ORIGINAL ARTICLE:

The effect of sambiloto tablet (AS201-01) on placental Chondroitin Sulfate A (CSA) expression of pregnant mice infected by *Plasmodium berghei*

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ABSTRACT

Objectives: To determine the effect of Sambiloto tablet (AS201-01) in reducing the placental Chondroitin Sulfate A (CSA) Expression of pregnant mice infected Plasmodiumberghei.
Materials and Methods: Experimental study using 24 pregnant mice were divided into 4 groups with randomization. Uninfected group, the placebo group, the Sambiloto tablet (AS201-01) group and the DHP tablet (as a standart drug) group. The last three groups, were infected with *P. berghei*on day 9th of pregnancy, and the treatment was started at day 11th of pregnancy, and samples were terminated at day 15th of pregnancy by surgery. Placental sampling were stained with Tunnel assay to measure placental CSA antibodies.
Results: The placental Chondroitin Sulfate A (CSA) expression. Uninfected group compared to Sambiloto tablet (AS201-01) groups was not significantly different ($p>0.05$), uninfected group compared with the other treatment groups differ meaningfully ($p<0.05$). Placebo group compared with all groups significantly different ($p<0.05$). Sambiloto tablet (AS201-01) group compared to uninfected group ($p>0.05$) was not significantly different, with another group was significantly different ($p<0.05$). DHP tablet group compared to all the groups was significantly different ($p<0.05$).
Conclusion: Placental Chondroitin Sulfate A (CSA)expression of mice infected by *Plasmodium berghei* treated with Sambiloto tablet (AS201-01) lower than DHP tablet.

Keywords: Placental malaria; Chondroitin Sulfate A (CSA); Sambiloto (AS201-01) tablet

ABSTRAK

Tujuan: mengetahui aktivitas tablet sambiloto (AS201-01) dalam menurunkan ekspresi CSA plasenta mencit bunting diinfeksi *P. berghei* dibandingkan dengan tablet DHP.
Bahan dan Metode: Eksperimental laboratorium, 24 ekor mencit bunting dibagi dalam 4 kelompok dengan randomisasi. Kelompok tidak diinfeksi, kelompok plasebo, kelompok AS201-01 dan kelompok tablet DHP. Pada hari ke-9 diinfeksikan *P. berghei*, hari ke-11 diberikan perlakuan, hari ke-15 pembedahan, diambil sampel plasenta, dilakukan pewarnaan IHC CSA dan dihitung ekspresi plasenta.
Hasil: Ekspresi CSA plasenta kelompok tidak diinfeksi dibandingkan kelompok AS201-01 tidak berbeda bermakna ($p>0.05$), kelompok tidak diinfeksi dibanding dengan kelompok perlakuan yang lain berbeda bermakna ($p<0.05$). Kelompok plasebo dibanding dengan semua kelompok berbeda bermakna ($p>0.05$). Kelompok AS201-01 dibandingkan Kelompok tidak diinfeksi p 0,15 ($p>0.05$) tidak berbeda bermakna, dengan kelompok yang lain berbeda bermakna ($p<0.05$). Kelompok tablet DHP dibandingkan dengan semua kelompok berbeda bermakna ($p<0.05$).
Simpulan: Ekspresi CSA plasenta mencit bunting diinfeksi *P. berghei* yang diberikan AS20-01 lebih rendah dibandingkan tablet DHP.

Kata kunci: Malaria placenta; Chondroitin Sulfate A (CSA); tablet sambiloto (AS201-01)

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INTRODUCTION

Malaria infection in pregnancy remains a global health problem because it increases maternal and fetal morbidity and mortality. Pregnant women are more easily infected with malaria than the general population. This is due to the weakness and decrease in body immunity obtained when suffering from malaria. Pregnancy will aggravate malaria, otherwise malaria will affect maternal and fetal pregnancies and increase morbidity and mortality for both. The impact of malaria on pregnancy in malaria endemic areas anemia risk 3-15%, low birth weight 13-70% and neonatal mortality 3-8%.[1, 2, 3, 4]

