia, cerebral malaria, pulmonary edema, puerperal sepsis and death from severe malaria and bleeding. Problems with newborns are low birth weight, prematurity, impaired fetal growth, malaria infection and death.

In pregnant women infected with malaria, parasitic erythrocytes are present in the placenta of the mother and the placenta is an ideal place for malaria parasites. Receptors in the placenta may bind to the malaria parasite so as to have the ability to increase the density and duration of infection in malaria. The presence of such receptors and changes in the immune system of pregnant women result in severe clinical symptoms in malaria in pregnancy.[4,5] Malaria treatment that is shown to be safe in infants and pregnant women and used as a major antimalarial drug is chloroquine. In Indonesia, chloroquine began to be used in 1946, but in 1974 there were reported cases of chloroquine-resistant *P. falciparum* from East Kalimantan. Until 1992 resistance was reported throughout Indonesia.[6,7] Whereas, *P. vivax* resistance to chloroquine in Indonesia first was reported in 1991 from Irian Jaya,[8] followed by reports from Sumatra,[9] Kalimantan,[10] and several other areas in Indonesia. artesunate based combination treatment was applied in Papua around 2003. However, a few years later it was reported from District Hospital in Mimika, Papua that there was an occurrence of 16% prematurity, 74% abortion, fetal death in uterus 9.6% and also resistance to artesunate.[11] Due to frequent occurrence of treatment resistance to Plasmodium, it is deemed necessary to continue to seek and develop antimalarial drugs.[12]

Sambiloto (*Andrographis paniculate Nees*) is one of a kind of medicinal plants used in traditional medicine that can flourish and have been cultivated in various worlds, including Indonesia.[9] One of the pharmacological effects of sambiloto, among others, is as antimalaria.[13] Macrophage migration inhibitory factor (MIF) is a lymphokine that is known to play a role in immune infections of parasites, bacteria, viruses and autoimmune diseases. MIF is a proinflammatory cytokine that is released from various cells (T cells, monocytes, macrophages, dendritic cells, B cells, neutrophils, eosinophils, mast cells) and is involved in the pathogenesis of sepsis, inflammatory diseases, and autoimmunity.[14] Several studies have shown the role of MIF in pathogenesis and severity of malaria.[15,16] It has been reported that women infected with malaria have MIF in placental plasma significantly higher than non malaria infected women.[17] In pregnancy, glucorticoids increase. All preliminary studies on the effects of sambiloto tablets on placenta of pregnant mice were primarily done in the cenotrophoblast and placental cytotrophoblast, but no studies have been done on the mechanism of the sambiloto tablets in the placental intervillous space. Therefore, we conducted a study on the Sambiloto tablets (AS201-01) that had previously been shown to have an effective antimalarial activity and were safely used in pregnancy by measuring MIF expression using immunohistochemistry, which was in the placental intervillous space compared with DHP as a standard drug used for malaria therapy at this time. We investigated in this study wheteher the provision of sambiloto tablets inhibited the formation of hemozoin that stimulated the formation of MIF, resulting in worsening condition of mother and fetus.

MATERIALS AND METHODS

This study was a laboratory experimental study in BALB/c strain (*Mus musculus*) with randomized samples and controls. This study was conducted at the Institute of Tropical Disease (ITD), Universitas Airlangga, and the Veterinary Pathology Laboratory of the Faculty of Veterinary Medicine, Universitas Airlangga. The study population was BALB/c strain (*Mus musculus*) mice in experimental laboratory units. The sample consisted of 24 pregnant mice weighing 25-35 g and fulfilling the inclusion and exclusion criteria. The sample was divided into 4 groups of 6 each, the K1, K2, K3, and K4 groups. K1 group was pregnant mice without *P. berghei* infection, K2: pregnant mice with *P. berghei* infection receiving placebo of Carboxymethyle Cellulose Sodium (CMC Na) 2x daily for 4 days (placebo group). K3: pregnant mice with *P. berghei* infection receiving sambiloto tablets (AS201-01) dose 25 mg andrographolide/kg BW twice daily for 4 days (AS201-01 group). K4: pregnant mice with a *P. berghei* infection receiving a dihydroartemisinin-piperaquine phosphate tablet 1x daily for 3 days (DHP group). On day 11 K2, K3, K4 underwent parasitemia If the results were positive, the treatments were given. On day 15 the

surgery was performed, placental samples were taken, preparations for paraffin blocks were made and stainig was performed to examine the IHC MIF.

