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ORIGINAL 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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Repeated exposure of artemisinin cause increasing of malaria severity that indicated the presence of sequestration and histopathological changes in some organs. Histopathological studies laid the groundwork for our understanding of the pathogenesis of malaria after repeated artemisinin exposure

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 artemisisnin 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 (P4), infected mice with *Plasmodium berghei* which untreated by artemisinin as a control (K1), infected mice with *Plasmodium berghei* which has been ever treated by artemisinin 4 times but untreated as a treatment control (PK). P4 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 K and PK 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 odema, haemosiderosis, thickened alveolar septa and inflammatory cell infiltration in the lung. Cast formation Glumerulonephritis, tubular necrosis, and congesti 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.

Key word: Artemisinin, *Plasmodium berghei*, histopathology, lung, kidney, cerebrum

INTRODUCTION

Malaria still be a health problem in the world. Every year, especially in the tropics, approximately two million people die and 800 thousand people die from severe malaria (Elias *et al*., 2012; Souza *et al*., 2012). Increased incidence of morbidity and mortality due to increased parasitic resistance and decreased efficacy of artemisinin antimalarial drugs and its derivatives (Afonso *et al*.,2006; Noedl *et al*.,2008; Wongsrichanalai and Meshnick,2008). 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 because there is no new drug to substitute artemisin for the treatment of malaria, so it can trigger the occurrence of severe malaria.

Severe malarial pathogenesis is associated with the presence of infected red blood cell cytoaderens 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, acute lung injury and acute respiratory syndrome (Haldar, 2007). Other Plasmodium species can also be found in various microvascular organs during infection as in primates and rodents (Singh *et al*.,2010; Craig *et al*.,2012) 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, because the spectrum of malaria in human is not known clearly how to mechanism of the pathology. Histopathological changes occur in multiple organs during the acute infection, but are not restricted to the organs where sequestration takes place. 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.

MATERIAL AND METHOD

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 *"Laboratorium hewan coba Pusat Veterinaria Farma"* (PUSVETMA) for Medical Research were housed conventionally with sterile bedding, food and *ad libitum* water.

For all experiments, *Plasmodium berghei* ANKA strain was used in this study was got from Tropical Disease Center of Airlangga University. We used 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 mland 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 fromtail vein of mice.After parasitemia > 2% of iRBC, it was used as donor and passaged on new 5 mice. After 48 hours post infection, the mice were exposed to artemisinin anti-malarial drug with the same ED_{99} dose for 3 consecutive days 4 times passages. 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 (K): mice after inoculation of $1x10^5$ iRBC *P.berghei* in 0.2 ml who were untreated with artemisinin. Treatment control group (PK): 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 (P4): 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. 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 per 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.

Statistics

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.

RESULT

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. Sequestrations of iRBCs in the microsvaculature occurred in the cerebellum, but were rare in the lung and there was no sequestration observed in the kidney.

Lung

The lung from all mice showed a severe 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 decrease of alveolar expansion in repeated artemisinin exposure group that treated with artemisinin (P4) was significantly different with control group (K) and control treatment group that ever exposed to repeated artemisinin with unapplied artemisinin (PK) $p < 0.05$. Alveolar congestion changes in all groups showed no difference $p > 0.05$. Hemosiderin in the lung showed an increase in the group (PK) that was significantly different with the control group (K) at $p < 0.05$ and did not differ significantly with the P4 group at $p > 0.05$. Septal congestion was not significantly different in all treatment groups. Pulmonary edema showed an increase in group (PK) that was significantly different with group (P4) at $p < 0.05$. Pulmonary histopathologic changes in the control and treatment groups showed in table 1 and figure 1.

Kidney

The kidney damage from all mice showed a severe histological changes, such as cast formation, glumerulonephritis, tubular necrosis, and congesti 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 (P4) compared with group (PK) which was significantly different at $p < 0.05$, while congestive showed a decrease in the control group (K) compared to repeated exposed artemisinin (PK) and (P4) groups. Results of statistical analyzes of renal histopathologic changes in the control and treatment groups as in Table 2 and Figure 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 across the same sites and also there was difference in the amount of necrosis (macroglia). Some areas were oedema, 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 group of recurrent exposure (PK) that was significantly different from the control group (K). 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 Figure 3. Sequestration of the cerebrum as shown in figure 3A

DISCUSSION

Plasmodium berghei infection in mice causes a change in histopathologic features in various organs. There were changes in lung, such as alveolar expansion, alveolar congestion, hemosiderin, septal congersti and edema. Decreasing of alveolar expansion features of the group infected with *Plasmodium berghei* that was exposed to artemisinin repeatedly and treated with artemisinin (P4) compared with the control group (K) infected with *Plasmodium berghei* and not treated with artemisinin and the control treatment group that infected with *Plasmodium berghei* and had been exposed to repeated artemisinin amd not treated artemisinin (PK). There is 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 imonoregulator that capable to inhibit

TH_1 in order to inhibit macrophages producing TNFα so that tissue damage is inhibited. Beside 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. With 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 (Lovegrove *et al*., 2008; Souza *et al*., 2012; Aitken *et al*., 2014). The increasing of lung edema in the treatment control group (PK) significantly different from the treatment group (P4) 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 (Epiphanio *et al*., 2010). The increase of hemosiderin in lung in KP group was significantly different with control group (K). 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. (2017) which

states that repeated exposure of artemisinin to *Plasmodium berghei* may increase the number of neutrophils. Increased the value of ED50 and ED90, decreased the PCT and RT and also changes in morphology dormant and vacuole formation (Maslachah *et al*., 2017).

Histopathology features in the kidney showed tubular dilatation, cast formation and glomerulonephritis that was not significantly different in all treatment group. This 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 and decreased cellular defense mechanisms (Elias *et al*., 2012). During increasing of infection cytokines and reactive oxygen species (ROS) cause increasing lipid peroxidation, nitric oxid, inflammation and decreasing antioxidant defense in tissues including the kidney (Sibiya *et al.,* 2017). The decreasing in tubular necrosis in the group (P4) compared with the group (PK) 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 group that expose repeatedly (PK) was significantly different from the control group (K) 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 (Desruisseaux *et al*., 2008). The presence of edema and necrosis in all treatment groups infected with

Plasmodium berghei in accordance with a study by Queiroz et al (2011) 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 erytrosite and leukocyte adhesion in the cerebral cortex which is evidence of a link between leukoscyte 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 (RBC) 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 (Dorovini *et al.,* 2011). 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 (Sherman *et al*., 2003; 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 and function (Maier *et al*., 2009). Parasites produce mediators that can trigger cytokine release from host cells including endothelial cells. Cytokines facilitate the cytoaderen 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 (Chakravorty *et a.l*,

2008). The supporting factors of parasite adhesion in host cell endothelium are macrophages, limphotoxins, and microparticle plasma platelets (Faille *et al.,* 2009).

Plasmodium chabaudi has several multigene families coding which the analogs have a high similarity to the genes of *P. vivax* (e.g. *pir* genes) for the adhesion of parasitic molecules (Cunningham *et al*., 2010; Lawton *et al*., 2012). the *pir* genes of *Plasmodium vivax* also exist in *P. falciparum* iRBC so as to increase adhesion to cell receptors such as ICAM-1 (Bernabeu *et al*., 2012). If *Plasmodium vivax* and rodent malaria parasites have a multigene family similarity, that may be potential to presence cytoadherence by the same host receptor so that it can be used to explain sequestration can occur in the same organ.

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 Memeriksa preparat histopatologi paru dan statistic analysis. All the research teams read the draft of the article.

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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 expantion	Alveolar congesti	Hemosiderin	Septal congesti	Oedema
K1	2.20^{b} ± 0.44	1.40 ^{a}±0.54	0.60 ^{a} ± 0.54	2.20 ^{a}± 0.44	2.00 ^{ab}± 0.70
P4	0.80^{a}± 0.44	2.40 ^{a}± 1.14	1.80 ^{ab}± 1.30	2.00 ^{a}± 0.70	0.80 ^{a}±0.83
PK	2.20 ^{b}± 0.44	2.60 ^{a}± 1.14	2.80 ^{b} ± 1.30	2.20 ^{a}± 0.44	2.40^{b}± 0.54

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				
	Congesti	Glomerulonephritis	Tubular necrosis	Cast formation	Tubular dilatation
K1	0.80 ^{a}± 0.44	2.20 ^{a}± 0.44	2.60 ^{ab}± 0.54	0.80 ^{a}±0.44	2.60 ^{a}±0.54
P4	2.40 ^{b} ± 0.54	2.80 ^{a}± 0.44	1.60 ^{a}± 0.54	0.00 ^{a}±0.00	1.60 ^{a}±0.89
PK	2.40 ^{b} ± 0.54	2.40 ^{a}± 0.54	2.80 ^{b} ± 0.44	0.80 ^{a}±0.83	2.80 ^{a}±0.44

Mean values with different superscripts within a column differ significantly ($p<0.05$)

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		
	Oedema	Necrosis	Haemorhagies
K1	0.00 ^{a}± 0.00	1.80 ^{a}± 0.44	0.20 ^{a}± 0.44
P4	0.20 ^{a}± 0.44	1.20 ^{a}± 0.44	0.40 ^{ab}± 0.54
PK	1.00 ^{a}± 1.00	2.00 ^{a}± 0.70	1.80 ^{b}± 1.30

Mean values with different superscripts within a column differ significantly ($p<0.05$)

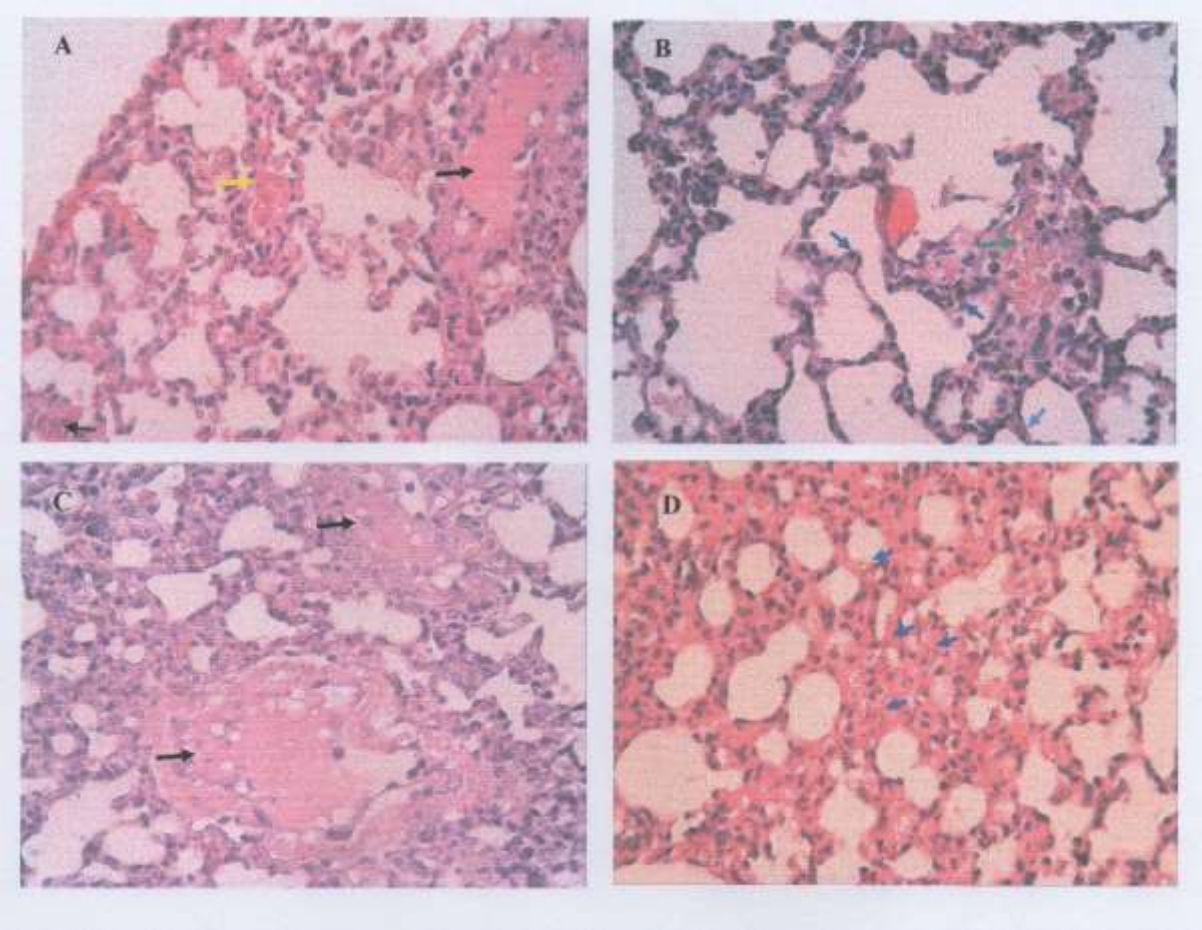


Figure 1. Representative images of the lung pathology are shown. The lungs from PK group (A) demonstrate septal congesti and some sequestration of parasites (yellow arrows) in the capillaries. The alveoli are filled with oedema fluid, RBC and neutrophils (black arrow) (400X, H&E stain). The lung from P4 (B) showed congestion of alveoli microvessels with RBC, pigment laden macrophages, and neutrophil (green arrow), also a number of haemosiderin(blue arrows) (400X, H&E stain). The alveoli from K1 are filled with oedema fluid (black arrow) (C). A number of haemosiderinfrom PK (D) are always seen (blue arrows) (400X, H&E stain).