In pregnant women infected with malaria, parasitic erythrocytes are found in the placenta of the mother because the placenta is an ideal place for the parasite. Sinsitiotrophoblast is a placental barrier that separates maternal and fetal blood will prevent hematogenous transmission from mother to fetus. Sinsitiotrophoblast produces a dominant receptor of placental, Chondroitin Sulfate A (CSA), which serves to bind antigen from the parasite, *Plasmodium falciparum* erythrocytes membrane protein-1 (PfEMP-1). The amount of CSA produced by the placenta depends on the accumulation of the parasite, the more severe the malaria infection, the higher the CSA receptor the placenta produces. The interaction of PfEMP-1 with CSA receptors produces both cellular and humoral immune responses and accumulation of the placenta parasite, thereby increasing apoptosis, necrosis and placental insufficiency.[5,6] Sinsitiotrophoblast necrosis, loss of microvillus and thickening of the trophoblast basal membrane will cause blood flow to the placenta and the fetus is reduced which may lead to abortion, premature birth, stillbirth or low birth weight.[7]

Therapy difficulties in pregnancy malaria are more commonly caused by the selection of drugs that are safe for the fetus and effective. Increased cases of parasitic resistance to first-line anti-malarial drugs are a problem in the world of health. The case of P. falciparum resistance to pyrimethaminesulfadoxine and Artesunat-amodiaquine, which is the first line of malaria treatment in Indonesia, also shows a treatment failure rate of >10%. Resistance can be caused by mutations that can reduce the sensitivity to antimalarial administration. The search for effective and safe antimalarial drugs is necessary; one of the alternatives is antimalarial drugs from natural ingredients.[8, 9]

In the community has been used sambiloto (*Andrographis paniculata*) as malaria therapy. Sambiloto with its active ingredients Andrographolide, is known to inhibit the growth of malaria parasites through inhibition of food vacuole system.[10,11] Clinical trials have been performed on both the main extracts, fractions and active compounds that have been isolated from the bitter plants (*Andrographis paniculata*Nees). With the toxicity test has provided a normal liver histopathologic picture of SGOT, SGPT, BUN and creatinine values in experimental animals.[12]

It has been observed on the effect of giving of bitter tablets (AS201-01) on fetal bone size and fetal morphology of mice, the result is that no fetal morphology difference or bone size of cranium, scapulae, costae, vertebrae, humerus, radius-ulna, femur and tibia -fibula infected fetal mice infected with *Plasmodium berghei* who get the ethyl acetate fraction of sambiloto with normal fetal mice fetus.[13] Administration of Sambiloto tablets (AS 201-01) may decrease the accumulation of placental malaria parasites in pregnant mice infected with *Plasmodium berghei* compared with placebo and DHP therapy.[14] It was found that administration of bitter tablets (AS 201-01) gave lower expression of TLR-4, COX-2 and Apoptosis indexes than with placebo and DHP therapy.[15, 16, 17]

The association between parasites and placenta is mediated by Chondroitin Sulfate A (CSA) with surface antigen of erythrocyte infected cells *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1) is an early process of pathological conditions in the placenta. It is therefore necessary to learn about the effect of giving of sambiloto on Chondroitin Sulfate A (CSA) expression on malaria placenta with immunohistochemically examination of Chondroitin Sulfate A (CSA).

In this study we used mice (Mus musculus) strain of BALBc bunting as a model infected by *P. berghei* with given sambiloto to see the effect of antimalarial on sambiloto and its effect on the fetus. This study using *Plasmodium berghei* because this parasite is a type of parasite on rodent. *Plasmodium berghei* infection in mice, resembling human P. falciparum infection, includes adhesion phenomena, the underlying mechanism of susceptibility and acquired immunity.[18, 19]

MATERIALS AND METHODS

Sambiloto tablet (AS201-01) is 96% ethyl acetate fraction of Sambiloto obtained from extracted Sambiloto leaf, fractionated with ethyl acetate and then formulated in tablet form that consist of *Andrografolide* 35 mg made in Institute of Tropical Disease, Airlangga University. DHP Tablet consist of Dihydroartemisinin 40 mg and Piparaquine phosphatase 320 mg (D-ARTEPP™) made by PT.Pharma laboratories, we used conversion dose from the human to mice.