Sambiloto (AS201-01) tablet is a 96% ethyl acetate fraction tablet obtained from sambiloto leaf, which was extracted, fractionated with ethyl acetate, and then formulated in tablet form. We used a dose of 25 mg andrographolide/kg BW. DHP tablets used a dose of 1.248 mg/kg/day. The asssessment of MIF expression used Immuno Reactive Score, which was the result of multiplication between the percentage score of immunoreactive cells with color intensity scores on imunnoreactive cells at IHC staining. Placental tissue, which consisted of syncytiotrophoblast cells that experienced DNA fragmentation, showed chromogene brown color.

Data were recorded in the data retrieval form. Data analysis used statistical application software. The normality test was carried out using the Kolmogrov-Smirnov test. If the data were normally distributed, then it was proceeded with ANOVA test. The level of significance used in this study was 0.05. Ethical feasibility was obtained from Research Ethics Commission, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia (No. 724-KE).

RESULTS AND DISCUSSION

Table 1. Body weight of female Balb/C mice strain

Bodyweight (gram)	K1	K2	K3	K4	P value
Mean	30.83	30.50	31.83	31.17	0.89
SD	1.72	3.08	4.26	2.48	

Notes: K1: Group not infected with *P. berghei*; K2: Placebo group; K3: Sambiloto tablet group (AS201-01); K4: DHP tablet group

The body weight characteristics of female mice before treatment are shown in Table 1. Data on mice weights before treatment were tested by the Kolmogorov-Smirnov normality test. Statistical calculations yielded a p value of 0.89 ($p>0.05$) which showed a normal distribution, indicating no significant differences in those female mice groups before treatment, which meant that the sample was homogeneous.

Figure 1 shows placental cells expressing MIF, indicated by brown chromogen color. The highest MIF expressions was in the group infected by *P. berghei* that received placebo, followed by the group infected with *P. berghei* receiveing DHP, the group infected with *P. berghei* receiving sambiloto tablet, and, lastly, the group that was not infected with *P. berghei*.

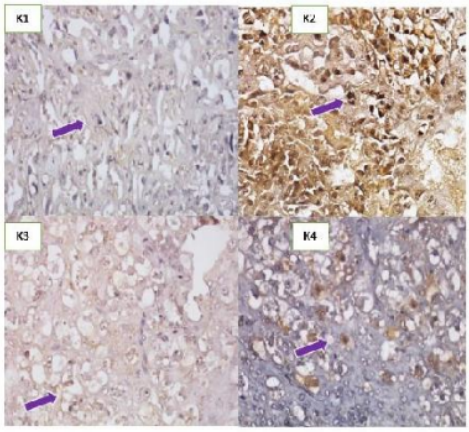

Figure 1. Differences in MIF expression in placental cytotrophoblast cells (arrows) in the treatment group. Notes: immunohistochemical staining, K1: pregnant mice group not infected with *P. berghei*; K2: pregnant mice group infected with *P. berghei* and receiving placebo of CMC Na tablets; K3: groups of pregnant mice infected with *P. berghei* and receiving sambiloto (AS201-01) tablets; and K4: groups of pregnant mice infected with *P. berghei* receiving DHP tablets.

Data on MIF expression in placental cells were tested by the Kolmogorov-Smirnov normality test and showed that the data were normally distributed. Statistical analysis was followed by Anova comparative test. The MIF expression in placental cells in each group are presented in Table 2.