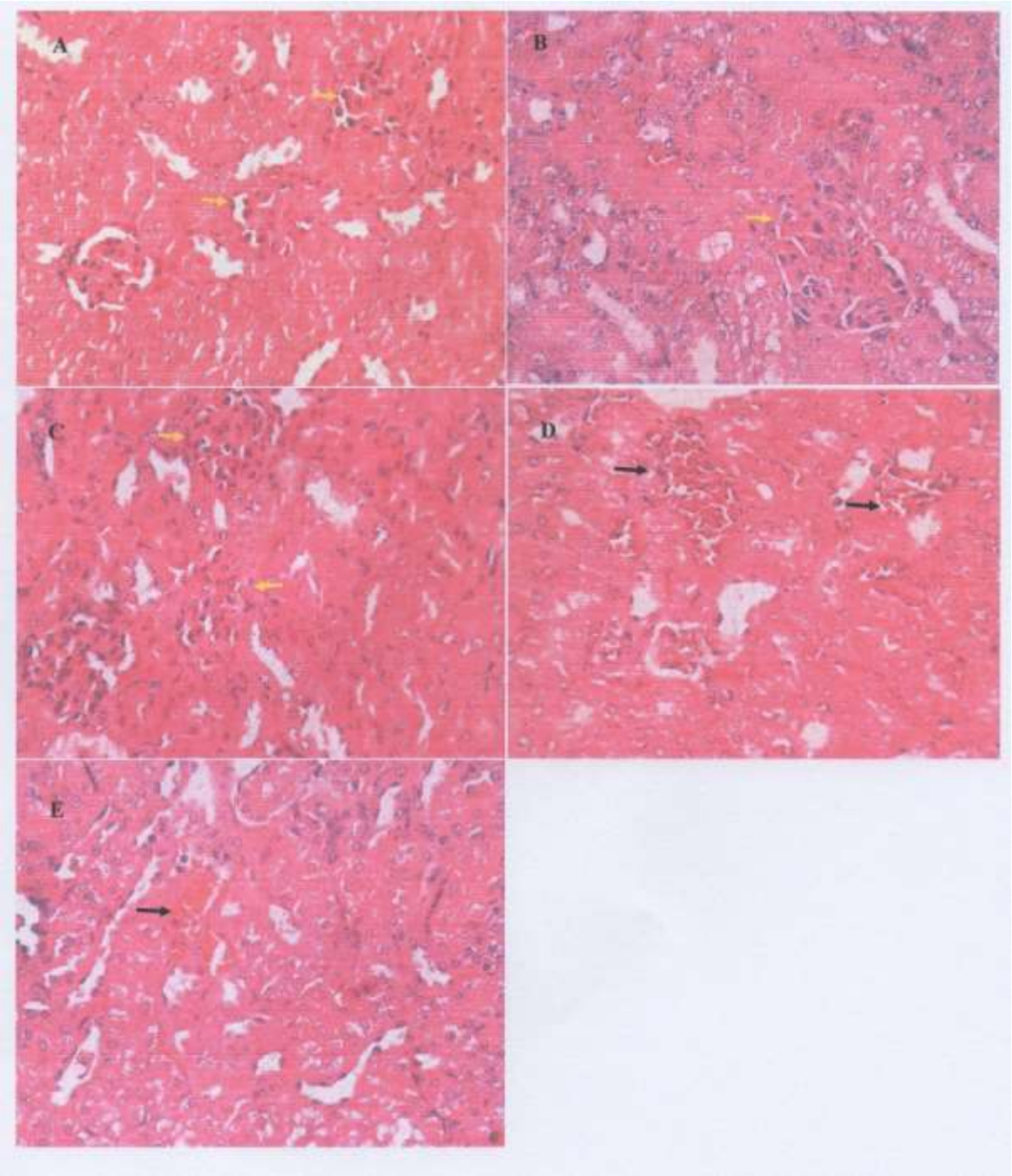


Figure 2. Representative images of the kidney pathology are shown. Glomerulonephritis (yellow arrow)with some mononuclear cells are seen in a renal glomerulus from PK group (A), P4 group (B), and K group (C)(400X,H&E stain)**.**A section of kidney tissue from PK group (D) and P4 group (E) showing congesti (yellow arrow)(400X,H&E stain).

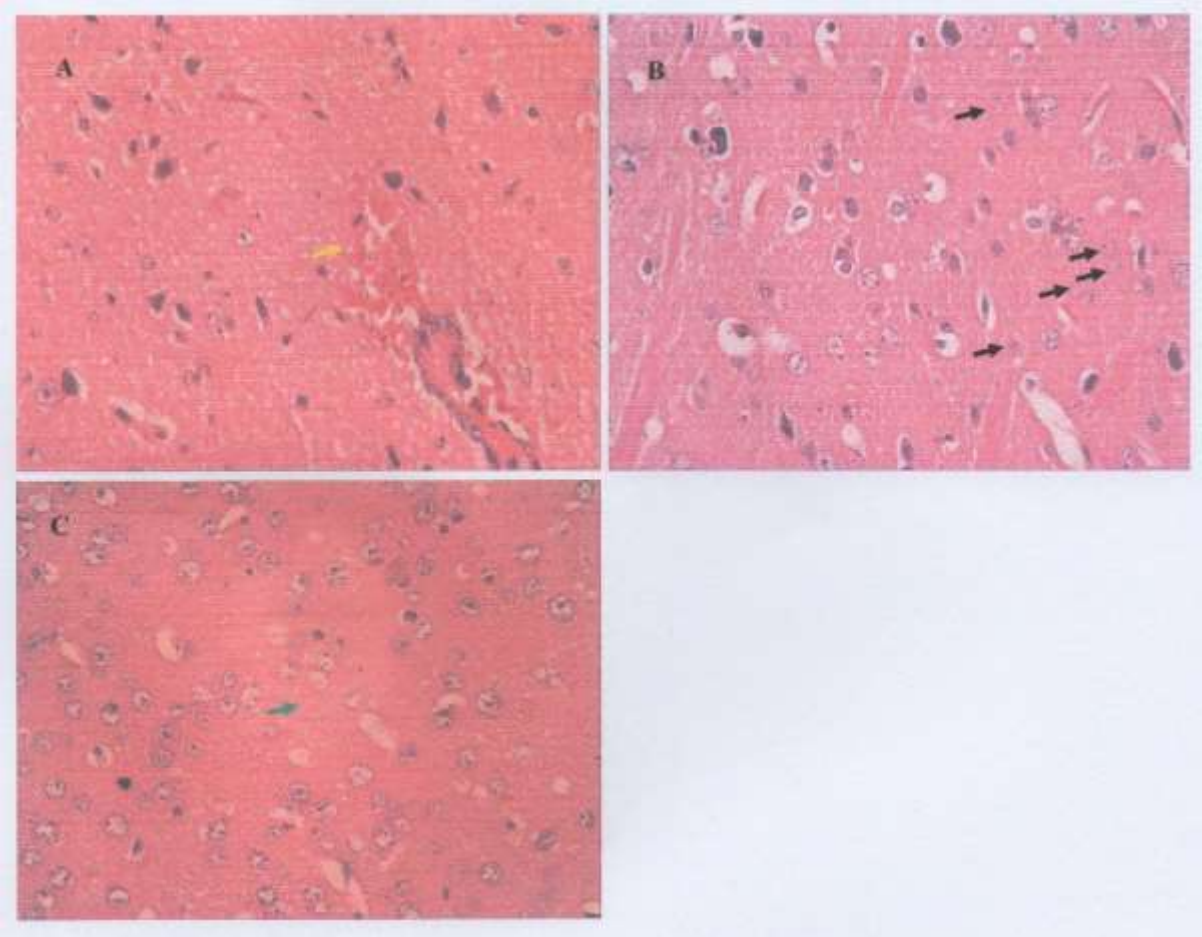
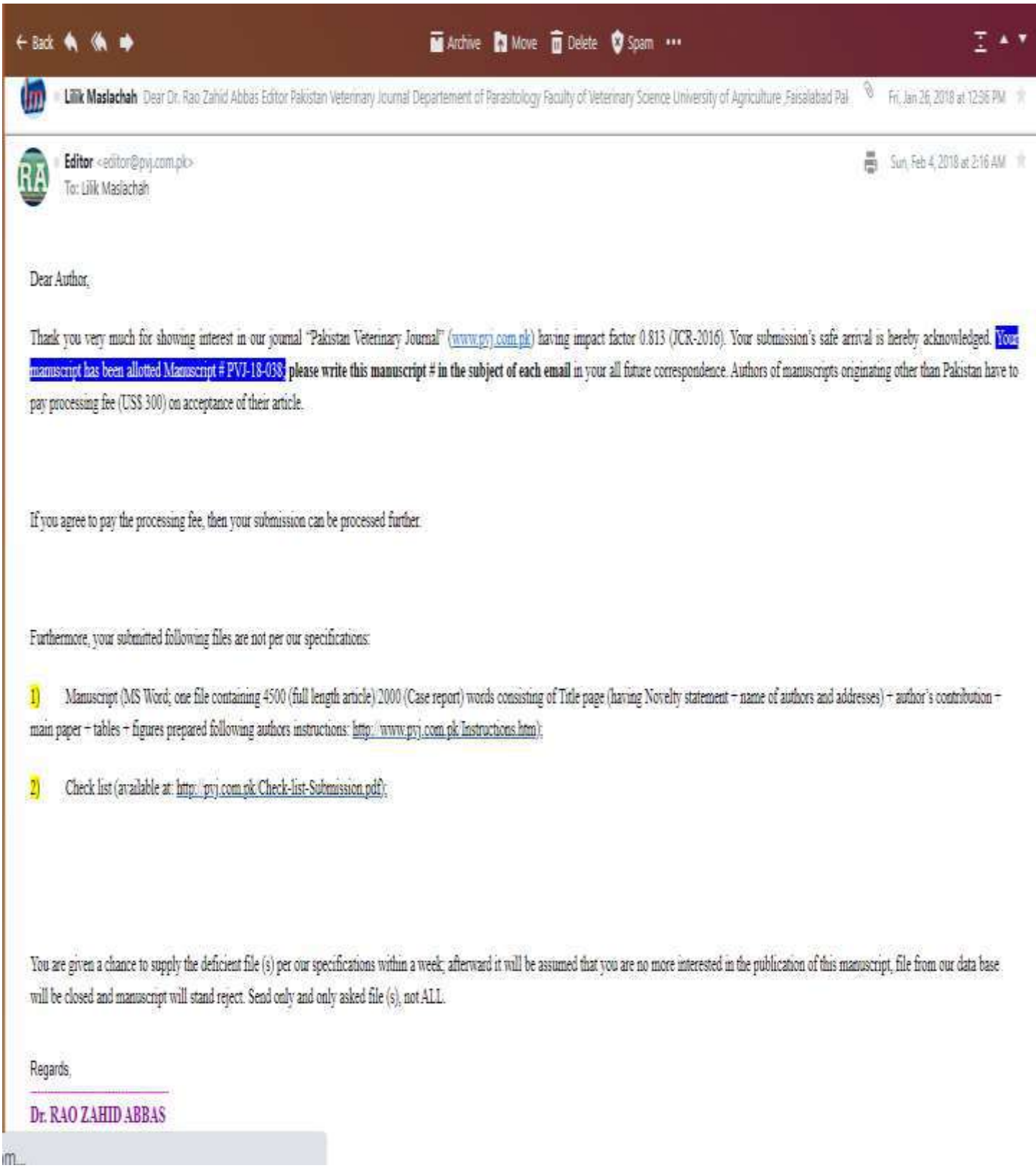


Figure 3. Representative images of the brain pathology are shown.A section of cerebrum tissue from PK group (A) showing haemorrhages with sequestration of parasites in the grissea substance, around vessels (yellow arrow). Necrosis of the macroglia cells can be seen in P4 group (B). The alba substance of cerebrum tissue from PKgroup (C)showing oedema(400X,H&E stain).



