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RESEARCH ARTICLE

Sequestration and Histopathological Changes of the Kidneys, Lungs and Brain of Mice Infected with *Plasmodium berghei* that Exposed to Repeated Artemisinin

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ABSTRACT

The purpose of this study was to determine the pathogenesis of malarial infection in rodent as in vivo model in humans due to repeated exposure of artemisinin through organ histopathological picture. Healthy adult *Albino swiss* mice with average weight of 20-30 g were used for the study. Fifteen mice were divided into three groups: mice were infected with *Plasmodium berghei* which has been ever treated with artemisinin up to 4 times than treated by artemisinin (T4), infected mice with *Plasmodium berghei* which untreated by artemisinin as a control (C), infected mice with *Plasmodium berghei* which has been ever treated by artemisinin 4 times but untreated as a treatment control (TC). T4 group was oral administered with artemisinin which was given with "4-day-test" (4-DT) with ED_{99} dose (200 mg/kg weight of mice) for 3 days which begins 48 hours after infection but C and TC group were given aquadest. The histopathology of the lung, kidney, and cerebrum tissues was studied by routine histology method with Haematoxylin-Eosin staining. Histological examination edema, haemosiderosis, thickened alveolar septa and inflammatory cell infiltration in the lung. Cast formation Glumerulonephritis, tubular necrosis, and congestion occurred in the cortex area of the kidney. The brain showed cerebral microvessels congested, haemorrhages and necrosis. Conclusions repeated artemisinin exposure with repeated passages in mice cause increasing of sequestration on the brain and lungs and increasing the histopathological changes of the lung, kidney, and cerebrum.

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INTRODUCTION

Malaria still be a health problem in the world. Every year, especially in the tropics, approximately two million people die (Souza *et al*., 2013). Increased incidence of morbidity and mortality due to increased parasitic resistance and decreased efficacy of artemisinin antimalarial drugs and its derivatives. Resistance and decreased efficacy of artemisinin and ACT partner drugs have been reported from the Greater Mekong Subregion of Myanmar (Myint *et al*., 2017) The results of the research by Maslachah (2013) showed an increase in inhibitory concentration of 50%, phenotypic changes of dormant form, faster growth after viabel of dormant form and mutation in *pfatpase6* gene on *Plasmodium falciparum* exposed to repeated artemisinin in vitro. The results of this study became an emergency that there will the development of resistance in vivo in humans and become a health problem in the world so it can trigger the occurrence of severe malaria.

Severe malarial pathogenesis is associated with the presence of infected red blood cell cytoadherence in endothelial cells causing microvascular sequestration of parasites and microvascular obstruction in vital organs (Barber *et al*., 2015). The presence of sequestration in important organs causes severe malaria symptoms in humans such as cerebral malaria, and respiratory distress (Milner *et al*., 2013; Milner *et al*., 2015). Other Plasmodium species can also be found in various microvascular organs during infection as *Plasmodium chabaudi* in mice (Brugat *et al*., 2013) such as in liver, lungs, spleen, and brain (Milner *et al*., 2014).

This study aimed to know how the effect of repeated artemisinin exposure on mice infected with *Plasmodiun*

berghei is associated with histopathological changes and sequestration in several organs. Experimental in vivo study using rodent malaria is used to support laboratorium study translation into clinical study. It can be used as a basic to predict and anticipate the spread of artemisinin antimalarial drug resistance in practical use in the clinic associated with impaired organ function in severe malaria.

MATERIALS AND METHODS

Ethical approval: This study was approved by the Animal Ethics Committees of Veterinary Medicine Faculty of Universitas Airlangga Surabaya, Indonesia (certificate number No. 464 KE).

Mice, parasites and drugs that used in the study: Male *Albino Swiss* strain aged 8-10 weeks and weight 20-30 g from the SPF unit at the Veterinaria Farma Center (PUSVETMA). *Plasmodium berghei* ANKA strain was got from Tropical Disease Center of Universitas Airlangga. Artemisinin Pro analysis (PA) from Sigma Chemical Co.

