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FOREWORD

Alhamdulillah, praised to Allah, Journal *Qanun Medika: Fakultas Kedokteran Universitas Muhammadiyah Surabaya* vol 04 no 02 has been published. It consists of 15 articles including 2 literature reviews, 1 case report and 12 research articles in the medical field. We would like to thanks our reviewers and editorial board members who helped us in this publication. In order to be internationalized, we only published articles written in English since July 2019. We hope that these articles can be read widely both by domestic and foreign readers.

> Thank you, Yelvi Levani, MD.,M.Sc Editor in Chief







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Research Article

Sequestration of erythrocytes infected with *Plasmodium berghei* ANKA in BALB/c mice treated with goat bile

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Abstract

Sequestration of Plasmodium berghei ANKA-infected erythrocytes occurs in BALB/c mice as characteristic of Plasmodium falciparum infection in humans. Animals' bile has been widely used for centuries in Traditional Chinese Medicine. Goat bile has been used in healing infectious and non-infectious diseases; however, no report on the use of goat bile against malaria infection and sequestration. The purpose of this study was to analyze the correlation between parasitemia and sequestration in the liver of P.berghei ANKA-infected BALB/c mice treated with goat bile. This research was an in vivo experimental study using the post-test control group design. The male BAL-B/c mice aged \pm 6 weeks, body weight 20-25 g were used. The mice were divided into five groups where Group 1-3 were mice treated with goat bile 25%, 50%, and 100%, respectively. Group 4-5 were negative (sterile water) and positive controls (DHP). Parasitemia was observed daily from each mouse and the number of sequestered infected erythrocytes on the endothelium of sinusoids. The data were analyzed using t independent test. Antimalarial activity of goat bile was shown by the lower parasitemia in goat bile-treated mice compared with the negative control. The average number of sequestration was goat bile concentration-dependent manner. The higher the concentration, the lower the number of sequestration. Sequestration was correlated with parasitemia (p=0,0001). Sequestration of *P.berghei* ANKA-infected erythrocytes correlated with parasitemia, and was goat bile concentration-dependent manner.



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INTRODUCTION

Malaria is a disease that remains a problem in the world, especially in endemic areas, such as Africa, Southeast Asia, and the Eastern Mediterranean. According to the World Malaria Report 2018, during 2017, there were 219 million new cases of malaria with a mortality rate of 435,000 worldwide (WHO, 2018). In Indonesia, the hyperendemic malaria areas were provinces of Papua, Maluku, North Maluku, and East Nusa Tenggara (Pusdatin, 2016).

Protozoan genus *Plasmodium* causes malaria. In general, five species of *Plasmodium* that infect humans are Plasmodium falciparum, Plasmodium vivax. *Plasmodium* ovale. Plasmodium malariae, and Plasmodium knowlesi (Wassmer et al., 2015). Four infecting Plasmodium species rodents that have been extensively used in in vivo rodent research are Plasmodium berghei, Plasmodium chabaudi, Plasmodium yoelii, and Plasmodium vinckei (De Niz & Heussler, 2018).

Clinical pathologies of P. falciparum in human infection are severe anemia (White, 2018), sequestration of infected erythrocytes (David et al., 1983), rosetting, and organ complications such as cerebral malaria, malaria in pregnancy, splenomegaly, hepatomegaly, hypoglycemia, pulmonary edema to death (Bartoloni & Zammarchi, 2012). Hepatomegaly is a common feature in malaria infection, especially in P. falciparum (Viriyavejakul et al., 2014). infection Sinusoidal dilatation is the most important factor contributing to the enlargement of the liver (Baheti, Laddha, & Gehlot, 2003). Sequestration is a characteristic of P. falciparum infection where infected erythrocyte as adhere to endothelial cells in microvasculature of vital organs such as the brain, lungs, spleen, placenta, eye, subcutaneous fat, heart, bone

marrow, intestine, liver which can cause various types of malaria severity (Brugat et al., 2014). Sequestration of *P. falciparum* in small blood vessels induces local blood flow impairment leading to disturbances and failure in various organs, including liver (MacKintosh, Beeson, & Marsh, 2004).