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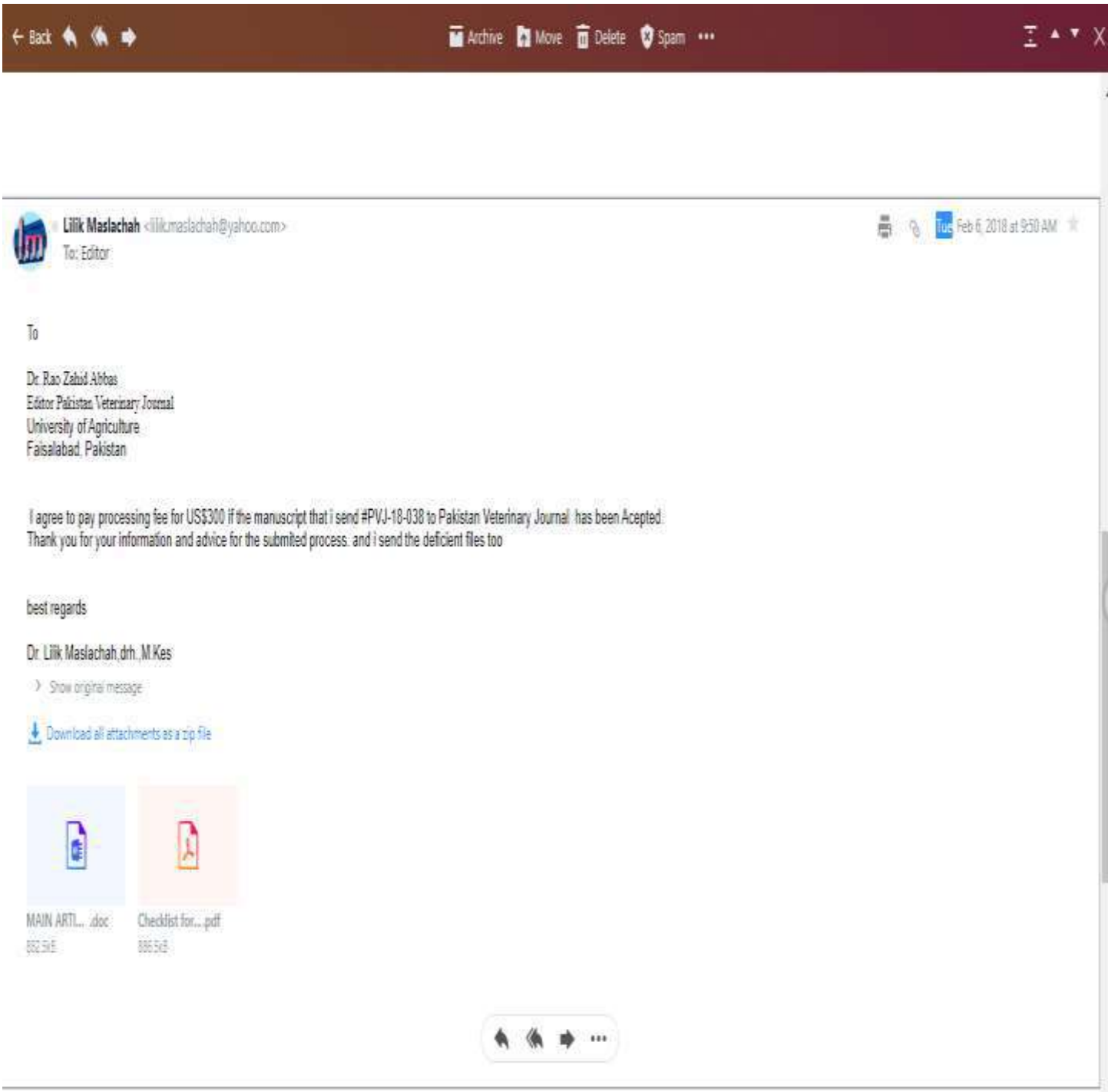
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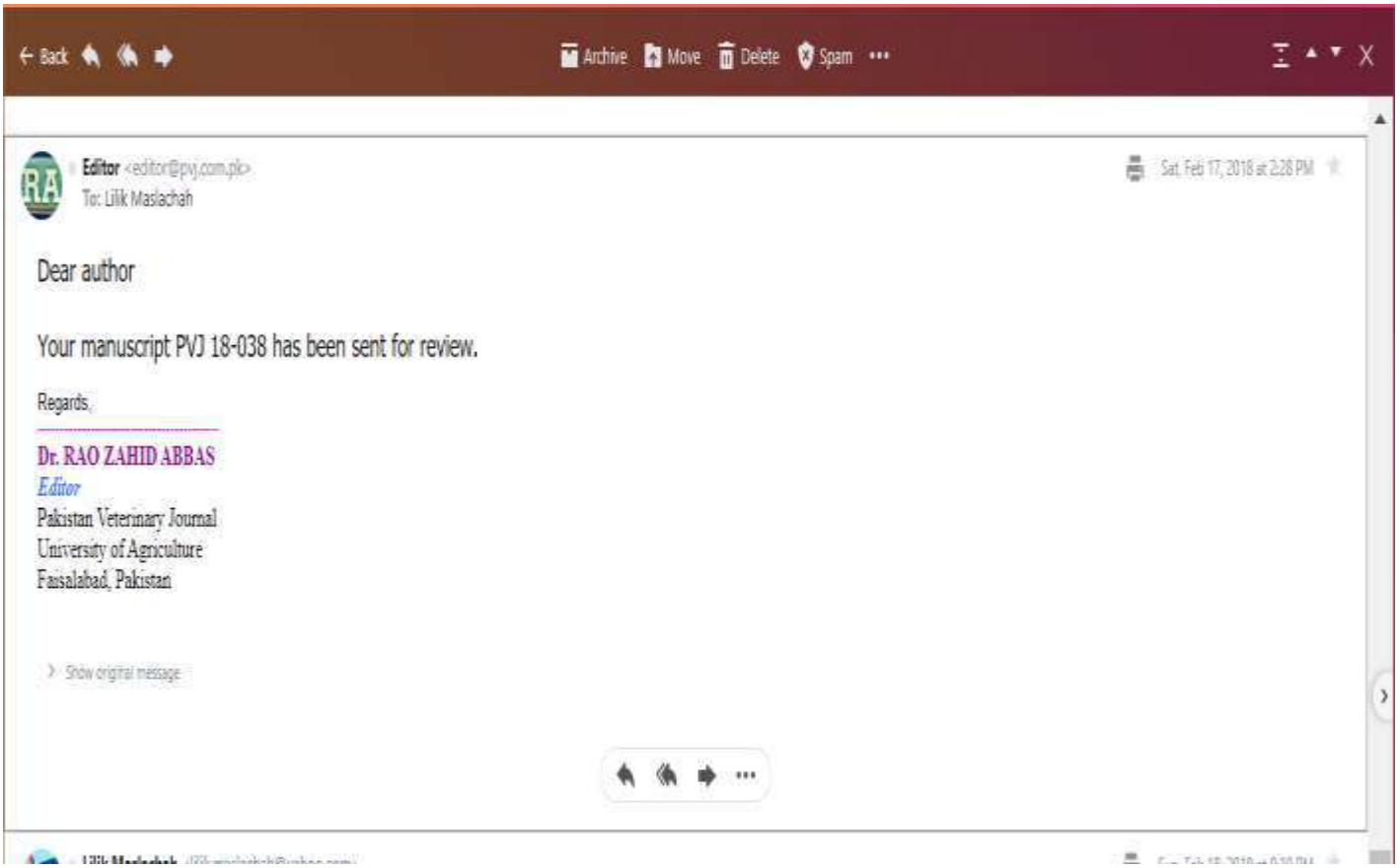
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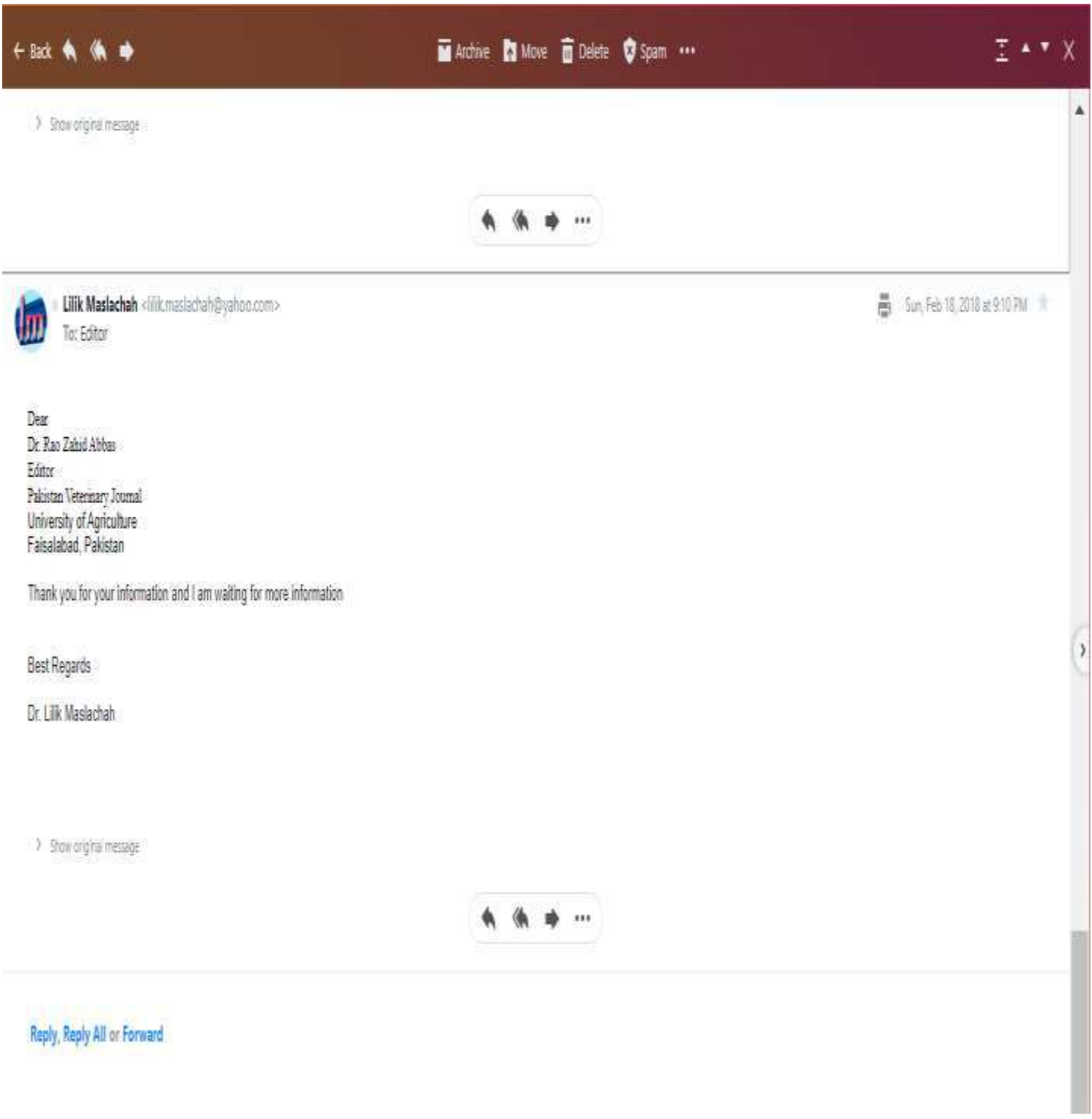
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Reviewer comments, if any:

The manuscript is poorly written with many grammatical and spelling mistakes. The histological images are of poor quality and resolution which makes it hard to observe the described changes in the organs especially of lung and kidney. Moreover, many changes e.g. edema and hemosiderosis in lungs, glomerulonephritis in kidney sections, cerebral edema and parasite sequestration in brain are not clearly visible. The authors shall select good quality sections with clearly reperesntative histological changes.

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Sequestration and histopathological changes of the kidneys, lungs and brain of mice infected with *Plasmodium berghei* that exposed to repeated artemisinin

Repeated exposure of artemisinin cause increasing of malaria severity that indicated the presence of sequestration and histopathological changes in some organs. Histopathological studies for our understanding of the pathogenesis of malaria after repeated artemisinin exposure**.**

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 artemisisnin 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 (P4), infected mice with *Plasmodium berghei* which untreated by artemisinin as a control (K), infected mice with *Plasmodium berghei* which has been ever treated by artemisinin 4 times but untreated as a treatment control (PK). P4 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 K and PK 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 odema, haemosiderosis, thickened alveolar septa and inflammatory cell infiltration in the lung. Cast formation Glumerulonephritis, tubular necrosis, and congesti 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.

Key word: Artemisinin, *Plasmodium berghei*, histopathology, lung, kidney, cerebrum

INTRODUCTION

Malaria still be a health problem in the world. Every year, especially in the tropics, approximately two million people die and 800 thousand people die from severe malaria (Elias

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Commented [ia3]: Congestion?

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et al., 2012; Souza *et al*., 2012). Increased incidence of morbidity and mortality due to increased parasitic resistance and decreased efficacy of artemisinin antimalarial drugs and its derivatives (Noedl *et al*.,2008; Wongsrichanalai and Meshnick,2008). 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 cytoaderens 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, acute lung injury and acute respiratory syndrome (Haldar, 2007). Other Plasmodium species can also be found in various microvascular organs during infection as in primates and rodents (Singh *et al*.,2010; Craig *et al*.,2012) 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.

MATERIAL AND METHOD

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 Airlangga University. Artemisinin Pro analysis (PA) from Sigma Chemical Co.

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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 (K): mice after inoculation of $1x10^5$ iRBC *P.berghei* in 0.2 ml who were untreated with artemisinin. Treatment control group (PK): 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 (P4): 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. 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.

Statistics

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.

RESULT

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 a severe 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 decrease of alveolar expansion in repeated artemisinin exposure group that treated with artemisinin (P4) was significantly different with control group (K) and control treatment group (PK) $p<0.05$. Alveolar congestion changes in all groups showed no difference $p> 0.05$. Hemosiderin in the lung showed an increase in the group (PK) that was significantly different with the control group (K) at $p <0.05$ and did not differ significantly with the P4 group at $p> 0.05$. Septal congestion was not significantly different in all treatment groups. Pulmonary edema showed an increase in control treatment group (PK) that was significantly different with group (P4) at $p <0.05$. Pulmonary histopathologic changes in the control and treatment groups showed in table 1 and figure 1.

Kidney

The kidney damage from all mice showed a severe histological changes, such as cast formation, glumerulonephritis, tubular necrosis, and congesti 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 (P4) compared with group (PK) which was significantly different at $p <0.05$, while congestive showed a decrease in the control group (K) compared to repeated exposed artemisinin (PK) and (P4) groups. Results of

Commented [ia9]: If the lungs are not properly perfused at necropsy they will collapse and may look like a pneumonic lung with increase in cellularity. The authors have not mentioned any of the perfusion methods in the materials and methods section, so how would they justify that this increase in cellularity was a pathological change and not an artefact due to lack of proper fixative protocol?

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statistical analyzes of renal histopathologic changes in the control and treatment groups as in Table 2 and Figure 2.