Selection of the artemisinin antimalarial drug resistance *in vivo* in the mice: Infections were initiated by intraperitoneal (i.p.) injection of $1x10^5$ infected red blood cell (iRBC) in 0.2 ml and then given artemisinin anti-malarial drug with "4-day-test '(4-DT) with ED_{99} dose (200 mg/kg weight of mice) was given for 3 days started at 48 hours after infection (D2). Parasitemia was monitored and calculated at 120 hours after infection and monitored by microscopic examination of Giemsa 20% stained blood smears that taken from tail vein of mice. After parasitemia >2% of iRBC, it was used as donor and passaged on new 5 mice. Each passage is exposed to artemisinin in the same way, dose, and time up to 4 times of drug exposure (Muregi *et al*., 2011). Mice were divided into 3 treatment groups: The control group (C): mice after inoculation of $1x10^5$ iRBC *P.berghei* in 0.2 ml who were untreated with artemisinin. Treatment control group (TC): Mice after inoculation of $1x10^5$ iRBC *P.berghei* 0.2 ml that had previously been treated four times with artemisinin in who were untreated with artemisinin. Treatment group (T4): Mice after inoculation of $1x10^5$ iRBC *P.berghei* in 0.2 ml that had previously been treated four times with artemisinin who were treated with artemisinin ED_{99} dose. The development of parasites was observed over 10^{th} day of infection in all treatments (Kiboi *et al*., 2009; Henriques *et al*., 2013).

Histological assessment: Mice were euthanized by Ketamin and were required for thoracotomy and direct cardiac perfusion with throughout circulation supplied by the left side of the heart. Needle were placed into the apex of the left ventricle, and the pump were turned of PBS buffer. Then the right auricle was cut immediately to allow the perfusate to exit the circulation until the fluid exiting were clear of blood then perfuse with formalin 10%. This technique is appropriate for harvesting brain and organs. This is the optimal method of tissue preservation because the tissues are fixed before autolysis begins.

The brain, left lobes of the lung, and left kidney from control and treatment groups were fixed in 10% neutral buffered formalin for 24 h at room temperature. Fixed organs were embedded in paraffin, sectioned (3-4 μm), and stained with hematoxylin and eosin routine protocols. Sections were examined microscopically and changes recorded using a standard non-linear semi-quantitative scoring system using a scale from 0 to 5 adapted from Shackelford *et al*. (2002). Significant findings were scored 0 (where no change was detectable), 1 when the least amount of change was detectable by light microscopy (usually <10% of tissue affected), 2 when change was readily detected but not a major feature (<20%), 3 when the change was more extensive and might be expected to correlate with changes in organ weight or function, 4 when up to 75% of tissue was affected by the change and 5 when the whole tissue was affected by a change which was likely to be functionally relevant. Organs from control group were always compared with those from treatment groups. The percentage of vessels in each organ containing iRBC was determined from 100 vessels.

Statistical analysis: Data are shown as means by XLSTAT. The non-parametric Kruskal Wallis test was used and P values below 0.05 were considered as statistically significant, than was followed by Dunn test.

RESULTS

The results of histopathologic examination showed the presence of histopathological changes that occur in several organs, some of which are in the organs where iRBC sequestered.

Lung: The lung from all mice showed asevere histological changes, such as edema, increasing cellularity of the alveolar septae and thickened alveolar septa and inflammatory cell infiltration in the lung, haemosiderin was observed in septum interalveolare and bronchial epithelial degenaration. The finding of sequestered parasites and tissue damage in the lungs was rare (Figure 1A). The statistical analysis showed that the alveolar expansion in repeated artemisinin exposure group that treated with artemisinin (T4) was significantly different with control group (C) and control treatment group (TC) $P<0.05$. Alveolar congestion changes in all groups showed no difference $P>0.05$. Hemosiderin in the lung showed an increase in the group (TC) that was significantly different with the control group (C) at $P<0.05$ and did not differ significantly with the T4 group at $P>0.05$. Pulmonary edema showed an increase in control treatment group (TC) that was significantly different with group (T4) at $P<0.05$. Pulmonary histopathologic changes in the control and treatment groups showed in Table 1 and Fig. 1.