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Similar clinical features are found in rodent malaria. Experimental cerebral malaria (ECM) in C57BL/6 mice infected with P. berghei ANKA showed sequestration of infected erythrocytes in the brain, as found in human cerebral malaria (Baptista et al., 2010). Sequestration of P. berghei ANKA-Infected erythrocytes in BALB/c mice are found in the spleen, lungs, and adipose tissue indicated that sequestration is associated with the severity of the disease (Franke-Fayard et al., 2010). Sequestration usually occurs when erythrocytes infected with the stages of adult trophozoites, schizonts, and young gametocytes (Mota & Rodriguez, 2017) as an attempt to escape from the immune system (Belachew, 2018). In fact, sequestration in P. berghei-infected mice evidenced by the presence of schizont-infected erythrocytes sequestration in the organ that expressing CD36+ markers (Franke-Fayard et al., 2010).

Animals' bile has been widely used for centuries Traditional Chinese Medicine (TCM) in for clinical practice (Li et al., 2016). Bile is secreted from hepatocyte involves in biliary system (Hundt M et al, 2018). Bile contains about 95% of water, bile salts, phospholipid bilirubin, cholesterol, amino acids, steroids, enzymes, porphyrins, vitamins, and heavy metals, and exogenous drugs, xenobiotics and toxic environments (Boyer, 2013) and a wide variety of antioxidants, bilirubin, glutathione, melatonin (N-acetyl-5vitamin E, and methoxytryptamin) (Wang & Carey, 2014). The functions of bile are to improve liver function, dissolve gallstones, inhibit bacterial and viral multiplication, promote cardiac chronotropsim,

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as well as exhibiting anti-inflammatory, antipyretic, anti-oxidant, sedative, anti-seizure, anti-allergic, anti-congestive, anti-diabetic and anti-plasmodic effects (Boyer, 2013).

Malaria control in Indonesia uses Artemisinincombination based therapy (ACT) as recommended by WHO (Kemenkes, 2016). However, some people of Indonesia consume goat gallbladder to treat malaria and to increase their stamina (Amalia, 2012). Goat bile has been used in healing several diseases such as optical atrophy, blindness, and diarrhea (Li et al., 2016; Wang & Carey, 2014). Until now, there is no report on the effect of goat bile to sequestration of malaria parasite-infected erythrocytes in the vital organs of infected mice. The effect of goat bile on the sequestration of P.berghei ANKAinfected erythrocytes in the liver of BALB/c mice is reported herein.

METHODS

Ethical approval

The proposal of this research has been reviewed by the Ethics Committee of Faculty of Medicine, Universitas Airlangga as described on the Ethical Clearance No. 110/EC/KEPK/ FKUA/2019.

Research Design

This research is an in vivo experimental study using the post-test control group design. After infected with *P. berghei* ANKA mice were divided into five groups. Group 1 was a positive control treated with 187.2 mg/kg body weight of Dihydroartemisinine Piperaquine or DHP (Mersi Farma, Sukabumi, Indonesia), Group 2 was negative control mice were only given with sterile water, Group 3-5 were given with 25% (GB25), 50% (GB50) and 100% goat bile (GB100), respectively.

Parasite infection in mice

Parasite used in this experiment was P. berghei ANKA obtained from the Department of Medical Parasitology, Faculty of Medicine, Universitas Airlangga. The BALB/c mice aged six weeks with average weight about 25 grams, healthy, and had never received any treatment before. Mice were acclimatized for one week before infection. Five donor mice were infected with 200µL per mouse of P. berghei ANKAinfected frozen blood. When parasitemia reached $\pm 20\%$ mice were sacrificed, the blood was collected by cardiac puncture and infected to test mice. Each test mouse was infected with 1x10⁶ infected erythrocytes. A four daytreatment was started on day two post-infection. Each mouse was given 0,5 mL/25-gram mouse of each concentration of goat bile.

Goat bile and DHP preparation

Goat gallbladders were bought from Pegirikan slaughterhouse Surabaya. The healthy Java goat was chosen as this strain of goat was usually consumed by the Javanese. Gallbladders were sprayed with 70% alcohol before bile removal with syringe. Goat bile were then pooled into sterile tube and diluted with sterile water to prepare 25% and 50% goat bile solutions. The working goat bile solutions were stored in a refrigerator during the course of experiment. The DHP was diluted with sterile water to prepare 187.2 mg/kg body weight of doses.

