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Research Article

The differences of parasitemia and lungs size in malaria-associated acute respiratory distress syndrome (MA-ARDS) and non-MA-ARDS in mice infected with *Plasmodium berghei* ANKA

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ABSTRACT

Malaria-associated acute respiratory distress syndrome (MA-ARDS) is characterized by extensive infiltration of leukocytes, microhemorrhages, vasogenic edema, changes in lung color, and a significant increase in the weight of the lung. This study was aimed to find out the differences in parasitemia and lung size in MA-ARDS and non-MA-ARDS in mice infected with *Plasmodium berghei* ANKA. Sixteen male BALB/c mice were infected with *P. berghei* ANKA, and daily parasitemia was observed on Giemsa-stained tail blood smears. Mice were sacrificed when parasitemia reached ±20%. Simultaneously eight uninfected mice were used as negative control (NEG). The statistical analysis was done using Kruskal Wallis, Mann Whitney U tests, and Spearman correlation test. The results showed that there were significant differences in parasitemia (p=0.001), weight (p=0.001), and lung length (p=0.021) between the MA-ARDS and non-MA-ARDS groups. Comparison of NEG and MA-ARDS resulted in a significant difference in lung size (p=0.05). When non-MA-ARDS compared with NEG groups, it showed a significant difference in lung width (p=0.001). However, there was no significant difference in lung weight and length (p>0.05). Spearman correlation test showed that there was a strong correlation between parasitemia with weight (p=0.000), length (p=0.001), and lung width (p=0.017). The findings indicated that parasitemia played a role in the development of MA-ARDS in mice infected with *P. berghei* ANKA and influenced the size of the lung.

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INTRODUCTION

Malaria is an infectious disease caused by a parasite of the genus *Plasmodium* and transmitted to humans by the bite of female *Anopheles* mosquito (Heny Arwati, Yotopranoto, Rohmah, & Syafruddin, 2018). In 2020, an estimated 3.2 billion people, almost half the world's population across 91 countries or territories, are still at high risk of malaria (CDC, 2021). It has been stated that malaria will remain a major health problem until 2025 in 107 countries in the world because around 300-500 million people are infected with malaria every year (Dimi, Arlin, & Alim, 2020). Indonesia is one of the countries at risk of malaria because accounting for 21% of the region's reported cases and 16% of malaria deaths (WHO, 2019). The rates of infected population and mortality are still high, especially in Eastern regions such as Papua, West Papua, NTT, Maluku, and North Maluku (Kementrian Kesehatan RI, 2018); however, by 2018, a total of 285 districts in Indonesia successfully achieved their target of eliminating malaria (WHO, 2019).

Malaria-associated acute respiratory distress syndrome (MA-ARDS) is severe malaria with a lethality rate of up to 80% despite anti-malarial treatment. It is characterized by a vast infiltration of leukocytes, microhemorrhages, and vasogenic edema in the lungs (Vandermosten et al., 2018). Approximately 80% of ARDS patients present with fluid around the lungs (pleural effusion) in addition to fluid in the airspaces (alveolar edema) and within the lung parenchyma (interstitial edema) (Melo & Bates, 2019). This disease occurs especially in malaria caused by *Plasmodium falciparum, Plasmodium vivax,* and *Plasmodium knowlesi* (Aisiku et al., 2016). Excessive pulmonary inflammation and alveolar-capillary membrane damage cause overwhelming vasogenic edema and severe hypoxemia (Vandermosten et al., 2018).