Table 2. Mean and standard deviation of MIF expression in pregnant mice placentas in the treatment groups

Groups	MIF Mean ± SD	P value
K1	1.37 ± 0.78a	0.00
K2	4.47 ± 1.03b	
K3	2.03 ± 0.77a	
K4	2.77 ± 0.64c	

Table 2 shows that mean MIF expression in group infected with *P. berghei* and received placebo was the highest and that in group not infected with *P. berghei* was the lowest. Mean MIF expression in group not infected with *P. berghei* was not significantly different from that in sambiloto (AS201-01) tablet group. Sambiloto (AS201-01) tablet group had MIF expression

results which were not significantly different from that in group not infected with *P. berghei* and in group receiving DHP tablet, but significantly different (significantly lower) compared with placebo group. MIF expression in sambiloto group was significantly lower than that in DHP tablet group.

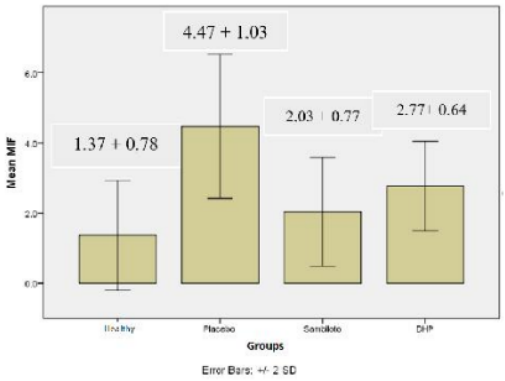

Figure 2. Mean MIF expression in each treatment group.

Figure 2 shows that the sambiloto tablet group had MIF expression almost the same as the group not infected with *P. berghei*, while the one with the highest MIF expression was the placebo group.

Plasmodium berghei parasite was used in this study because this parasite has similarities with those that infect mammals in terms of, including, life cycle, morphology, growth stage, genome organization, and metabolic pathway.[18] In addition, *P. berghei* genome shows high similarity, both in the structure and gene content, with the genome of the human malaria parasite, *Plasmodium falciparum*.[19] Macrophage Migration Inhibitory Factor (MIF) is a lymphokine produced by monocytes, macrophages, dendritic cells, neutrophils, and pituitary cells that are regulated by immune stimulation and function directly as pro-inflammatory cytokines by activating or promoting TNF-α cyto-kine expression, IL-1, IL-2, IL-6, IL-8, IFN-γ, nitric oxide release, and the induction of cyclooxy-genase (COX)-2 pathway.[20] Cytokines play a major role in the immune response. Increased production of IFN-γ by mononuclear cells derived from intervillus is associated with protection system in endemic areas.

It was reported that multigravida women with negative placental malaria had higher IFN-γ compared to primigravida. This proves that these cytokines play an important role in the protection of placental malaria. Increased TNF-α and IFN-γ on the placenta showed that placental malaria stimulated an immune response involving Th-1 (TNF-α, IFN-γ, IL-1β) and Th-2 (IL-6, IL-10). Increased IL-10 plays an important role in regulating the negative effects of Th-1 in pregnancy. Increased levels of IL-8 are associated with IUGR. Macrophage Migration Inhibitory Factor (MIF) plays a role in the response to malaria during pregnancy based on its ability to activate macrophages and overcome the immunosuppressive effects of gluco-corticoids, thus helping in cleaning parasites. Infiltration of immune cells into IVS during placental malaria has been associated with undesirable effects, such as prematurity and LBW. This in turn is associated with an increased risk of neonatal death.

Characteristics of research subjects

This study used 24 Balb/C strains female mice that met the inclusion criteria of uniformity between body weight, which was 25-35 grams, and age. Statistical analysis showed that the p value in the study subjects was 0.89 (p> 0.05), which meant there were no significant differences between groups of mice before treatment. In other words, those groups were homogeneous. This study used mice that had their first pregnancy. It is known that pregnant women are more susceptible to malaria than non-pregnant women.[21] It is almost a century ago that primigravidas tended to be more vulnerable than multigravidas, because women gained immunity to malaria during successive pregnancies, especially in areas with high transmission.[22] In addition to children, pregnant women, especially primigravida[23] and non-immune humans, such as travelers, have a high risk of developing severe malaria.[24] The difference in parity is related to the ability to produce cytokines in the placenta. In multigravida the ability to produce IFN-γ cytokines is higher, so that they are able to eliminate malaria compared to primigravida who produce slower and fewer IFN-γ cytokines. IFN-γ cytokines stimulate monocytes to excrete proinflammatory cytokines which will eliminate malaria parasites by increasing macrophage phagocytic activity and T-cell stimulation. In addition, in malaria-infected multigravida the rate of inhibition of parasite attachment to Chondroitin Sulphote-A (CSA) is higher than in malaria-infected primigravida.[22]