This research used Mice BALB/C strain, from LPPT-Gajah Mada University. The weight between 25-30 g and maintained at Animal Laboratory of Institute of Tropical Disease, Airlangga University. Permission and approval for animal studies were obtained from Faculty of Veterinary Medicine, AirlanggaUniversity, No:560-KE/2016. The parasite that infected mice in this research was *Plasmodium berghei* ANKA strain, which originally obtained from Eijkman Institute for Molecular Biology, Jakarta. This parasite has been maintained at Institute of Tropical Disease, Universitas Airlangga.

Experimental study using 24 pregnant mice were divided into 4 groups (n=6) with randomization. Uninfected group (pregnant mice without infection of *P. berghei*), the placebo group (pregnant mice that treated with placebo), the Sambiloto tablet (AS201-01) group (pregnant mice treated with Sambiloto tablet) and the DHP tablet group (pregnant mice that treated with DHPas a standart drug). The last three groups, were infected with *P. berghei*on day 9th of pregnancy, and the treatment was started at day 11th of pregnancy, then were terminated at day 15th of pregnancy by surgery. Placental sampling were stained with Tunnel assay to measure placental apoptosis index.

Apoptosis index calculation used*Immuno Reactive Score*that was the result of multiplying the score of percentage Immunoreactivecells with a score of color intensity on the cell imunnoreaktifwithTunnel assay staining. Placental tissue that consist of syncytiotrophoblast cells undergoing DNA fragmentation will be labeled as brown chromogen.[8]

Data of this study analyzed using one way Analysis of variance (Anova) and statistical significance was calculated at p 0.05. This research was carried out at the Institute of Tropical Disease (ITD), Airlangga University and at Pathology Laboratory of Veterinary, Faculty of Veterinary Airlangga University

RESULTS AND DISCUSSION

In this study, 24 pregnant mice that performed surgery on the 15th day, then we took placental sampling to make preparations and stained with Tunnel assay, reagents would labeled the free 3-OH group in the end of the molecule DNA which fragmented in the apoptosis process colored as brown chromogen.

According to Figure 1, the number of placental CSA Expression (brown chromogen) in placebo group higher than the others group (Sambiloto tablet (AS201-01) group, DHP tablet group and uninfected group). The placental CSA Expression of uninfected group was lowest than another's treated group (Placebo, Sambiloto (AS201-01) and DHP) but between treated groups; Sambiloto tablet (AS201-01) group was the lowest.