Protein-rich pulmonary edema is one of the causes of ARDS that causes severe hypoxemia, and impaired carbon dioxide excretion is associated with large numbers of neutrophils, monocytes; denuded epithelial cells; and proinflammatory markers, including cytokines, proteases, oxidants, and procoagulant factors (Matthay & Zemans, 2011). Accumulation of those cells and formation of edema in the lung contributed to the increased lung weight, and in addition, the total lung weight can be used as a better parameter for the quantification of MA-ARDS (Van den Steen et al., 2013). The color of the lungs of mice infected with *Plasmodium* changed to greyish-brown due to bleeding and increased hemozoin formation (Deroost et al., 2013). Regardless of the *Plasmodium* species, the clinical manifestations of malaria are highly variable, and many factors influence it. The level of parasitemia is related to the severity or malignancy of malaria infection (Avrina et al., 2011). The experimental studies on MA-ARDS in *P. berghei* infection have been reported previously (Vandermosten et al., 2018; Gonzales et al., 2015); however, the differences of parasitemia and lung size in MA-ARDS and non-MA-ARDS in mice infected with *P. berghei* ANKA has not been explored. Pulmonary edema is one of the ARDS signs that caused the increase of lung weight, and parasitemia is an indicator of malaria severity. Therefore, this study was aimed to find out the differences in parasitemia, lung weight, length, and width between MA-ARDS and non-MA-ARDS in *P. berghei* ANKA infection in mice compared with those uninfected mice.

METHODS

Ethical approval

This research proposal has been reviewed and approved by the Ethical Committee from the Faculty of Dental Medicine, Universitas Airlangga, as mentioned on the Ethical Clearance certificate No 159/HRECC.FODM/IV/2021.

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Research Design

This research is an in vivo experimental study with a post-test control group design. Mice were divided into three groups; each group consisted of 8 male mice aged ± 7 weeks and weighing ±25 grams. Group 1 was negative control (NEG) that were not infected with *P.berghei* ANKA; Group 2 and 3 were infected with *P. berghei* ANKA. Group 2 was the MA-ARDS group where mice were expected to develop an MA-ARDS, Group 3 was a non-MA-ARDS group in which mice were without any ARDS symptoms. Observation of lung size was performed at the end of the experiment.

Parasite infection in mice

Parasite *P. berghei* ANKA was obtained from the Department of Medical Parasitology, Faculty of Medicine, Universitas Airlangga. Four healthy BALB/c mice were used as donor mice which were infected with 200µL of frozen blood infected with *P. berghei* ANKA. When parasitemia reached ±20%, the blood was collected by cardiac puncture and infected to the test mice. Each mouse was injected intraperitoneally with $1x10^6$ of infected erythrocytes.

Determination of parasitemia

The degree of parasitemia in donor and test mice was determined daily on Giemsa-stained tail blood smears and calculated based on the number of infected erythrocytes. The smears were examined under a light microscope at 100x magnification. Parasitemia was calculated by the following formula (Laboratory Identification of Parasitemia of Public Health Concern, 2020):

$$\% \text{ Parasitemia} = \frac{\text{Number of infected erythrocytes}}{\text{Total number of erythrocytes counts}} \text{ X } 100\%$$

Determination of MA-ARDS and lung removal

When parasitemia in the infected mice reached >15%, mice were sacrificed, and lungs were removed prior to measurement of their weight, length, and width. The mice were considered MA-ARDS when macroscopically pleural effusion was observed around the lungs, lungs underwent edema and greyish-brown in color (Melo & Bates, 2019; Deroost et al., 2013). The lungs were collected, and the weight was measured using an analytic balance scale, while the length and width were measured using a ruler.

Statistical analysis

The difference between parasitemia and the mice's lung weight, length, and width in the MA-ARDS group was compared with those of non-MA-ARDS and NEG groups by using the Kruskal Wallis test. The comparison between experimental groups was made by employing the Mann-Whitney U test, and the correlation between parasitemia and lung size was determined using the Spearman correlation test. The confidence interval of 95% (α=0.05) was employed, and the results were considered statistically significant when the p-value was less than 0.05.

RESULTS

MA-ARDS and parasitemia

Physical examination of the appearance of mice on the third-day post-*P. berghei* ANKA infection showed that they were still moving actively. However, at day 5 or 6, the mice started to shivering, the four hinds, both ears, and tail were started to be pale and moved less actively. In this appearance, the MA-ARDS-developed mice could not be distinguished from non-MA-ARDS. The observation of mice post scarification found that the chest cavity was full of pleural effusion, and the lungs were

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greyish-brown in color. Only 3 out of 8 mice developed MA-ARDS. The lung color in the MA-ARDS group was virtually different from that of non-MA-ARDS and uninfected mice (NEG), as shown in Figure 1. This figure shows the freshly reddish lung color of the uninfected mouse (A), the greyish-brown of mouse lung with MA-ARDS (B), and dark brown of the non-MA-ARDS mouse (C). Furthermore, observation and counting of parasitemia on Giemsa-stained tail blood smears resulted in the mean of parasitemia in the MA-ARDS group was higher (31.03%) than that of the non-MA-ARDS group (12.51%). Statistical analysis of parasitemia in MA-ARDS and non-MA-ARDS groups was significantly different with p=0.001 (Table 1).