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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 oedema, 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 (PK) that was significantly different from the control group (K). 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 Figure 3. Sequestration of the cerebrum as shown in figure 3A

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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 (P4) compared with the control group (K) and the control treatment group (PK). 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 imonoregulator that capable to inhibit TH_1 in order to inhibit macrophages producing TNFα so that tissue damage is inhibited. Beside 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. With 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*., 2012; Aitken *et al*., 2014). The increasing of lung edema in the control treatment group (PK) significantly different from the treatment group (P4) 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 (Epiphanio *et al*., 2010). The increase of hemosiderin in lung in control treatment group (KP) was significantly different with control group (K). 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.* (2017) which states that repeated exposure of artemisinin to *Plasmodium berghei* may increase the number of neutrophils. Increased the value of ED50 and ED90, decreased the PCT and RT and also changes in morphology dormant and vacuole formation (Maslachah *et al*., 2017).

Histopathology features in the kidney showed tubular dilatation, cast formation and glomerulonephritis 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 and decreased cellular defense mechanisms (Elias *et al*., 2012). During increasing of infection cytokines and reactive oxygen species (ROS) cause increasing lipid peroxidation, nitric oxid, inflammation and decreasing antioxidant defense in tissues including the kidney (Sibiya *et al*., 2017). The decreasing in tubular necrosis in the treatment group (P4) compared with the control treatment group (PK) 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).

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Commented [ia16]: As discussed above, artemisinin has anti-inflammatory properties then how glomerulonephritis is justifiable in all groups under study?

The increasing of hemorrhage in cerebrum in the control treatmen group (PK) was significantly different from the control group (K) 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 (Desruisseaux *et al*., 2008). The presence of edema and necrosis in all treatment groups infected with *Plasmodium berghei* in accordance with a study by Queiroz *et al* (2011) 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 erytrosite and leukocyte adhesion in the cerebral cortex which is evidence of a link between leukoscyte 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 (Dorovini *et al.*, 2011). 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 and function (Maier *et al*., 2009). Parasites produce mediators that can trigger cytokine release from host cells including endothelial cells. Cytokines facilitate the cytoaderen 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 (Chakravorty *et a.l*, 2008). The supporting factors of parasite adhesion in host cell endothelium are macrophages, limphotoxins, and microparticle plasma platelets (Faille *et al.*, 2009).

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Plasmodium chabaudi has several multigene families coding which the analogs have a high similarity to the genes of *P. vivax* (e.g. *pir* genes) for the adhesion of parasitic molecules (Cunningham *et al*., 2010; Lawton *et al*., 2012). the *pir* genes of *Plasmodium vivax* also exist in *P. falciparum* iRBC so as to increase adhesion to cell receptors such as ICAM-1 (Bernabeu *et al*., 2012). If *Plasmodium vivax* and rodent malaria parasites have a multigene family similarity, that may be potential to presence cytoadherence by the same host receptor so that it can be used to explain sequestration can occur in the same organ.

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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.

Acknowledgements

The authors would like to thank to the Ministry of Higher Education on Research and Technology (*Kemenristek Dikti*) for the PUPT research fund support 2016 with contract number is 018 / SP2H / LT / DRPM / HI / 2016/ 17 February 2016.

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.

REFFERENCES

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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 expantion	Alveolar congesti	Hemosiderin	Septal congesti	Oedema
K	2.20^{b} ± 0.44	1.40 ^{a}±0.54	0.60 ^{a} ± 0.54	2.20 ^{a}± 0.44	2.00 ^{ab}± 0.70
P4	0.80^{a}± 0.44	2.40 ^{a}± 1.14	1.80 ^{ab}± 1.30	2.00 ^{a}± 0.70	0.80 ^{a}±0.83
PK	2.20 ^{b}± 0.44	2.60 ^{a}± 1.14	2.80 ^{b} ± 1.30	2.20 ^{a}± 0.44	2.40^{b}± 0.54

Mean values with different superscripts within a column differ significantly ($p<0.05$)

Commented [ia26]: What is the meant by alveolar congestion and septal congestion? As the blood vessels supplying alveoli are present in the interalveolar septae/tissue.

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				
	Congesti	Glomerulonephritis	Tubular necrosis	Cast formation	Tubular dilatation
K	0.80 ^{a}± 0.44	2.20 ^{a}± 0.44	2.60 ^{ab}± 0.54	0.80 ^{a}±0.44	2.60 ^{a}±0.54
P4	2.40 ^{b} ± 0.54	2.80 ^{a}± 0.44	1.60 ^{a}± 0.54	0.00 ^{a}±0.00	1.60 ^{a}±0.89
PK	2.40 ^{b} ± 0.54	2.40 ^{a}± 0.54	2.80 ^{b} ± 0.44	0.80 ^{a}±0.83	2.80 ^{a}±0.44

Mean values with different superscripts within a column differ significantly ($p<0.05$)

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		
	Oedema	Necrosis	Haemorhagies
K	0.00 ^{a}± 0.00	1.80 ^{a}± 0.44	0.20 ^{a}± 0.44
P4	0.20 ^{a}± 0.44	1.20 ^{a}± 0.44	0.40 ^{ab}± 0.54
PK	1.00 ^{a}± 1.00	2.00 ^{a}± 0.70	1.80 ^{b}± 1.30

Mean values with different superscripts within a column differ significantly ($p<0.05$)

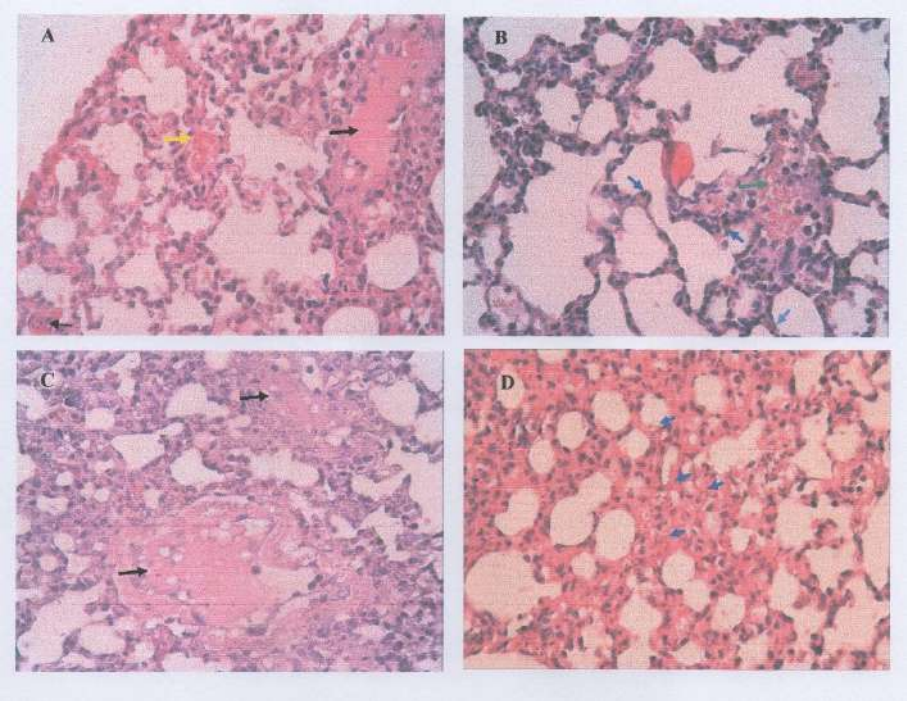


Figure 1. Representative images of the lung pathology are shown. The lungs from PK group (A) demonstrate septal congesti and some sequestration of parasites (yellow arrows) in the capillaries. The alveoli are filled with oedema fluid, RBC and neutrophils (black arrow) The lung from P4 (B) showed congestion of alveoli microvessels with RBC, pigment laden macrophages, and neutrophil (green arrow), also a number of haemosiderin (blue arrows)). The alveoli from K are filled with oedema fluid (black arrow) (C). A number of haemosiderin from PK (D) are always seen (blue arrows) (400X, H&E stain).

Commented [ia27]: Congestion? It is hard to appreciate the described changes in the figure. Most of the alveoli does not contain edema fluid. Sequestered parasites are difficult to observe in the image.

Commented [ia28]: Which pigment? Hemosiderin? In figure B, Hemosiderin is not visible, even a blue arrow seems to be placed at a pneumocyte.

Commented [ia29]: Most of the alveoli are empty?

Commented [ia30]: The images are of poor quality and resolution.

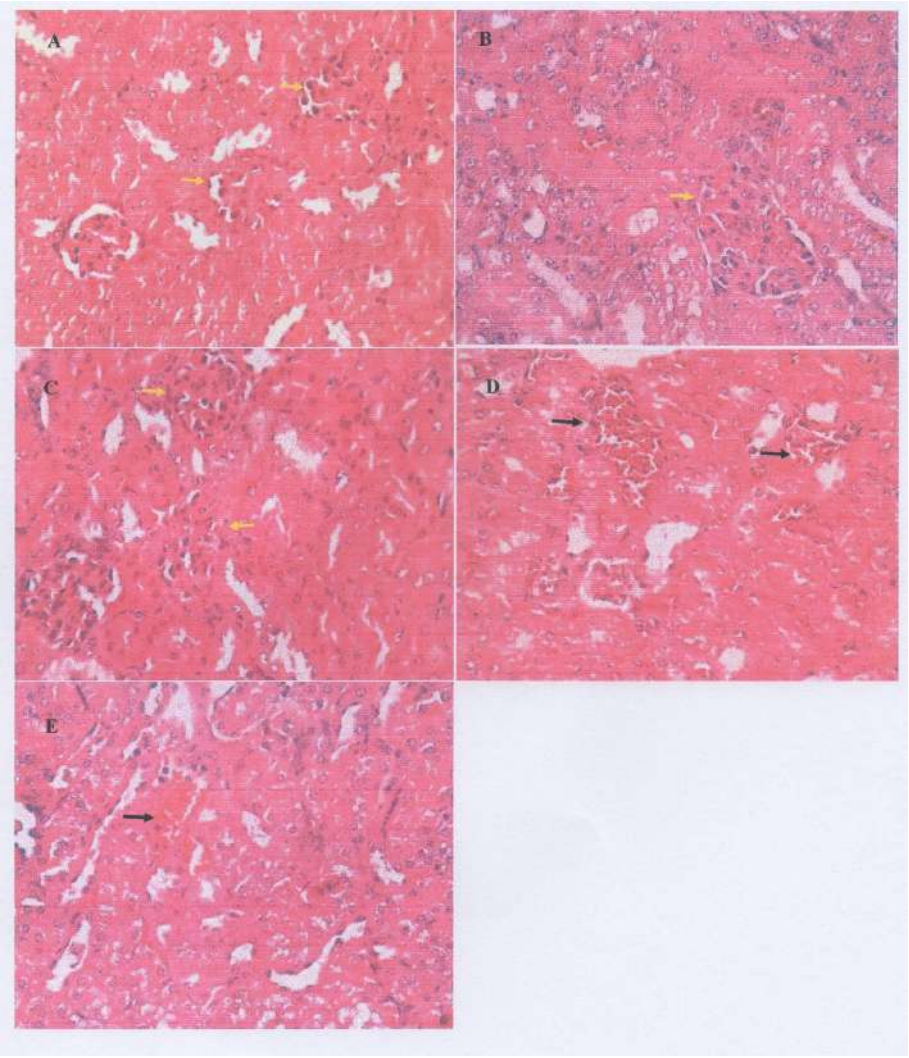


Figure 2. Representative images of the kidney pathology are shown. Glomerulonephritis (yellow arrow) with some mononuclear cells are seen in a renal glomerulus from PK group (A), P4 group (B), and K group (C) A section of kidney tissue from PK group (D) and P4 group (E) showing congesti (black arrow) (400X,H&E stain).

Commented [ia31]: The said change is not visible. Use good quality images.