Kidney: The kidney damage from all mice showed severe histological changes, such as cast formation, glumerulonephritis, tubular necrosis, and congestion occurred in the cortex area of the kidney. We also observed tubular dilatation in the kidney but kidney damage in all mice even in the absence of sequestration. The results of statistical analysis showed that tubular

dilatation, cast formation and glomerulonephritis were not significantly different in all treatment groups P>0.05, but in tubular necrosis showed a decrease in group (T4) compared with group (TC) which was significantly different at P<0.05, while congestive showed a decrease in the control group (C) compared to repeated exposed artemisinin (TC) and (T4) groups. Results of statistical analyzes of renal histopathologic changes in the control and treatment groups as in Table 2 and Fig. 2.

Cerebrum: The major histopathological changes in postmortem cerebrum tissue are cerebral microvessels congested with iRBCs, hemorrhage and necrosis. Every 100 microvessels, we found several cells of sequestered parasites in the cerebrum with pigmented parasites. There was difference in the distribution of parasites or in the percentage of vessels parasitized and amount of necrosis (macroglia). Some areas were edema, which occur predominantly in the cortex of the cerebrum, but there was no difference. Inflammatory cell infiltration is a variable finding. The histopathologic changes of the cerebrum showed an increasing hemorrhagic in the control treatment group (TC) that was significantly different from the control group (C). The histopathological changes of edema and necrosis showed no significant difference in all treatment groups. Results of statistical analyzes of histopathological changes in the control and treatment groups as shown in Table 3 and Fig. 3. Sequestration of the cerebrum as shown in Fig. 3D.

DISCUSSION

Plasmodium berghei infection in mice causes a change in histopathologic features in various organs. Decreasing of alveolar expansion features of the group infected with *Plasmodium berghei* that was exposed to artemisinin repeatedly and treated with artemisinin (T4) compared with the control group (C) and the control treatment group (TC). Decreasing of alveolar expansion in the administration of antimalarial drug artemisinin in mice infected with *Plasmodium berghei* because of the function of artemisinin as an anti-inflammatory and immunoregulator that capable to inhibit TH_1 in order to inhibit macrophages producing TNFα so that tissue damage is inhibited. Besides that, artemisinin's ability to inhibit TH_{17} to produce polymorphonuclear (PMN) causes acute infection, tissue damage can also be inhibited and artemisinin's ability to activate T reg (IL10, TGF_B) so that it can increase immune tolerance (Shi *et al*., 2015). Alveolar congestion and septal congestive changes occur in all groups. This is due to Plasmodium parasite infection can induce inflammatory cells that can cause changes in pulmonary microcirculation as indicated by endothelial cell cytoplasm swelling and edema in lung interstitium tissue. Systemic inflammatory response increasing distal organ damage, Infected monocytes and erythrocytes attached to the capillary blood vessels, and alveolar capillary membrane barriers are damaged causing edema in the septal or lung insterstitials so that the lung is damaged (Souza *et al*., 2013; Aitken *et al*., 2014). The increasing of lung edema in the control treatment group (TC) significantly different from the treatment group (T4) due to *Plasmodium berghei* who had been exposed to repeated anti-malarial artemisinin drugs may increase lung damage associated with its ability to activate the dependent CD36 as infected red blood cell mediator (iRBC) sequestration, since the presence of blockade on CD36 as mediated sequestration that may increase the ability of mononuclear phagocytosis so that it can be effective to clean the parasite through non opsonic phagocytosis (Lagase *et al.*, 2016). Microvascular obstruction due to sequestration of parasites and the presence of endothelial adhesion by inflammatory responses as well as the release of proinflammatory mediators (adhesion molecules, cytokines, chemokines) leads to increased edema in the lung (Van den Steen, 2013), In addition, pathological changes in lung in the form of hemorrhagic edema due to increased VEGF circulation (Canavese *et al*., 2014; Hempel *et al*., 2014). The increase of hemosiderin in lung in control treatment group (TC) was significantly different with control group (C). The results of this study indicate that in *Plasmodium berghei* who have been exposed to repeated anti-malarial artemisinin drugs give a more severe pathogenicity effect, this is in accordance with Maslachah *et al*. (2017a) which states that repeated exposure of artemisinin to *Plasmodium berghei* may increase the number of neutrophils in mice. Other study show exposure to artemisinin with repeated passages in mice increased the value of ED50 and ED90, decreased the parasite clearance time (PCT) and recrudescence time (RT) and also changes in morphology dormant and vacuole formation (Maslachah *et al*., 2017b).