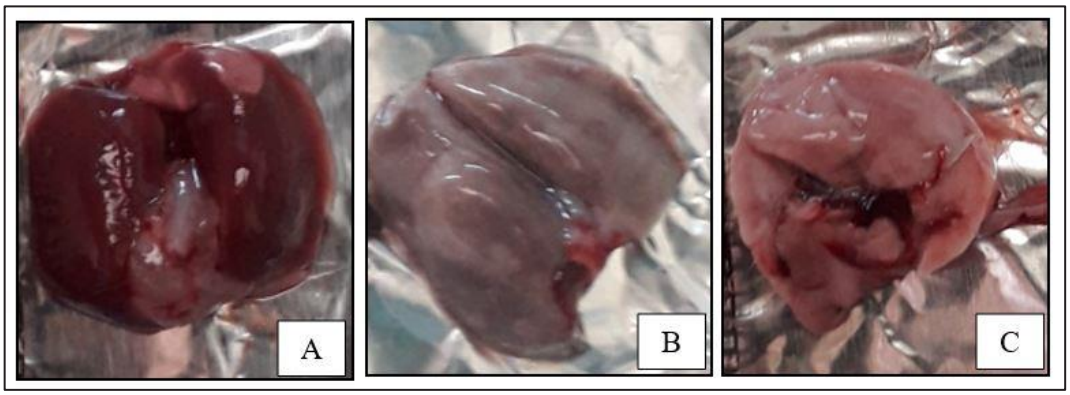

Figure 1. Representative picture of the lungs of mouse infected with *P. berghei* ANKA. The lung of mouse in negative control is freshly reddish (A), in MA-ARDS is greyish-brown (B), and in non-MA-ARDS is dark brown.

Table 1. The differences in parasitemia, lung weight, length and width of MA-ARDS compared with non-MA-ARDS in mice infected P. Berghei ANKA and negative control

	NEGATIVE CONTROL	MA-ARDS	NON MA-ARDS	p*
Parasitemia		31.03±1.68	12.51 ± 1.16	0.001
Lung weight (g)	0.265 ± 0.006	0.343 ± 0.005	0.262 ± 0.006	0.000
Lung length (cm)	1.24 ± 0.056	1.56 ± 0.086	1.30 ± 0.037	0.014
Lung width (cm)	1.18 ± 0.179	1.312 ± 0.047	1.18 ± 0.029	0.001

*Statistical analysis using Kruskal Wallis and Mann Whitney, n=16. Significance p<0.05

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Weight, length, and width of lungs

Comparison of lung size in the MA-ARDS and non-MA-ARDS groups resulted in significant differences in the weight (p=0.001) and length (p=0.021) of lungs; however, the width of the lungs was not (p=0.059). When the size of lungs in MA-ARDS was compared with that in NEG resulted in a significant difference in weight (p=0.001), length (p=0.010,) and width (p=0.005). Further, a comparison of non-MA-ARDS with NEG showed only the width of lungs was significantly different (p=0.001), but not in the weight (p=0.599) and length (p=0.442) of lungs. Furthermore, the Spearman correlation test resulted in a strong correlation between parasitemia and the weight (p=0.000), length (p=0.001), and width (p=0.017) of lungs (Table 1).