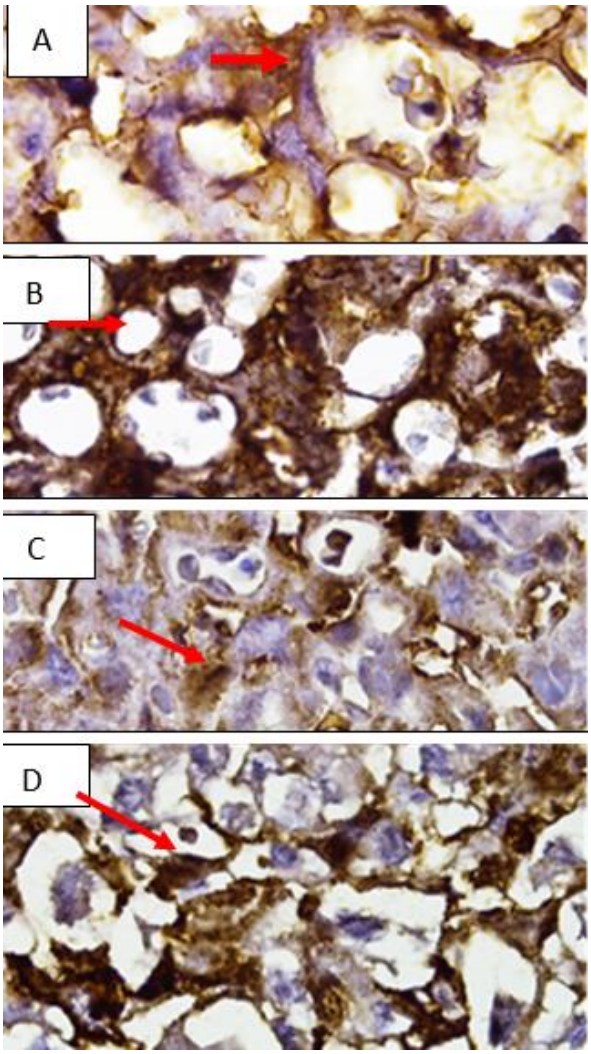

Figure 1. Immunohistochemical staining (Tunnel assay) of the placenta of mice infected by *P. berghei* to detect apoptosis index, took by Nikon light microscope with a magnification 1000x H600L Case (A) Uninfected group (B) Placebo group (C) Sambiloto tablet (AS201-01) group (D) DHP tablet group, showed as brown chromogen color as DNA fragmentation.

The data of placental CSA Expression analyzed with one way Anova. The results of placental apoptosis index in each group shown in Table 1.

Table 1. Mean and standard deviation placental CSA expression in pregnant mice in each treatment group.

Group	CSA Mean±SD	95% CI	p Value
K1	7.57±1.08 [a]	6.43 – 8.70	0.000
K2	11.60±0.49 [b]	11.09 – 12.11	
K3	7.70±1.55 [a]	6.07 – 9.33	
K4	9.57±1.74 [c]	7.74 – 11.39	

Notes: The letters are different in the same column differ significantly p<0.05. Table 1 The mean and standard deviation of plasental CSA expression. K1: Uninfected group; K2: Placebo group; K3: Sambiloto group (AS201-01), DHP group

In the statistical data Table 1 shows that the average of CSA expression in group of bitter tablets (AS201-01) is 7,70±1,55 while mean of tablet group DHP 9,57±1,74. The mean expression of CSA of the bitter tablet group (AS201-01) was lower than that of the DHP tablets. The mean of the placebo group was 11.60±0.49 and the uninfected group mean was 7.57±1.08. The mean group of bitter tablets (AS201-01) was lower than in the placebo group and the mean DHP tablet group was also lower than in the placebo group. The mean of the uninfected group was lower than the treatment group.

Comparisons of CSA Expression in *Plasmodium berghei* infected pregnant mice that received the bitter tablets (AS201-01), DHP tablets, placebo and uninfected groups in multiple comparisons were as follows: Uninfected group compared with Plasebo group and DHP tablet group showed significant difference that is p value 0.00 (p<0.05), while compared with bitter tablet (AS201-01) no significant difference was p 0.862 (p>0, 05). The placebo group compared with the uninfected group, the bitter tablets (AS201-01) and DHP tablets were found to have a significant difference of p value 0.000 and p 0.014 (p<0.05).The group of bitter tablets (AS201-01) compared with the uninfected group showed no significant difference of p value 0.862 (p>0.05). While the group of sambiloto tablets (AS201-01) with placebo and DHP tablets found significant difference that is p value 0.000 and p value 0.023 (p<0.05). Group of DHP tablets compared with uninfected group, placebo and bitter tablets (AS201-01) there was a significant difference of p 0.016; P 0.014 and p 0.023 (p<0.05).