Commented [ia32]: Poor quality of images

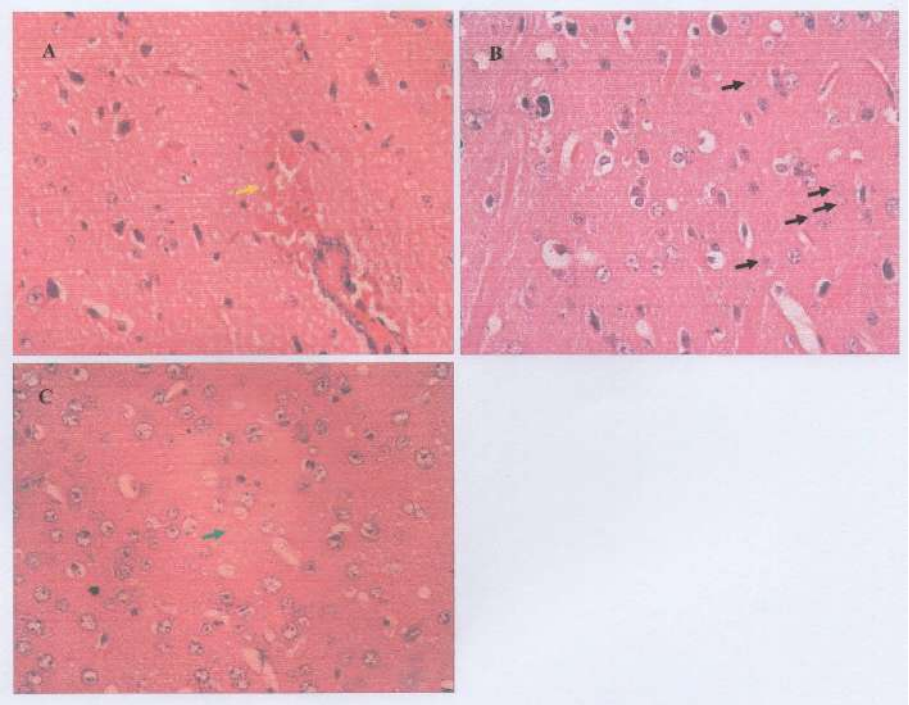
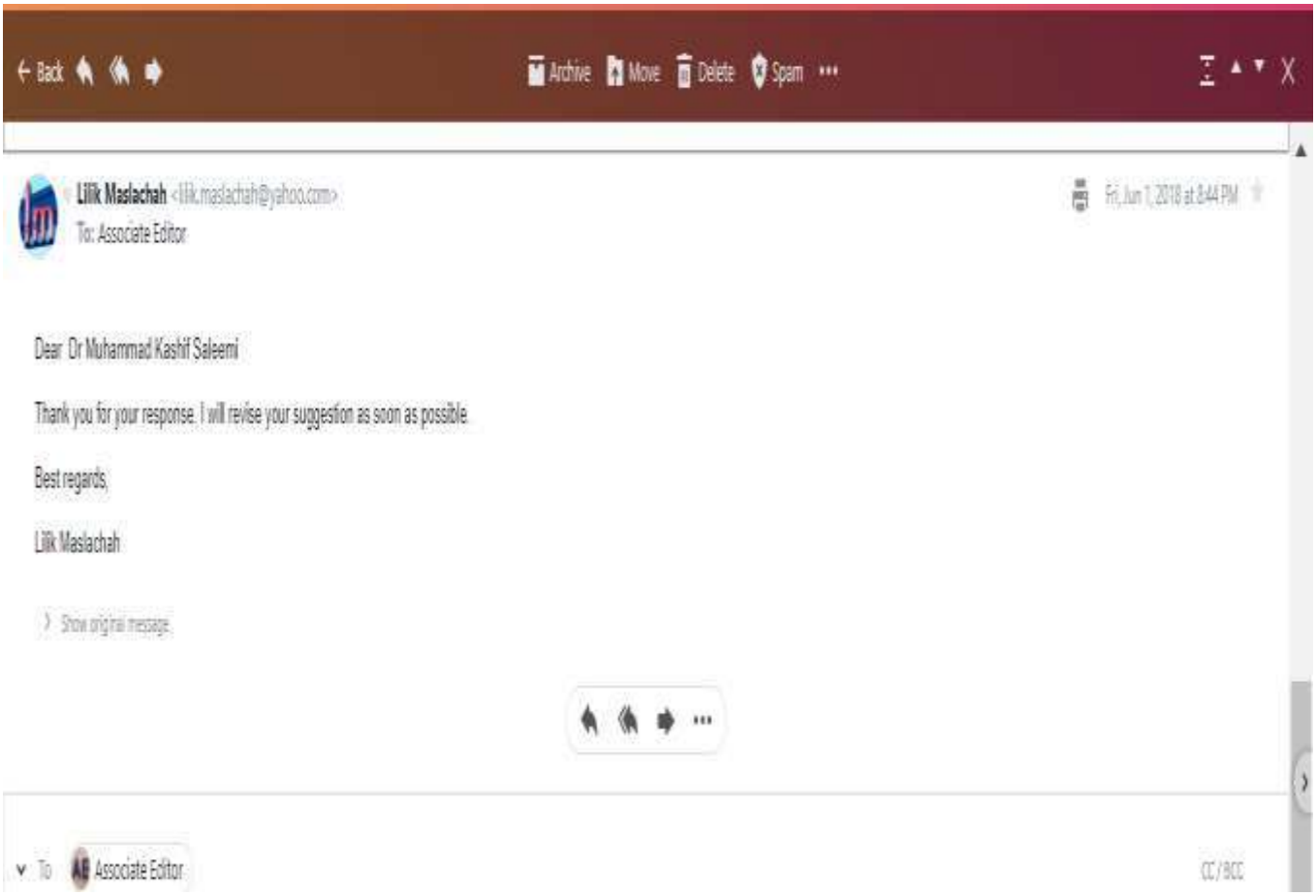


Figure 3. Representative images of the brain pathology are shown. A section of cerebrum tissue from PK group (A) showing haemorrhages with sequestration of parasites in the grissea substance, around vessels (yellow arrow). Necrosis of the macroglia cells can be seen in P4 group (B). The alba substance of cerebrum tissue from PK group (C) showing oedema (400X,H&E stain).

Commented [ia33]: Parasite sequestration is not clearly visible. Cerebral Malaria is usually associated with accumulation of inflammatory cells but the images here does not show the presence of inflammatory cells.

Commented [ia34]: Edema is not visible



CHECKLIST-REV- I

Line	Before	Line	After Revised
12	Artemisisnin	12	Artemisinin
16	P4	16	T4
17	K	17	C
18	PK	18	TC
24	Congesti	24	Congestion
34-35	Every year, especially in the tropics, 33 approximately two million people die and 800 thousand people die from severe malaria (Elias *et al*., 2012; Souza *et al*., 2012).	34	Every year, especially in the tropics, approximately two million people die (Souza *et al*., 2013).
35-37	Increased incidence of morbidity and mortality due to 35 increased parasitic resistance and decreased efficacy of artemisinin antimalarial drugs and its 36 derivatives (Noedl *et*	34-38	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

	al.,2008; Wongsrichanalai and Meshnick,2008).		partner drugs have been reported from the Greater Mekong Subregion of Myanmar (Myint *et al*., 2017)
45	Cytoaderens	45	Cytoadherence
47-50	acute lung injury and acute respiratory syndrome (Haldar, 2007). Other Plasmodium species can also 48 be found in various microvascular organs during infection as in primates and rodents (Singh *et* 49 *al*.,2010; Craig *et al*.,2012)	47-50	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)
87	Mice were euthanized by Ketamin. The brain	87-93	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 were 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.
115	decrease of alveolar expansion	124	alveolar expansion
127	Congesti	136	Congestion
142	oedema,	151	Edema
149	3A	158	3D
159	Imonoregulator	168	Immunoregulator
166-168	With infected monocytes and erythrocytes attached to the capillary 166 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*., 2012; Aitken *et al*., 2014).	175-178	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)
179	(Epiphanio *et al*., 2010).	189	(Canavese *et al*., 2014; Hempel *et al*.,2014).

183-186	Maslachah *et al.* (2017) which states that repeated exposure of 183 artemisinin to *Plasmodium berghei* may increase the number of neutrophils. Increased the 184 value of ED50 and ED90, decreased the PCT and RT and also changes in morphology dormant 185 and vacuole formation (Maslachah *et al*., 2017).	193-198	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).
192-194	This leads to increased hypoxia microenvironment and and decreased cellular defense mechanisms (Elias *et al*., 2012)	204-207	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).
195	Oxid	208	oxide,
207	(Desruisseaux *et al.,* 2008).	220	(Greiner *et al*., 2015)
209	Queiroz *et al* (2011)	222	Martin *et al* 2016
211	Erytrosite	224	Erythrocytes
212	Leukoscyte	225	Leukocyte
220	(Dorovini *et al.,* 2011).	233	(Milner *et al*.,2015)
227-228	to alter the morphology, physiology and function (Maier *et al*., 2009)	242-243	morphology, physiology, function and contribute to the pathological changes seen in severe malaria (Utter *et al*., 2017).
230	Cytoaderen	244	Cytoadherence
231-232	involved in the 231 inflammatory response and apoptosis (Chakravorty *et a.l*, 2008).	246-248	involved in the inflammatory response and apoptosis. The leakage into the perivascular space affects astrocytes and pericytes leading to BBB impairment (Storm *et a.l*, 2014).
233-234	limphotoxins, and microparticle 233 plasma platelets (Faille *et al.*, 2009).	249-253	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).
313-314	Lagase HAD, Anidi UI, Craig JM, Limjunyawong N, Poupore AK, Mitzner W and Scott LA. 313 2016. Journal of Leukocyte Biology 99 (5): 659-671.	314-317	Lagase HAD, Anidi UI, Craig JM, Limjunyawong N, Poupore AK, Mitzner W and Scott LA. 2016. Recruited monocytes modulate malaria induced lung injury through CD36 mediated clearance of sequestered infected erythrocytes. Journal of Leukocyte Biology 99 (5): 659-671.
326-327	Milner AD, Jr Whitten OR , Kamiza S *et al*. 2014. The systemic pathology of cerebral 326 malaria in African children. Front Cell Infect Microbiol 4 :104.	336-338	Milner Jr AD, Whitten OR, Kamiza S, Carr R, Liomba G, Dzamalala C, Seydel BK, Molyneux EM and Taylor ET. 2014. The systemic pathology of cerebral malaria in African children. Front Cell Infect Microbiol 4 :104.
339-341	Shackelford C, Gerald long, Wolf J, Okerberg C, and Herbert R. 2002. Toxicologic Pathology 339 Quantitative Toxicologic Pathology Qualitative and Quantitative Analysis of 340 Nonneoplastic Lesions in Toxicology Studies . Toxicologic pathology 30(1): 93–96.	349-351	Shackelford C, Gerald long, Wolf J, Okerberg C, and Herbert R. 2002. Qualitative and Quantitative Analysis of Nonneoplastic Lesions in Toxicology Studies . Toxicologic Pathology 30(1): 93–96.
351-352	Souza MC, Silva JD, Padua TA, Capelozzi VL, Rocco PRM, Henriques MG. 2013. Respiratory 351 Physiology & Neurobiology. 186:65-72.	358-360	Souza MC, Silva JD, Padua TA, Capelozzi VL, Rocco PRM, Henriques MG. 2013. Early and late acute lung injury and their association with distal organ damage in murine malaria. Respiratory Physiology & Neurobiology. 186:65-72.
353-354	Van den Steen EP, Deroost K, Deckers J, Herck EV, Struyf S and Opdenakker G. 2013. Trends 353 in Parasitology 29(7): 346-358.	366-368	Van den Steen EP, Deroost K, Deckers J, Herck EV, Struyf S and Opdenakker G. 2013. Pathogenesis of malaria associated acute respiratory distress syndrome. Trends in Parasitology 29(7): 346-358.
272-368	**REFFERENCES**	272-368	References replace with recently published articles (2013-2018) except in materials and methods

370-384	**Table**	374-387	Table has been revised
386-411	**Figure 1,2 and 3**	390-420	Figure 1,2 and 3 have been replaced with good quality and clearly histological changes

Sequestration and histopathological changes of the kidneys, lungs and brain of mice infected with *Plasmodium berghei* that exposed to repeated artemisinin

Repeated exposure of artemisinin cause increasing of malaria severity that indicated the presence of sequestration and histopathological changes in some organs. Histopathological studies for our understanding of the pathogenesis of malaria after repeated artemisinin exposure**.**

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.

Key word: Artemisinin, *Plasmodium berghei*, histopathology, lung, kidney, cerebrum

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.

MATERIAL AND METHOD

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 Airlangga University. 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 were 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.

Statistics

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.

RESULT

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 a severe 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 figure 1.

Kidney

The kidney damage from all mice showed a 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 Figure 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 Figure 3. Sequestration of the cerebrum as shown in figure 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. Beside 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).

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 a.l*, 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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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^{b} ± 0.44**	**1.40 ^{a}±0.54**	**0.60 ^{a} ± 0.54**	**2.20 ^{a}± 0.44**	**2.00 ^{ab}± 0.70**
P4	**0.80^{a}± 0.44**	**2.40 ^{a}± 1.14**	**1.80 ^{ab}± 1.30**	**2.00 ^{a}± 0.70**	**0.80 ^{a}±0.83**
TC	**2.20 ^{b}± 0.44**	**2.60 ^{a}± 1.14**	**2.80 ^{b} ± 1.30**	**2.20 ^{a}± 0.44**	**2.40^{b}± 0.54**

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 ^{a}± 0.44	2.20 ^{a}± 0.44	2.60 ^{ab}± 0.54	0.80 ^{a}±0.44	2.60 ^{a}±0.54
P4	2.40 ^{b} ± 0.54	2.80 ^{a}± 0.44	1.60 ^{a}± 0.54	0.00 ^{a}±0.00	1.60 ^{a}±0.89
TC	2.40 ^{b} ± 0.54	2.40 ^{a}± 0.54	2.80 ^{b} ± 0.44	0.80 ^{a}±0.83	2.80 ^{a}±0.44

Mean values with different superscripts within a column differ significantly ($p<0.05$)

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 ^{a}± 0.00	1.80 ^{a}± 0.44	0.20 ^{a}± 0.44
P4	0.20 ^{a}± 0.44	1.20 ^{a}± 0.44	0.40 ^{ab}± 0.54
TC	1.00 ^{a}± 1.00	2.00 ^{a}± 0.70	1.80 ^{b}± 1.30

Mean values with different superscripts within a column differ significantly ($p<0.05$)