Table 1: The results of scoring histopathological changes of lung mice that infected with *Plasmodium berghei* in the control group and treatment groups that exposed to repeated artemisinin

Group	Mean±SD				
	Alveolar expansion	Alveolar congestion	Hemosiderin	Septal congestion	Edema
K	2.20±0.44 ^{b}	1.40±0.54 ^{a}	0.60±0.54 ^{a}	2.20±0.44 ^{a}	2.00±0.70 ^{ab}
P4	0.80±0.44 ^{a}	2.40±1.14 ^{a}	1.80±1.30 ^{ab}	2.00±0.70 ^{a}	0.80±0.83 ^{a}
TC	2.20±0.44 ^{b}	2.60±1.14 ^{a}	2.80±1.30 ^{b}	2.20±0.44 ^{a}	2.40±0.54 ^{b}

Mean values with different superscripts within a column differ significantly (P<0.05).

Table 2: The results of scoring histopathological changes of kidney mice that infected with *Plasmodium berghei* in the control group and treatment groups that exposed to repeated artemisinin

Group	Mean±SD				
	Congestion	Glomerulonephritis	Tubular necrosis	Cast formation	Tubular dilatation
K	0.80±0.44 ^{a}	2.20±0.44 ^{a}	2.60±0.54 ^{ab}	0.80±0.44 ^{a}	2.60±0.54 ^{a}
P4	2.40±0.54 ^{b}	2.80±0.44 ^{a}	1.60±0.54 ^{a}	0.00±0.00 ^{a}	1.60±0.89 ^{a}
TC	2.40±0.54 ^{b}	2.40±0.54 ^{a}	2.80±0.44 ^{b}	0.80±0.83 ^{a}	2.80±0.44 ^{a}

Mean values with different superscripts within a column differ significantly (P<0.05).