Effect of Macrophage Migration Inhibitory Factor (MIF) on the placenta

Figure 1 shows placenta cells expressing MIF, from the highest to the lowest were group infected with *P. berghei* receiving placebo, group infected with *P. berghei* receiving DHP, group infected with *P. berghei* receiving sambiloto tablets, and finally group not infected with *P. berghei*. Several studies have shown the

role of MIF in the pathogenesis and severity of malaria.[16] Women with positive placental malaria have significantly higher levels of intervillous plasma MIF than women without placental malaria.[17]

Studies show that MIF increases 300 to 500 times in placental intervillus blood plasma compared to peripheral blood plasma. In women with positive placental malaria, there was a significant increase in intervillous MIF levels compared to women with negative placental malaria. Likewise, intervillous blood mononuclear cells produce higher levels of MIF than peripheral blood mononuclear cells. Increased levels of MIF in the intervillous blood can play an important role in the activation of macrophages by overcoming the immunosuppressive effects of corticoid hormones in the placenta and, thus, ultimately helping to cleanse the malaria parasite. The same study showed that MIF was expressed in various cellular compartments of the placenta, such as the syncytiotrophoblast, cytotrophoblast, extrophilic trophoblast, Hofbauer cell, stromal cell, amniotic epithelial cell, and mononuclear cells in the intervillous space. Comparison between positive placenta malaria and negative malaria placenta showed much higher levels of MIF expression in amnionic epithelial cells and mononuclear IVB cells. This observation shows that intervillous and syncytiotrophoblast mononuclear cells can be an important source of production of MIF in placental intervillus.[25]

Neres's research on pregnancy outcomes and placental pathology in mice infected with *P. berghei* revealed that plasmodium-infected placenta was associated with decreased fetal survival, inhibited fetal growth, impaired infant growth, and increased disease severity in pregnant women. In addition, this study found that chondroitin sulfate A (CSA) and hyaluronic acid (HA), known as *P. falciparum* adhesion receptors to human placenta, were also involved in malaria infection in mice placenta. Decreasing maternal blood flow in the placenta is a major factor in placental malaria which can worsen the innate inflammatory response to red blood cells infected with plasmodium and trigger the severity of placental pathology. This experimental model provides an opportunity to identify cell component and molecular pathogenesis of severe malaria placenta and to investigate the inflammatory response that causes observed abnormal fetal and placental blood circulation.[26]

Brabin's study on malaria in pregnancy revealed that chronic infection in the placenta was associated with a significant reduction in birth weight and a low risk of severe fetal birth, including prematurity and stunted fetal growth. Chronic placental malaria infection is more common in primigravida than multigravida.[27]

Meanwhile, acute infection was statistically related to the risk of a low incidence of IUGR and without a significant increase in the incidence of prematurity. The incidence of acute infection at the end of pregnancy plays a role in the incidence of prematurity. This indication is related to the initial reports of the incidence of abortion and prematurity in the malaria epidemic.[21]

Histological changes related to decreasing infant birth weight in relations with malaria have been investigated in a large series of placental samples from areas with high transmission of malaria, which showed a high incidence of malaria infection. These findings suggest that mononuclear inflammation of the intervillous space is the main mechanism to explain malaria-related births and is associated with weight loss, especially growth disorders. These findings also confirm that malaria infection through the placenta in semi-immune women contributes to LBW, prematurity, where preterm infants are more likely to die than IUGR infants.[28] MIF levels differ significantly in IVB, peripheral, and umbilical plasma, with the result that plasma IVB has the highest MIF level and peripheral plasma has the lowest levels. Women who were positive for placental malaria had significantly higher plasma IVB MIF levels than women with negative placental malaria. However, this relationship is not seen in peripheral or umbilical cord MIF levels. In addition, the likelihood of stillbirth and low birth weight is significantly higher in high placental MIF levels, which supports the hypothesis that increased placental MIF concentrations can be associated with an increased risk of poor birth outcomes.[17]