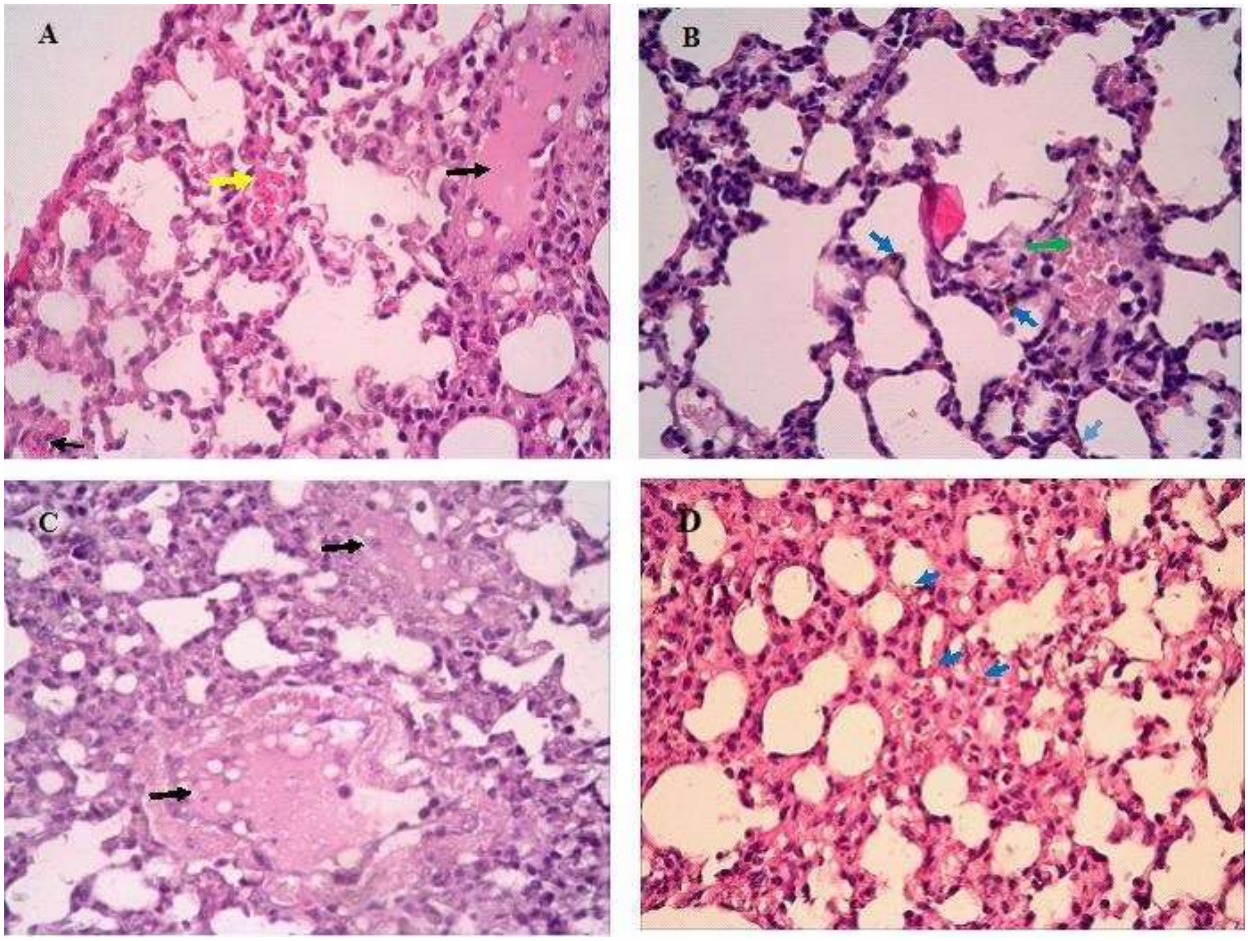


Figure 1. Representative images of the lung pathology are shown. The lungs from TC group (A) demonstrate septal congestion and some sequestration of parasites (yellow arrows) in the capillaries. The alveoli are filled with edema fluid, RBC and neutrophils (black arrow) The lung from T4 (B) showed congestion 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).

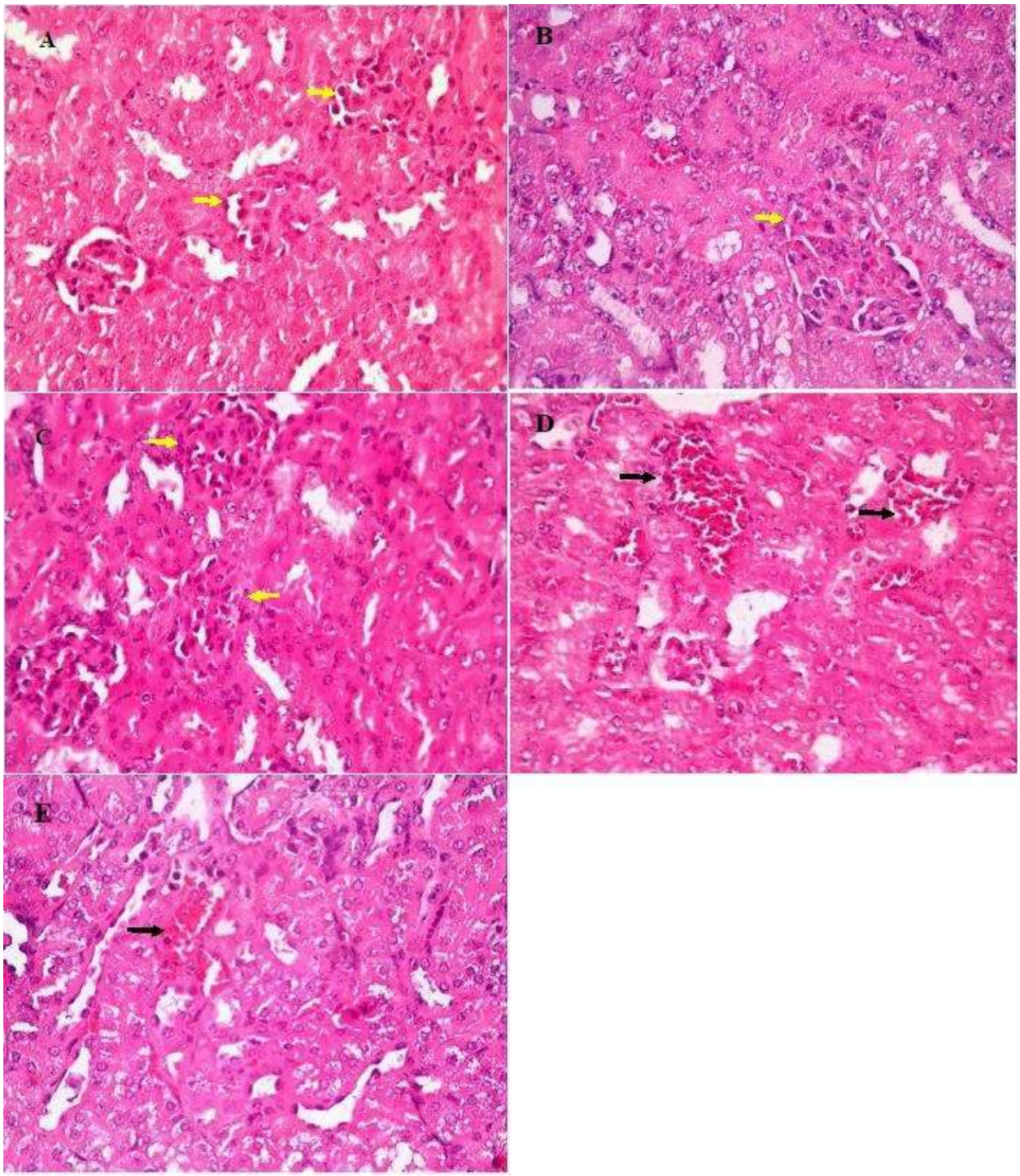


Figure 2. Representative images of the kidney pathology are shown. Glomerulonephritis (yellow arrow) with some mononuclear cells are seen in a renal glomerulus from TC group (A),

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).

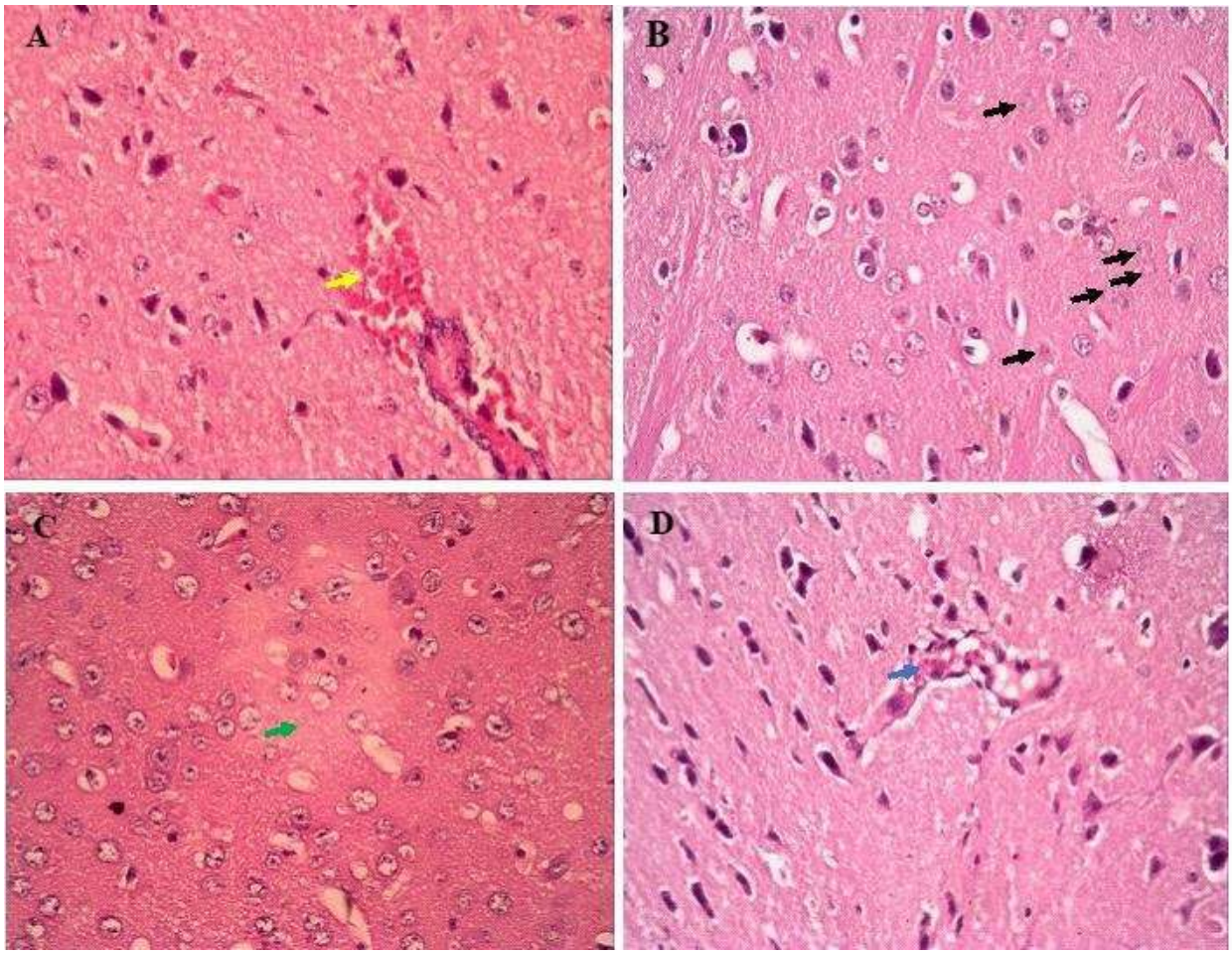
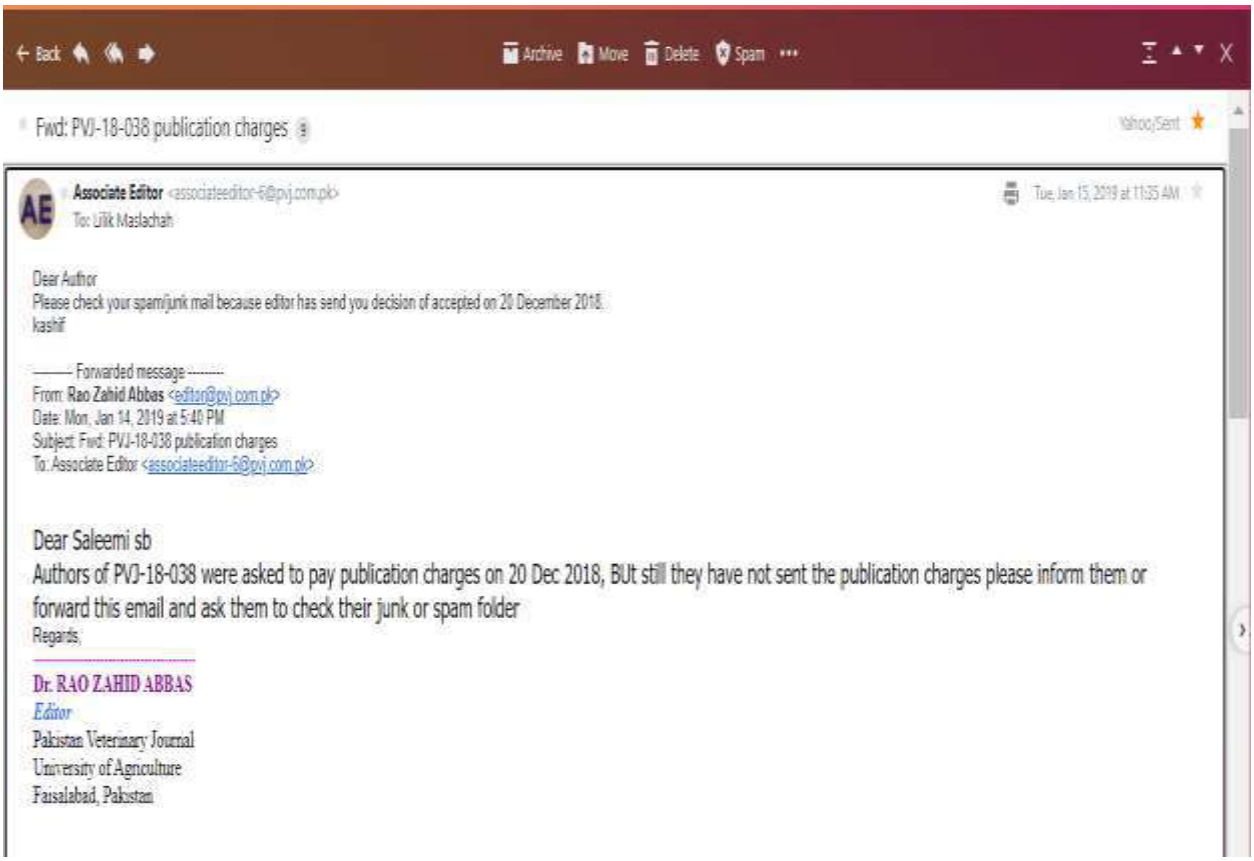