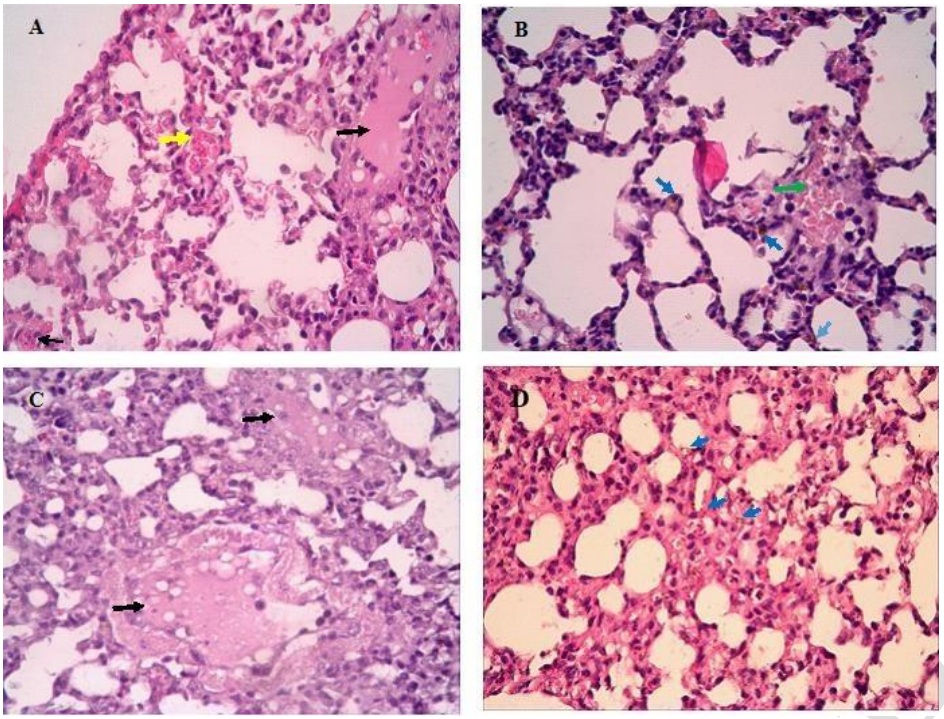

Fig. 1: Representative images of the lung pathology are shown. The lungs from TC group (A) demonstrate septal congestion and some sequestration of parasites (yellow arrows) in the capillaries. The alveoli are filled with edema fluid, RBC and neutrophils (black arrow) The lung from T4 (B) showed congestion of alveoli microvessels with RBC, pigment laden macrophages, and neutrophil (green arrow), also a number of haemosiderin (blue arrows)). The alveoli from C are filled with edema fluid (black arrow) (C). A number of haemosiderin from TC (D) are always seen (blue arrows) (400X, H&E stain).

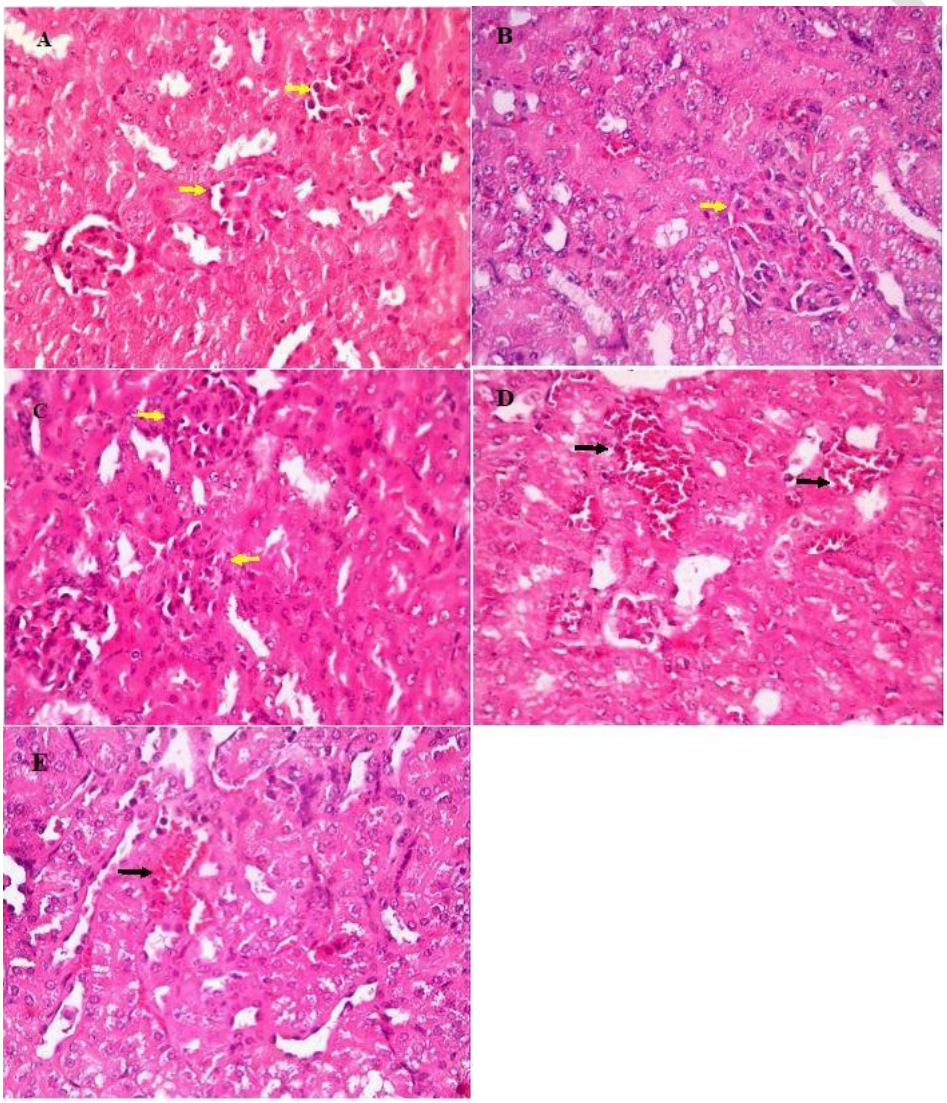

Fig. 2: Representative images of the kidney pathology are shown. Glomerulonephritis (yellow arrow) with some mononuclear cells are seen in a renal glomerulus from TC group (A), T4 group (B), and C group (C) A section of kidney tissue from TC group (D) and T4 group (E) showing congestion (black arrow) (400X,H&E stain).