Malaria in pregnancy is the sequestration of parasites in the placenta that often cause severe infections. In 1996 placental receptor Chondroitin Sulfate A (CSA) was known by Fried and Duffy, and in 2003 PfEMP-1 parasitic ligand was identified. Since then large-scale efforts have been invested in making adhesion inhi-biting vaccines between CSA and PfEMP-1.[21, 22, 23]

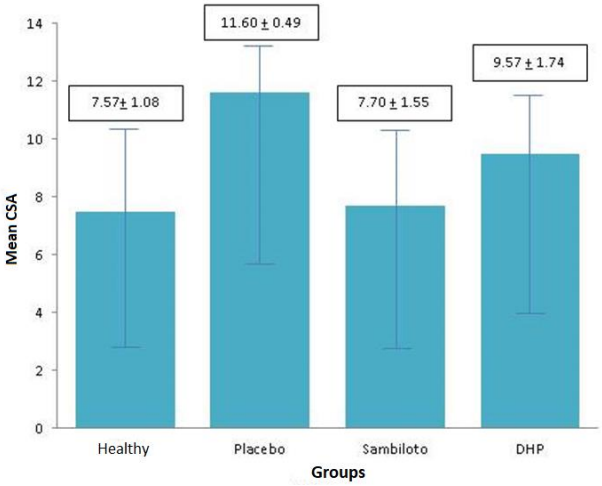

Figure 2. Mean CSA expression in each group: Uninfected group; Placebo group; Sambiloto (AS201-01) group and DHP group

In study shows that the interaction of PfEMP-1 parasitic ligand with CSA receptor secreted by sinsitiotrophoblast is considered important in the development of placental malaria. Several studies have shown the role of CSA in mediating iRBC adhesion in the placenta. CSA expression is higher in syncytiotrophoblasts in placenta infected with malaria and these receptors also play a role in iRBC sequestration in the placenta.[24]

The sequestration results of iRBC also cause micro circular obstruction that forms the basis of multiorgan dysfunction in severe malaria infections. Infected erythrocytes form a rosette which is an important sequestration mechanism in the blood in the placenta that affects the fetal-maternal circulation and results directly to the fetus. Sequestration due to citoadhesi is considered a key point in the pathogenesis of placental malaria.[7,25,3]

Sequestration in the intervillous space (IVS) will cause chemokine secretion that produces inflammatory cells and cytokine production associated with poor outcome of pregnancy.[26,27] Although placental malaria is largely asymptomatic, there is a correlation between peripheral and placental parasitemia at delivery. Placental malaria can be categorized as acute, chronic, or previous infection using specific pathological criteria.[28] Chronic placental malaria is associated with an increased risk of IUGR, whereas placental malaria is associated with premature risk in malaria endemic areas.[29] The presence of CSA bonds with parasitic ligand PfEMP-1 is the key to how the mechanism of placental malaria can induce IUGR and premature. Placental malaria will decrease the function of the syncytiotrophoblast so that the

transport of nutrients from mother to fetus will be inhibited.[6]

Effect of *P. berghei* infection to plasental Chondroitin Sulfate A (CSA) Expression

P. berghei infection triggers a response to the immune system that is naturally acquired and immunity. Natural immune already existed from birth that occurs without contact with the malaria parasite first. The main natural immune response to protozoal infection is phagocytosis. The acquired immunity is obtained after contact with the malaria parasite, comprising cellular immunity carried out by T lymphocytes and humoral immunity performed by B lymphocytes.[5]

Placental malaria is characterized by the presence of infected erythrocytes attached to the placenta via CSA receptors. This infected adhesion of erythrocytes causes the entry of macrophages and leukocyte cells into the intervillous space. In placental malaria there is also a deposition of hemozoin pigment which is a product of the metabolism of malaria parasites to hemoglobin in infected erythrocytes. In *P. berghei* infection, inflammation of infected erythrocytes occurs via PEXEL (Plasmodium export element) or VST (Vacuolar transport signal) to CSA receptors in the placenta of mice.[27, 29]

During a malaria infection a placenta will produce CSA receptors in response to parasites in the placenta. A CSA bond with a parasitic ligand will lead to an early change in diploma of pathology. The amount of CSA expressed depends on the number of placental parasites. The higher parasite more CSA expressed by the placenta. A CSA bond with a parasitic ligand will result in Reactive Oxygen Species (ROS). In placental malaria, the production of ROS will cause destruction of trophoblast cells to cause premature and IUGR.[29]