Determination of parasitemia

Parasitemia of infected donor and test mice were determined daily by counting the infected erythrocytes on Giemsa-stained thin blood smears of mouse tail blood. Parasitemia was calculated using the following

 $formula = \frac{number of infected erythrocytes}{total number of erythrocytes} \ge 100\%$



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Observation of sequestration

Test mice were anesthetized by intraperitoneal injection of ketamine prior to liver removal. Livers were then fixed in 10% formaldehyde. Fixed organs were embedded in wax, sectioned (5 μ m), and stained with hematoxylin eosin HE. The sequestrations of P. berghei-infected erythrocytes on endothelial cells of liver microvasculature were observed quantitatively on 10 fields of view or 100 sinusoids microscopically at 1000x magnification (Olympus CX21, Tokyo, Japan).

Statistical analysis

The difference of the parasitemia and the number of sequestrations were compared with negative and positive controls were analyzed using t dependent test. The correlation of parasitemia and number of sequestration was analyzed using Pearson correlation test.

RESULTS

Parasitemia

Based on **Figure 1**, normal parasitemia was shown in negative control which did not received any drug administration. On the other hand, parasites in mice treated with DHP were



Figure 1. Parasitemia on day 5 of mice infected with *P. berghei* ANKA treated with goat bile compared with positive and negative controls. GB25: goat bile 25%, GB50: goat bile 50%, GB100: goat bile 100%. NEG: negative control (sterile water). POS: positive control (187.2 mg/kg body weight of DHP).

completely eliminated, indicated a potent anti-malaria drug. There was no significant difference of parasitemia between the negative control and GB treatment group (p>0,05). However, parasitemia of the GB25, GB50 and GB100 were lower than that of negative control. This result indicated that GB possessed antimalarial activity.

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Sequestration

Figure 2 shows the average number of sequestrations of P. berghei ANKA-infected erythrocytes decreased along with the increase of concentration of goat bile. Statistical analysis of sequestration was shown in Table 1. The difference of sequestration in the liver of mice treated with GB25 and GB50 was not significant compared with negative controls (p>0.05), while GB100 was significant. In contrast, the comparison between positive controls with GB25, GB50 showed significant differences (p <0.05). However, there was no significant difference between positive control and GB100. The significant difference was obviously seen between negative and positive controls. The sequestration of P.berghei ANKA-infected erythrocytes in current research showed that BALB/c mice



Figure 2. Sequestration of *P. berghei* ANKA-infected erythrocytes treated with goat bile compared with positive and negative controls. GB25: goat bile 25%, GB50: goat bile 50%, GB100: goat bile 100%. NEG: negative control (sterile water). POS: positive control 18.72 mg/kg body weight.

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without goat bile treatment (negative control) similar to that of mice treated with GB25 (p=0,450) and GB50 (p=0,702, **Table 1**) where average number of sequestrated *P.berghei* AN-KA-infected erythrocytes in negative control, GB25, and GB50-treated mice were 16.5,19.05 and 15.3, respectively. Sequestration in mice treated with GB100 was significantly different with the negative control. The sequestration in the liver of mice is shown in **Figure 3**. This figure proved the sequestration of *P. berghei* ANKA-infected erythrocytes that occurred in the liver of BALB/c mice. Kupffer cells, hemozoin particles, and clumps also adhered to the liver endothelium. The Pearson correlation test for the correlation between sequestration and parasitemia resulted in a significant correlation with p=0.001 (significance at p<0.01).