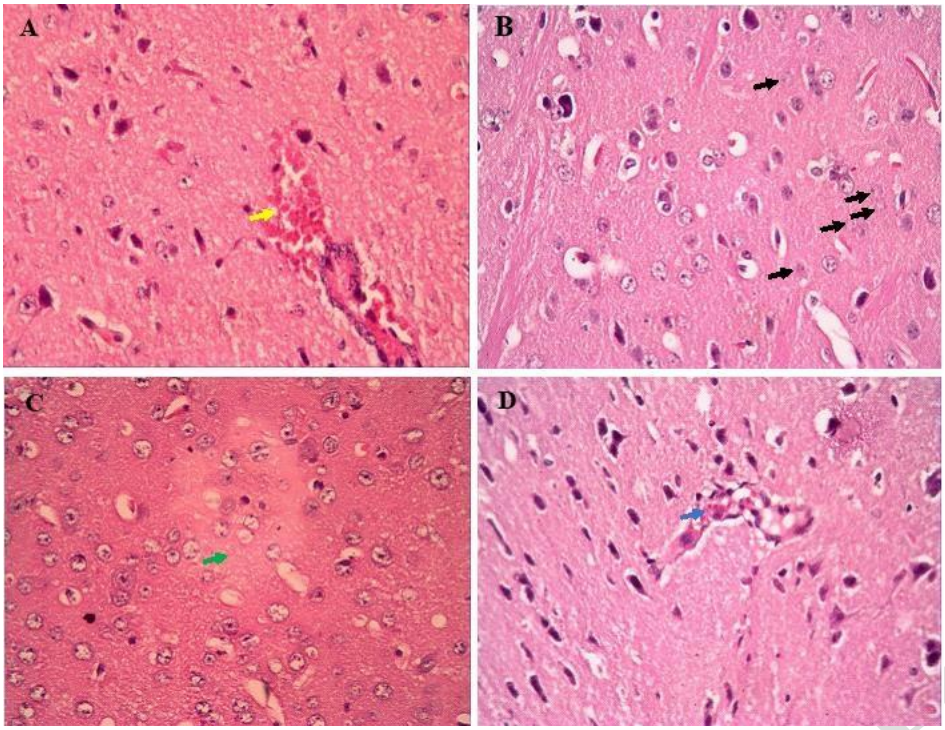

Fig. 3: Representative images of the brain pathology are shown. A section of cerebrum tissue from TC group (A) showing haemorrhages in the grissea substance, around vessels (yellow arrow). Necrosis of the macroglia cells can be seen in T4 group (B). The alba substance of cerebrum tissue from TC group (C) showing edema. TC group (D) showing parasitized red blood cells (PRBC) (blue arrow) (400X,H&E stain).

Table 3: The results of scoring histopathological changes of brain mice that infected with *Plasmodium berghei* in the control group and treatment groups that exposed to repeated artemisinin

Group	Mean±SD		
	Edema	Necrosis	Haemorrhage
K	0.00±0.00 [a]	1.80±0.44 [a]	0.20±0.44 [a]
P4	0.20±0.44 [a]	1.20±0.44 [a]	0.40±0.54 [ab]
TC	1.00±1.00 [a]	2.00±0.70 [a]	1.80±1.30 [b]

Mean values with different superscripts within a column differ significantly (P<0.05).