Comparison of placental Chondroitin Sulfate A (CSA) expression between uninfected group, placebo group, sambiloto tablet (AS201-01) group and DHPtablet group

The mean of CSA expression on placental placental group (K2) was 11,60±0,49, mean of CSA expression index of group of sambiloto tablets (AS201-01) (K3) that is 7,70±1,55 and CSA expression DHP tablets group is 9.57±1.74. The Placental Chondroitin Sulfate A (CSA) expression on the Sambiloto tablet group (AS201-01) (K3) was 7.70±1.55 and the DHP (K4) tablet group of 9.57±1.74 lower than the placebo group (K2) with results Average 11.60±0.49. Different test results from the post hoc table obtained p value 0.00 ($p<0.05$), this indicates that there is a significant difference between the placebo group (K2) and the Sambiloto tablet group (AS201-01) (K3) and the DHP tablet group (K4). It is understandable that in the placebo group the parasite growth did not experience obstacles, so the number of parasites increased; the more formed the bond between PfEMP-1 and CSA. With the increasing number of parasites, the placebo group's CSA expression is higher than that of the bitter tablets and DHP tablets.

From this study, mean of CSA expression on placental cell of group of tablet of sambiloto (AS201-01) (K3) is 7.70±1.55 lower than the DHP tablet group (K4) that is 9,57±1,74 with result ($P<0.05$), so it can be concluded that there was a significant difference between group of bitter tablets (AS201-01) (K3) and DHP tablet group (K4). How Sambiloto tablets (AS201-01) can decrease CSA expression of the placenta is lower than DHP tablets, this may be due to the activity of Sambiloto with the main active ingredient Andrografolide has many mechanisms such as antimalarial, anti-inflammatory, immuno-modulatory and as an antioxidant.

Sambiloto as antimalarial can decrease CSA expression through obstacles in hemoglobin degradation process and heme detoxification, so it does not form hemozoin, in which the hemozoin is a food for plasmodium. With increased heme and unformed hemozoin will be a hazard to the plasmodium that will die. As a result the number of parasites will decrease and eventually the expressed CSA also decreases. This is consistent with the results of previous research that Andrographolide has activity as schizontocidal and acts on parasitic food vacuoles through inhibition of heme polymerase process in the observation of voodola morphology of *P. falciparum* in vitro food using Transmission Electron Microscope.[30]

Sambiloto as an anti-inflammatory and immunomodulatory may inhibit inflammatory cytokines such as TNFα and macrophages, which act as a death activator in the extrinsic pathway of apoptosis, consequently TNFR-1 and adapter proteins will be activated in small amounts so that caspase 8 and caspase 3 are also slightly activated Decrease the amount of apoptosis. This is consistent with previous research that *Andrographis paniculata* may suppress the production of IL-2, the proliferation of T-cells in lymphocytes and inhibit the maturation of dendritic cells, and the emergence of antigens.[31] *Andrographis paniculata* as an immunomodulator is also reported to inhibit the production of TNFα and IL-12 in macrophages.[32] In addition it is reported that administration of bitter extract from stems and leaves 25 mg /kg in mice can stimulate anti-bodies in mice.[33]

Sambiloto as antioxidants can decrease the production of Reactive Oxygen Species (ROS) generated from the bond between PfEMP-1 with the CSA, in which ROS will be entered into the path of the intrinsic (mitochondrial), reduce the production of proteins and anti-apoptotic Bcl-2, activates protein pro-apoptotic Bax/Bak so forth cytochrome C from mitochondria activates procaspase 9 into caspase 9 which then activates caspase 3 and there was apoptosis through the intrinsic pathway. This is consistent with a previous study using pregnant mice infected with *P. berghei* also proved that the bitter apoptotic index lowers than DHP and placebo, and no significant difference with the uninfected group.[15] Sambiloto has antioxidant activity (preventing oxidative damage), where the compound Andrographolide on this bitter Vinayak reported by Verma and can significantly increase the activity of antioxidant enzymes (Catalase, Superoxide dismutase, and gluthation-S transferase). Additionally Andrographolide can weaken PMA (phorbol-12-myristate-13-acetate) which can induce accumulation of ROS andfMLP (N-formyl-metionyl-leucyl-phenyalanine) whichinduce adhesion netrophil in mice. Bitter also can maintain endothelial function and maintaining the balance of nitric oxide / endothelin.[34]