DISCUSSION

In this study, based on the physical examination of mice during sacrification, only 3 out of 8 mice developed MA-ARDS. The small number of mice that developed MA-ARDS related to the mouse strain and the strain of parasite used in this experiment. This disease is often associated with cerebral malaria (CM), acute renal failure, and high parasitemia (Gachot et al., 1995; Jindal et al., 2002). Malaria-associated pathogenesis is considered multi-factorial, with both host and *Plasmodium* factors playing critical roles (Epiphanio et al., 2010). Experimental MA-ARDS in *P. berghei* infection have been reported in different strains of mouse and parasite because the development of MA-ARDS in mice was highly dependent on the strain of mice and parasite. When C57BL/6 and BALB/c strains of mice were infected with *P. berghei* NK65-E strain of parasite, only male and female C57BL/6 mice developed MA-ARDS, but not BALB/c mice. Furthermore, when the DBA/2 strain of mice was infected with *P. berghei* ANKA strain, only a lower degree of lung pathology as well as BALB/c mice (Vandermosten et al., 2018).

ARDS is one of severe clinical presentation of malaria infection along with acute lung injury (ALI), cerebral malaria (CM), pregnancy-associated malaria (PAM), and severe anemia (SA). Malaria-associated ALI and ARDS are both lung disorders with similar features as occurred in *P. berghei* ANKA-infected DBA mice. Vascular endothelial growth factor (*VEGF*) is a critical host factor for the onset of malaria-associated ALI. In those mice that developed ALI, VEGF levels increased significantly by day 7 post-infection, but not in BALB/c mice infected with *P. berghei* ANKA because these mice did not develop ALI (Epiphanio et al., 2010).

The MA-ARDS-developed mice found in this study were characterized by the appearance of pleural effusion in the mouse chest cavity and the greyish-brown of lung color. The greyish-brown color of the lungs is caused by bleeding and the increased hemozoin formation (Deroost et al., 2013). Hemozoin (malaria pigment) is a disposal product formed from the digestion of red blood cells by malaria parasites (Soniran, Idowu, Ajayi, & Olubi, 2012). Hemozoin and hemozoin-containing parasites are associated with MA-ARDS and induce pulmonary inflammation (Deroost et al., 2013). Hemozoin is deposited as a brownish granule and caused blockages of small blood vessels in the liver, kidney, spleen, lungs, brain, and heart (Soniran et al., 2012; Franke-Fayard et al., 2010; Deroost et al., 2013; Van den Steen et al., 2013)

Parasitemia in the MA-ARDS group of mice (31.03%) was significantly different from that in the non-MA-ARDS group (12.51%). The MA-ARDS is associated with high parasitemia (Moura et al., 2017). The increase of parasitemia is accompanied by the increase of hemozoin released by schizont-infected erythrocytes when ruptured, therefore as explained above,

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that disposition of hemozoin in various organs causing weight gain and discoloration of the organ.

As found in this experiment, the weight, length, and width of lungs correlated with parasitemia. The disposition of hemozoin and hemorrhages increased lung weights and massive edema, and the hemozoin concentration in the lungs was highly correlated with lung weight and the presence of alveolar edema (Deroost et al., 2013). The lung edema in ARDS is non-cardiogenic pulmonary edema (NCPE), ultimately resulting from capillary permeability secondary to cellular damage, inflammatory cascades, and overinflation by mechanical ventilation resulting in endothelial permeability (Gonzales et al., 2015). The increased lung weight is also due to fluid accumulation causes alveolar collapse, especially in the dependent areas, for example, in the dorsal basal areas of the lungs (Regaller & Richter, 2010). In this study, the higher the parasitemia, the higher the lungs' weight, length, and width. However, a high degree of parasitemia cannot be a reference for MA-ARDS but is characterized by the presence of pleural effusion in the chest cavity, increased lung size, and greyish-brown in lung color.

The limitation of this study was the difficulty of obtaining mice that developed MA-ARDS because the strain of mouse (BALB/c) and parasites (ANKA) used in this experiment were resistant to the experimental cerebral malaria which related to MA-ARDS (Epiphanio *et al.,* 2010), therefore developed a lower degree of lung pathology (Vandermosten et al., 2018).

CONCLUSION

The MA-ARDS in this study pathologically was low; however, parasitemia and lung size between MA-ARDS and non-MA-ARDS were significantly different. High parasitemia correlated with weight, length, and width of the lung in MA-ARDS in BALB/c mice infected with *P. berghei* ANKA.

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