Histopathology features in the kidney showed tubular dilatation and cast formation suggests that *Plasmodium berghei* infection in mice can lead to increased proinflamatory molecules and oxidative stress products that play an important role in the pathogenesis of renal damage. Loss of renal endothelial integrity during complex infections is associated with elevated heme toxic, oxygen and reactive species nitrogen, as well as proinflammatory molecules, resulting in decreased O2 deliveries to cells and tissues. This leads to increased hypoxia microenvironment, renal perfusion decrease, acute tubular necrosis and decreased cellular defense mechanisms can contribute to the occurrence of acute kidney injury (Bezerra *et al*., 2017). During increasing of infection cytokines and reactive oxygen species (ROS) cause increasing lipid peroxidation, nitric oxide, inflammation and decreasing antioxidant defense in tissues including the kidney (Sibiya *et al*., 2017). The decreasing in tubular necrosis in the treatment group (T4) compared with the control treatment group (TC) indicates that the ability of artemisinin act as anti-inflammatory so that it can inhibit the exacerbation of the proinflamatory response during infection so that tubular necrosis can be inhibited (Shi *et al*., 2015).

The increasing of hemorrhage in cerebrum in the control treatmen group (TC) was significantly different from the control group (C) due to *Plasmodium berghei* that had been exposed to repeated anti-malarial artemisinin drugs give heavier pathogenic effects that could increase inflammation in blood vessels and extravasation of red blood cells in some regions of the brain such as the cerebellum, as well as bleeding that occurs due to capillary thrombus and granuloma in the sub cortical region, the corpus callosum cerebellum. This is closely related to the cause of the increasing perivascular hemorrhages (Greiner *et al*., 2015). The presence of edema and necrosis in all treatment groups infected with *Plasmodium berghei* in accordance with a study by Martin *et al* 2016 that in mice infected with *Plasmodium berghei* showed histopathologic features of the brain in the form of cerebral edema, congestion, parenchymal haemorrhage, glial cell proliferation, accumulation of erythrocytes and leukocyte adhesion in the cerebral cortex which is evidence of a link between leukocyte recruitment, blood brain barrier permeability and chemokin production in malaria infection. Cerebral malaria in humans and rodent is roled by IFN (αB) receptor 1 (IFNAR1) that triggered by CD8 + T cell (Ball *et al*., 2013).

The sequestration of erythrocytes that infected with plasmodium (iRBC) in brain microvascular and other tissues through the cytoaderens of the endothelium plays an important role in the pathogenesis of malaria. Sequestration of iRBC in important organs has a major effect on organ function. Parasitic sequestration can be found in the brain, lungs, limpha, liver, kidney, small intestine, heart and fat tissue (Milner *et al*.,2015). In this study, sequestration is found in the brain and slightly in the lungs and in the kidney is not found. This might be cause by the differences in adhesion molecules and / or the use of parasitic ligands and mechanisms of pathogenesis as well as the immune response of organs (Brugat *et al.*, 2014).

In *Plasmodium falciparum* sequestration is mediated by the interaction between the parasitic ligand Pf EMP1 that located on the iRBC surface and various receptors such as ICAM1, VCAM 1, CD36, CD31 and CSA (El-Assaad *et al*., 2013). The interaction between iRBC and

not passive endothelial, the parasite protein interacts with the host RBC to alter the morphology, physiology, function and contribute to the pathological changes seen in severe malaria (Utter *et al*., 2017). Parasites produce mediators that can trigger cytokine release from host cells including endothelial cells. Cytokines facilitate the cytoadherence by increasing the regulation of ligand expression located on the host cell surface, and this interaction will activate the cascade signaling and regulate genes involved in the inflammatory response and apoptosis. The leakage into the perivascular space affects astrocytes and pericytes leading to BBB impairment (Storm *et al.*, 2014). The supporting factors of parasite adhesion in host cell endothelium are macrophages, lymphotoxins, and microparticle plasma platelets, intercellular adhesion molecule 1(ICAM-1), P selectin and vascular adhesion molecule 1 so several novel molecules including α3B1, VE-cadherin, ICAM2, junctional adhesion molecule B (JAM-B), laminin and cellular fibronectin (Mahamar *et al*., 2017; Ho *et al*., 2018).

Conclusions: Repeated artemisinin exposure with repeated passages in mice cause the increasing sequestration in the brain and lungs and increasing the histopathology changes of the lung, kidney, and cerebrum.

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Authors contribution: LM.: as head of research project, coordinating research design, data analysis, compiling manuscript and corresponding author. TVM. Examine the histopathological preparations of the brain and kidneys, LRY: Examine the histopathological preparations of the lungs and statistic analysis. All the research teams read the draft of the article.

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