Figure 3. Representative images of the brain pathology are shown. A section of cerebrum tissue from TC group (A) showing haemorrhages in the grissea substance, around vessels (yellow arrow). Necrosis of the macroglia cells can be seen in T4 group (B). The alba substance of cerebrum tissue from TC group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



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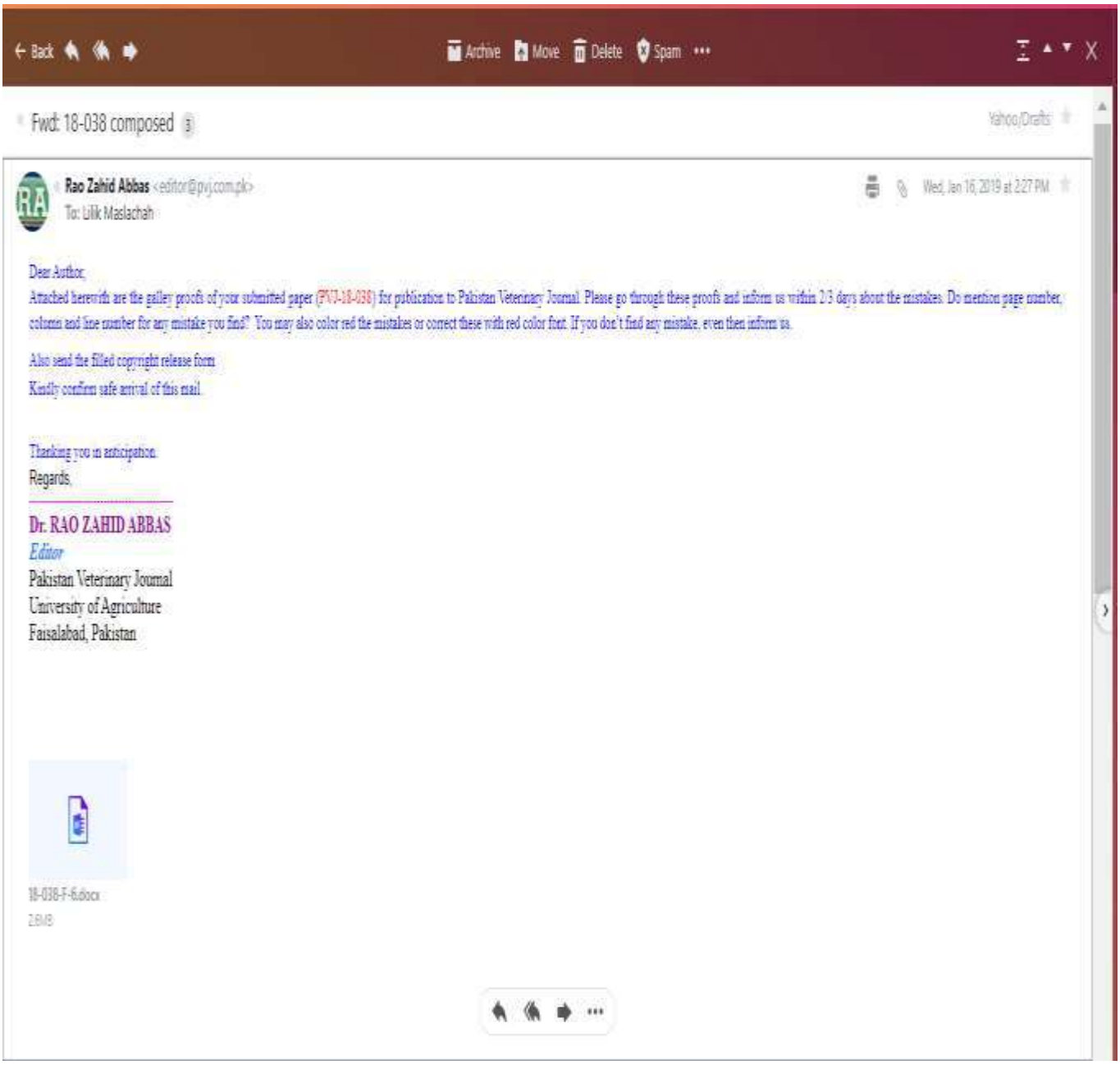

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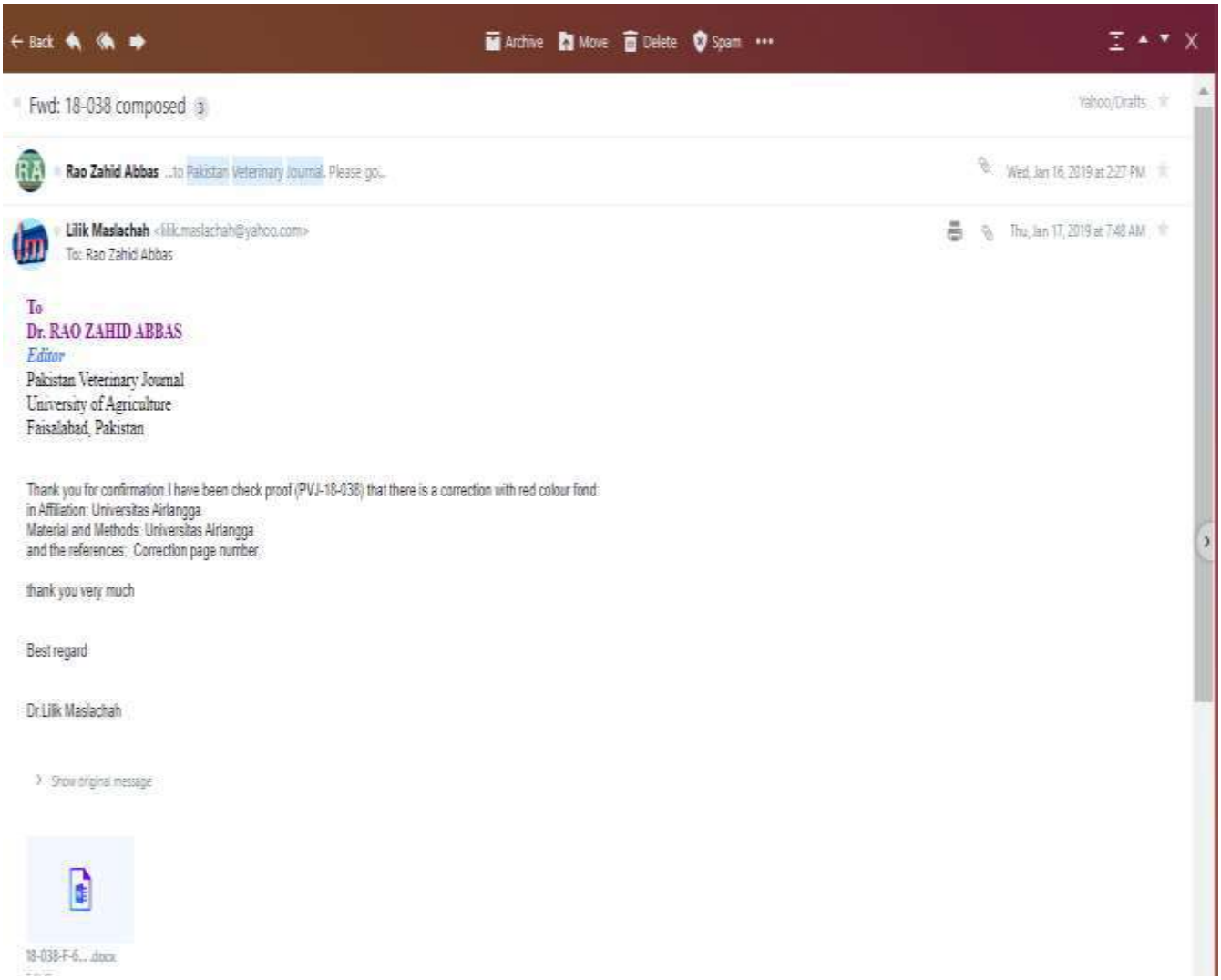

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RESEARCH ARTICLE

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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A B S T R A C T

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).

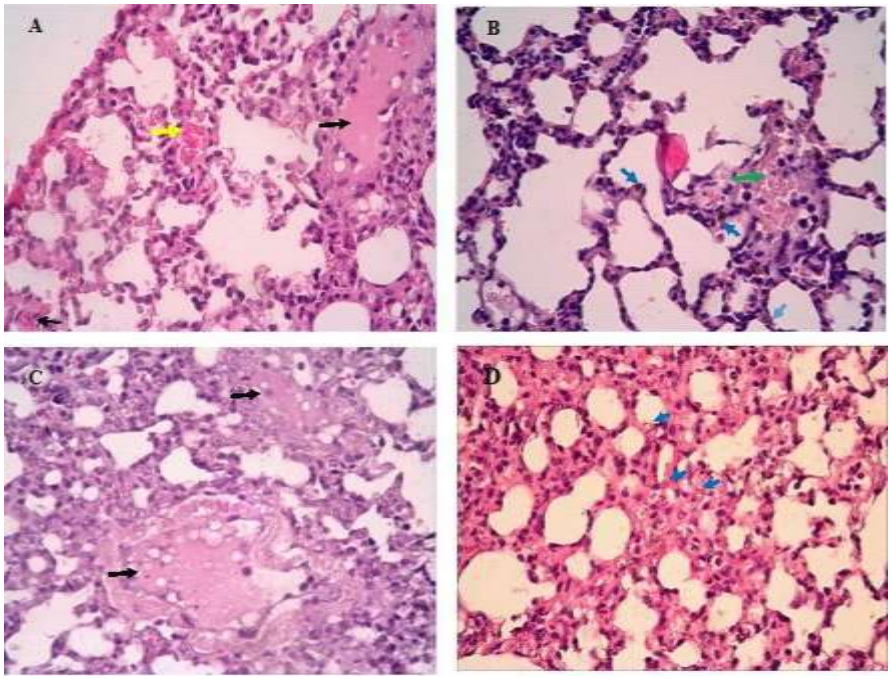

Fig. 1: Representative images of the lung pathology are shown. The lungs from TC group (A) demonstrate septal congestion and some sequestration of parasites (yellow arrows) in the capillaries. The alveoli are filled with edema fluid, RBC and neutrophils (black arrow) The lung from T4 (B) showed congestion 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).

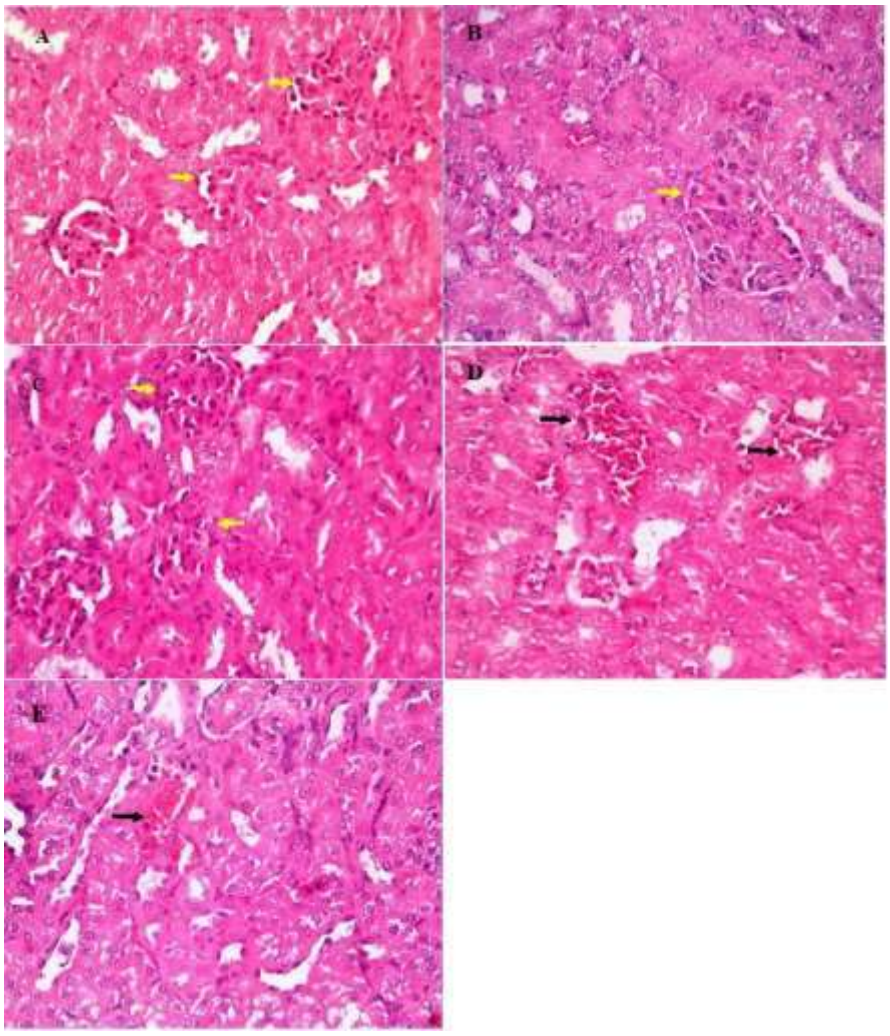

Fig. 2: Representative images of the kidney pathology are shown. Glomerulonephritis (yellow arrow) with some mononuclear cells are seen in a renal glomerulus from TC group (A), T4 group (B), and C group (C) **A section of kidney tissue from TC group (D) and T4 group (E) showing congestion (black arrow)**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