Comparison of MIF expression in placenta infected with *Plasmodium berghei* between groups receiving placebo, sambiloto tablets (AS201-01), and DHP

After the Kolmogorov-Smirnov normality test was performed, the mean expression of MIF in the three groups showed normally distributed data with a value of $p>0.05$. Statistical analysis was continued with Anova comparative test and p value <0.00 which showed a significant difference between the three groups. Placental MIF expression in pregnant mice group infected with *P. berghei* receiving placebo, compared with the group infected with *P. berghei* that received sambiloto (AS201-01) tablets and group with DHP tablets, showed significant differences. Placental MIF expression in group that received placebo was the highest. The group of pregnant mice infected with *P. berghei* and receiving sambiloto (AS201-01) tablets, compared to group with DHP tablet, did not show significant differences. Sambiloto (AS201-01) tablet group had lower placental MIF expression than DHP group.

The administration of sambiloto (AS201-01) tablets in this study showed a decrease in the expression of MIF in the placenta of infected pregnant mice compared to the placebo group and the DHP tablet group. It can be concluded that the administration of standardized sambiloto tablets can affect the decrease in MIF expression in the placenta of pregnant mice infected with *P. berghei*.

A study in Zambia (2017) examined the safety and efficacy of three artemisin-based combination drugs, the mefloquine-artesunate (MQAS), DHP and artemeter-lumefantrine (AL), in second and third trimester pregnant women with *P. falciparum* malaria infection. This study showed that both AL and DHP were well tolerated in second and third trimester pregnant women, with low treatment failure. DHP is well tolerated and has low treatment failure.[26]

The study conducted by Zein et al, who compared 5 types of drugs, the chloroquine, artemisinin, sambiloto extract, combination of chloroquine and sambiloto, and combination of artemisinin and sambiloto in vitro against *Plasmodium falciparum*, obtained results that a single dose of sambiloto extract, chloroquine and artemisinin and their combinations could kill parasites at a dose of 0.5 ug/ml and the effect increased with an increase in the optimum dose of 200 ug/ml.[31] This study showed that at low doses, sambiloto acted as an antioxidant that attracted free radicals that were harmful to the parasites. Andrographolide is a potent attractor of Reactive Oxygent Species (ROS).[32] However, with an increasing dose of sambiloto extract, the antioxidant effect turned into anti malaria. In this study, it was observed in vitro that the potential for anti-malaria between the combination of sambiloto extract with chloroquine and artemisinin was similar to sambiloto extract alone. Sambiloto extract inhibits plasmodium in the ring stage, both alone and in combination.[31]

A 2014 study in Thailand on the antimalarial effects of sambiloto extract on mice infected with *P. berghei* revealed that sambiloto extract had antimalarial potential and its combination with chloroquin could be more effective. This study showed that the sambiloto extract at a dose of 100 mg/kg did not cause mortality in mice. This study also showed that the sambiloto extract dose of 100 mg/kg had a 65% prophylactic effect on the inhibition of parasite growth, while chloroquine was stronger in inhibiting parasite growth by up to 90%. For curative effect, 100 mg/kg of sambiloto extract can inhibit parasite growth by up to 75% and chloroquine can inhibit parasite growth by up to 90%. The combination of sambiloto and chloroquine extract at a dose of 100 mg/kg can inhibit parasite growth by up to 99%. This study showed that sambiloto extract, alone or in combination with chloroquine, had an antimalarial effect.[34]

CONCLUSION

Macrophage Migration Inhibitory Factor (MIF) expression in pregnant mice placenta infected with *P. berghei* and receiving sambiloto (AS201-01) tablet therapy was not different compared to the expression in the group receiving dihydroartemisinin-piperaquine phosphate (DHP) therapy.

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