DHP tablet is a standard combination drug, which has been done a lot of research, proven to reduce parasitemia in blood is very significant compared with other drug combinations. DHP tablets are a combination consisting of 40 mg of dihydroartemisisnin and 320 mg piperaquine phosphate in the form of a single dose. This drug is an active metabolite of artemisisnin that works quickly eliminates parasites in the body, while piperaquine has a long half-life for 23 days (19-28 days). Artemisinin and piperaquine both have antimalarial effects as skizontocides, inhibiting nutritional input into the parasitic food vacuole and also inhibiting hemoglobinase, thus inhibiting the activation of inflammatory cytokines or death activators that may activate caspase in the extrinsic pathway of apoptosis.[8] However, it turns out that bitter tablets (AS201-01) which still contain a variety of other active compounds other than the main component of Andrografolide have extensive activities as antimalarial, anti-inflammatory, antioxidant and immunomodulatory that we suspect work synergistically inhibits *P. berghei* activity so as to have A stronger ability to decrease CSA in placental expression compared to the DHP tablet group.

The results of this study are same with the results of research on the effect of tablets sambiloto Morphology Fetal Mice Balb/C pregnant who got *Plasmodium berghei* infection. In this study, fetal morphological abnormalities were obtained from cranium, scapulae, costae, vertebrae, humerus, radius-ulna, femur and tibia-fibula in the CMC Na (placebo) and Dihydroartemisin-inpiperaquine phosphate tablets compared with the uninfected group. In the group of bitter tablets obtained fetal morphology that is not different from the uninfected group.[13]

Comparison of placental Chondroitin Sulfate A (CSA) expression between Sambiloto tablet (AS201-01) group with uninfected group

Expression and function of Chondroitin Sulfate A (CSA) in normal placental conditions is not known with certainty. The growth of trophoblast and into the invasion of the endometrium is the most important part during the placentation and is strictly regulated by local immune factors. Abnormal placentation may lead to early miscarriage or preeclampsia and intrauterine growth restriction leading to fetal and maternal health disorders. Chondroitin sulfate is involved in cell migration and invasion, a very important process during placentation. Van Sinderen's study showed that chondroitin sulfate produced locally stimulates migration and the invitation of syncytiotrophoblast (ST) or extravillous trophoblast (EVT) and shows that interleukin 11 (IL-11) and leukemia inhibitory factor (LIF) regulate the differentiation of villous cytotrophoblasts against invasive phenotypes at least Partly through Chondroitin sulfate.[35]

In this study the average of placental CSA expression in the K1 group is uninfected group is 7,57$\pm$1,08 and mean on K3 is Sambiloto tablet group (AS201-01) is 7,70$\pm$1,55 with different test on analysis Statistics obtained p 0.862 (p>0.05). It can be concluded that CSA expression of both groups did not differ significantly, CSA expression in placental malaria, similar to that of pregnant mice without *P. berghei* infection (Uninfected group).

This is in line with the results of the study on the ratio of placental parasite accumulation and placental histopathology in infected mice infected with *P. berghei*sambilototablets and DHP tablets, ie no parasite accumulation in the uninfected group, whereas in the tablet group of sambiloto still obtained parasite although not as much Accumulation in the placebo group.[14] CSA expression on the placenta will depend on the accumulation of the diplasenta parasite. The higher the parasite the higher the CSA receptor produced by the syncytiotrophoblast, and vice versa. In the use of fraction tablets EA-96 (AS201-01) there was lower parasite accumulation than other groups or almost the same as for uninfected group, so CSA expression was also lower.

CONCLUSION

Placental Chondroitin Sulfate A (CSA) of mice infected by *P. berghei* treated with sambiloto tablet (AS201-01) lower than DHP tablet.

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