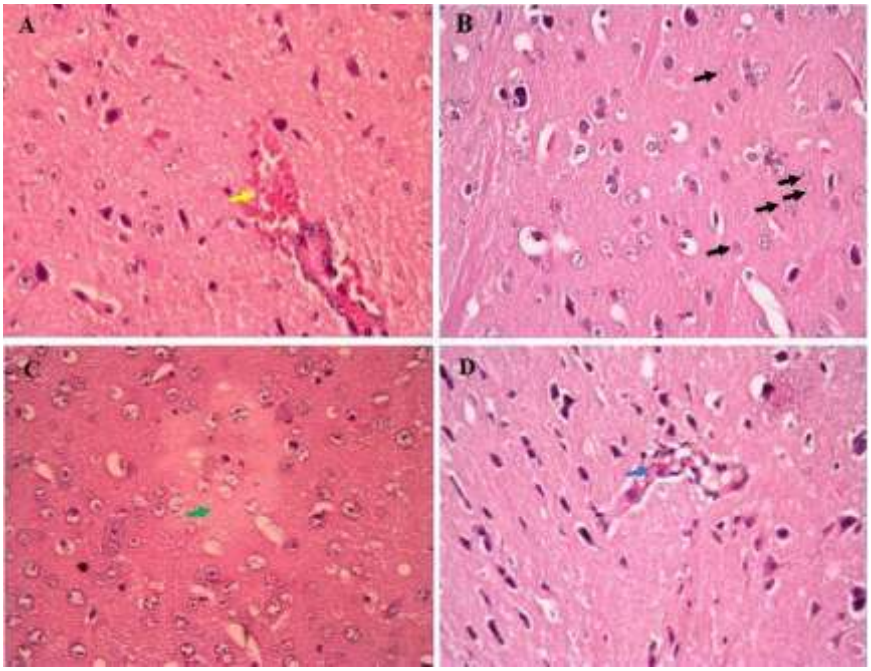

Fig. 3: Representative images of the brain pathology are shown. A section of cerebrum tissue from TC group (A) **showing haemorrhages in the grissea substance, around vessels (yellow arrow). N**ecrosis of the macroglia cells can be seen in T4 group (B). **The alba substance of cerebrum tissue from TC 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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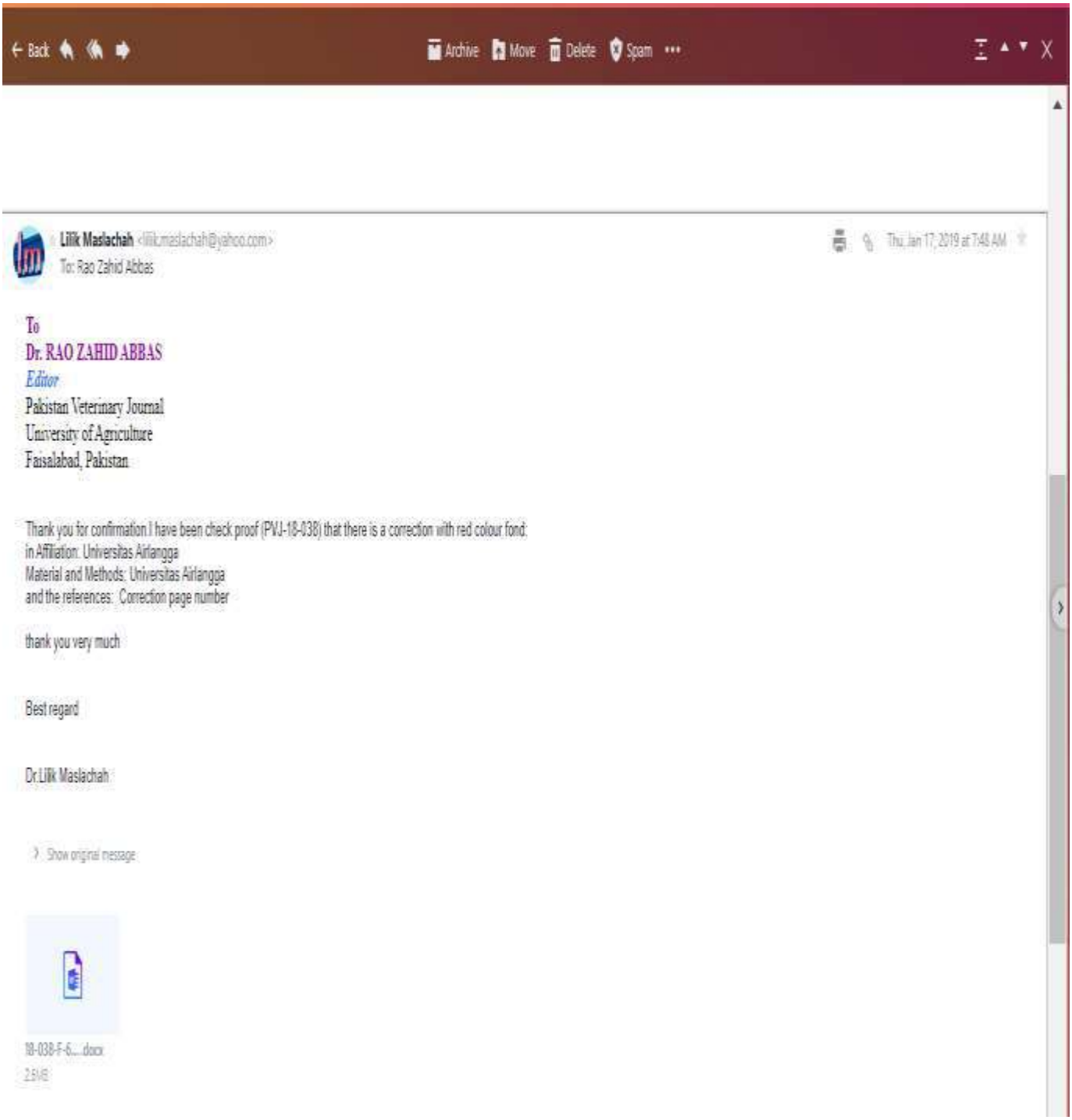

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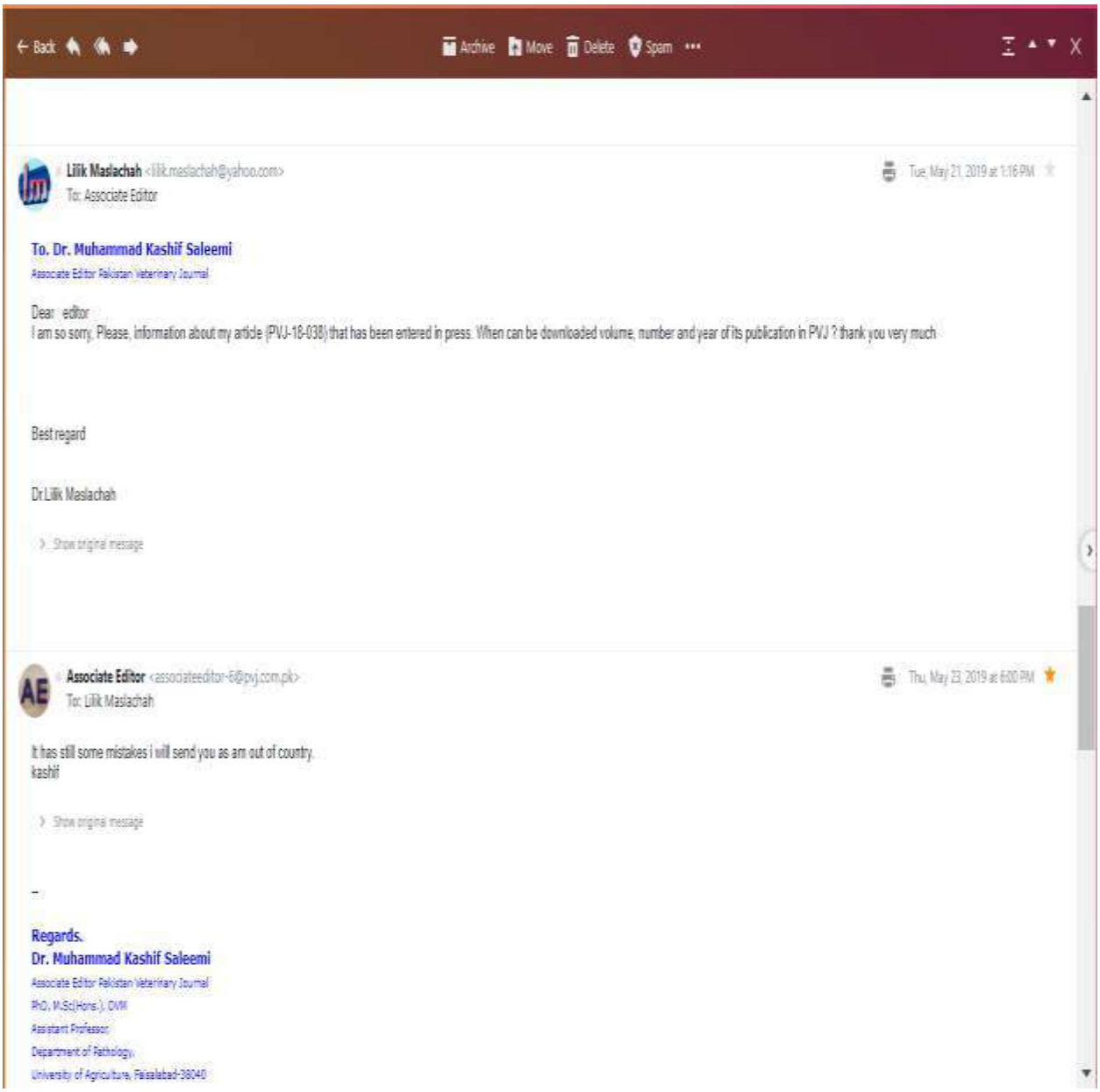

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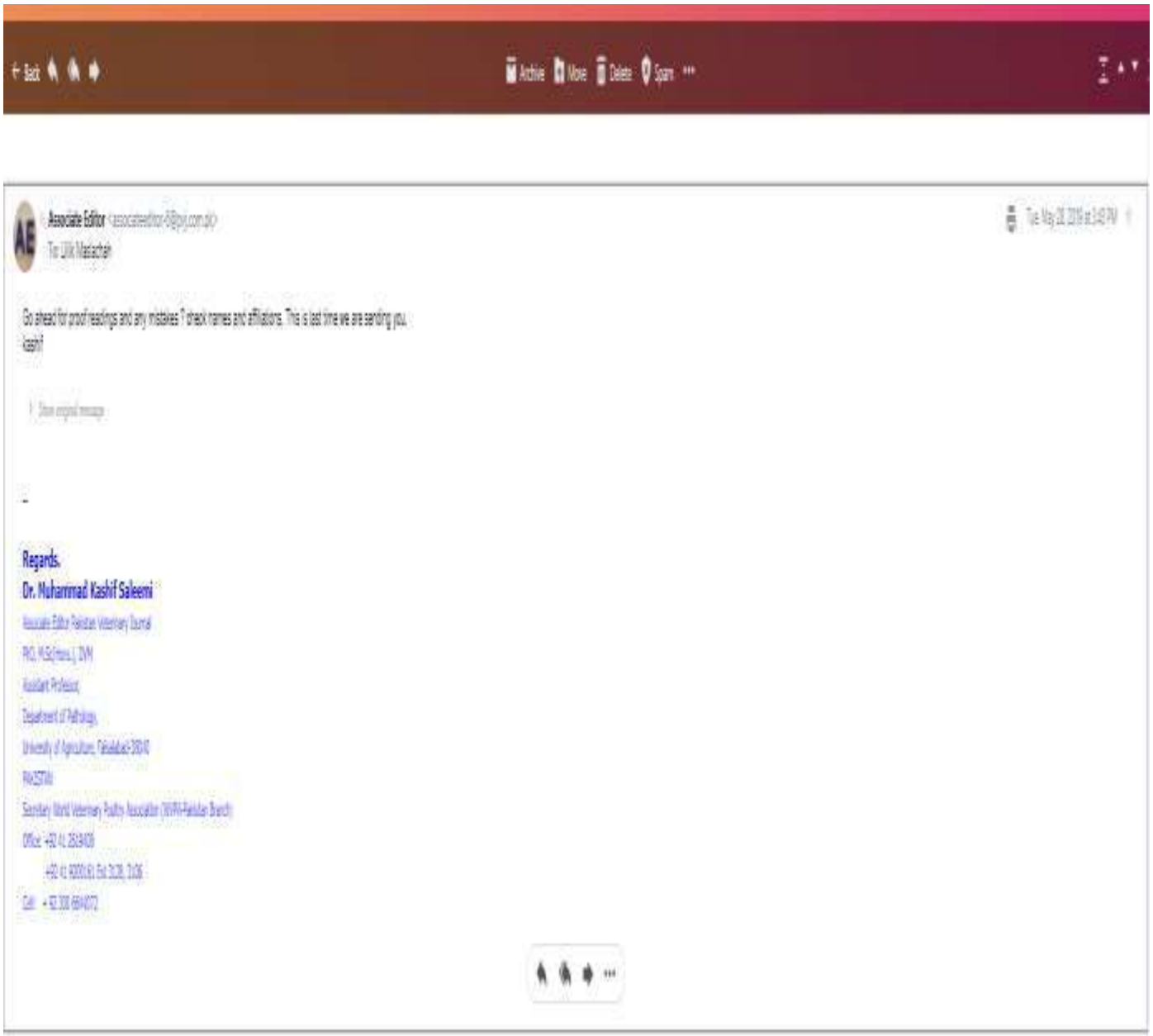
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