Table 1. The average number of sequestration of *P.berghei* ANKA- infected erythrocyte in BALB/c mice liver treated with goat bile compared with negative (NEG) and positive (POS) control group

Gro	up of mice	Mean± SD	р
NEG	GB 25	19.5 ± 7.314	0.450
	GB 50	$15.3~\pm~4.320$	0.702
	GB 100	9 ± 4.733	0.034*
	POS	5.5 ± 3.620	0.003*
POS	GB 25	19.5 ± 7.314	0.002*
	GB 50	$15.3~\pm~4.320$	0.002*
	GB 100	9 ± 4.733	0.181
	NEG	16.5 ± 5.822	0.003*

Statistical analysis using independent sample t test, n = 5. *Significance p< 0.05



Figure 3. Representative photomicrograph of HE-stained *P. berghei* ANKA-infected erythrocytes sequestration in the liver of mice: A and B: several infected erythrocytes in sinusoid; C and E: infected erythrocytes sequestered on endothelium; D zoomed of picture C; F zoomed of picture E. a. infected erythrocytes adhered to endothelial (sequestration), b. Hemozoin, c. Kupffer cell.



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DISCUSSION

The effects of goat bile on the alteration of parasitemia and the average number of infected erythrocyte sequestration have been observed in this study. Figure 1 shows the effect of goat bile on parasitemia in mice treated with GB25,GB50, and GB100, which were lower than that in negative control. This result indicated that goat bile possessed antimalarial activity against P.berghei ANKA in BALB/c mice. Parasitemia in positive control was the lowest among GB-treated mice that reached to zero, indicating that DHP is a potent antimalarial drug. Bile acids are potent stimulators of suicidal erythrocyte death (eryptosis) in vitro because bile acid can induce the stimulation of Ca²⁺ entry (Lang et al., 2016). The low parasitemia may caused by eryptosis due to the entry of Ca^{2+} . Then, the erythrocytes lysed and lead to parasite malnutrition.

The higher average number of sequestration of *P.berghei* ANKA-infected erythrocyte in 100 sinusoids was shown in the GB25 treated mice that werenot significantly different compared with negative control, indicated the slight effect of goat bile to the sequestration. However, GB100 gives the effect significantly different similar to DHP in positive control that reduced sequestration of *P.berghei* ANKAinfected erythrocytes in the liver of BALB/c mice. These results suggested that the effect of goat bile to sequestration *P.berghei* ANKAinfected erythrocytes was a concentrationdependent manner.

Sequestration is a unique phenomenon that usually occurs in *P.falciparum*-infected erythrocyte in humans. Some studies have reported that sequestration has also occurred in *P.berghei*-infected C57BL/6 mice, BALB/c mice, Wistar rats, and SHR/NCrIBR rats (Franke-Fayard, 2005). The *P.berghei* ANKA-infected erythrocyte adheres to the endhotelial cells of microvasculature through the CD36+ (Franke-Fayard et al., 2010) and ICAM-1 (Cunningham et al., 2017) receptors in C57BL/6 and BALB/c mice. The ligand on *P.berghei* ANKA-infected erythrocytes was unknown (Cunningham et al., 2017; Franke-Fayard et al., 2010). Bile acids have the ability to increase nitric oxide (NO) (Nakajima et al., 2000) lead to the reduction of adhesion molecule on endothelial cells (Gao et al., 2018). The higher concentration of goat bile increased NO and reduced the expression of the adhesion molecule caused the lower number of infected erythrocyte sequestration.

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Bile acids play a dual role due to their amphiphatic properties, which are hydrophobic and hydrophilic. Hydrophobic bile acids are strong cytotoxic acids, fully ionized at physiological pH values (Begley et al., 2005). The greater hydrophobicity the greater cytotoxic effect (Hofmann & Eckmann, 2006). The cytotoxic effect is played by hydrophobic deoxycholic acid (DCA) and chenodeoycholic (CDCA). Hydrophilic bile acids are cytotoxic inhibitor, which played by ursodeoxycholic acid (UDCA) and tauroursideoxycholic acid (TUDCA) (Hofmann & Eckmann, 2006; Mello-Vieira et al., 2013). The DCA, CDCA, and TUDCA increased Ca²⁺ in a concentration dependent manner (Nakajima et al., 2000).

CONCLUSION

Goat bile antimalarial activity in BALB/c mice infected with *P. berghei* ANKA and sequestration of infected erythrocytes in a concentration-dependent manner suggested that goat bile is a potential antimalarial therapy that may developed into a potent antimalarial drug through a series of more specific and intensive research.

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