Jurnal ILMU DASAR. vol 26 no 1 Januari 2025. Hal. 1-7

Jurnal ILMU DASAR (JID)
https://jurnal.unej.ac.id/index.php/JID

Interleukin-6 as an Indicator for Acute Toxicity of DBL2β-PfEMP1 Recombinant protein as a Peptide-based Malaria Vaccine Candidate

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Article info

Keywords
adaptive immunity, DBL2β-PfEMP1, malaria pathogenesis peptide-based malaria vaccine, recombinant protein,

Article history
Received 11-06-2024
Accepted 20-08-2024

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ABSTRACT

Malaria caused by *Plasmodium spp* is an important health problem, and vaccination could be essential for disease prevention. One potential protein candidate is the Duffy binding-like 2β (DBL2β)-*Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). The protein is responsible for malaria pathogenesis by mediating binding to intercellular adhesion molecule-1 (ICAM-1), a receptor on the host cells. This study aimed to investigate the acute toxicity of the DBL2β-PfEMP1 recombinant protein as a basis for developing a peptide-based malaria vaccine based on body weight and interleukin-6 (IL-6) concentration. The study used male and female Wistar rats, which were divided into treatment and control groups after two weeks of acclimatization. Rats in the treatment group were injected with 750 µg DBL2β-PfEMP1 recombinant protein, and the control group was injected with NaCl 0.9%. Any indications of clinical toxicity symptoms were closely monitored within 4 hours of injection up to 24 hours. Observations were conducted daily for 14 days and included body weight and toxicity symptoms such as rising fur, tremors, salivation, diarrhea, weakness, draping, excitability, twitching, and death. Blood was collected on days 5, 7, and 14 for IL-6 examination using the ELISA method. Rats were euthanized on day 14. Data were analyzed using an ANOVA test. There was no significant weight loss as well as weight gain and toxicity symptoms during 14 days after treatment in all groups. There was an increase IL-6 levels on day 14 in all groups. However, statistical analysis did not show a significant difference between the control and treatment groups ($p>0.05$). This study showed that the DBL2β-PfEMP1 recombinant protein has no acute toxicity in Wistar rats, implying its safety and potential as a peptide-based malaria vaccine.

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INTRODUCTION

Malaria is an infectious disease caused by *Plasmodium spp*. It is a significant global health problem. WHO has reported an estimated of 249 million cases of malaria worldwide in 2022. The number of cases were increasing up to 5 million compared to 2021. There were approximately 631.000 death due to malaria globally in 2020, the number was increasing by 10% compared to 2019 (WHO, 2023). Globally, malaria can be found in 85 endemic countries, mostly in African and also Asia. Indonesia is at the second rank of malaria cases in Asia with the distribution of malaria cases is concentrated in the Eastern part of Indonesia, such as East Nusa Tenggara, Papua, and West Papua (WHO, 2018).

Malaria is an important indicator of the Sustainable Development Goals (SDGs) for goal 3 good health and well-being, especially target 3.3 Communicable diseases because the disease causes a decrease in work productivity. Indonesia's Ministry of Health has a target to eliminate malaria by 2030 (Kemenkes, 2022).The government carries out various programs to control malaria, including early detection, prompt treatment, surveillance, vector control, advocacy, information, and education (Kemenkes, 2022).

Five species cause malaria in humans, namely *Plasmodium. falciparum, P. vivax, P. ovale, P. malaria,* and *P. knowlesi* (WHO, 2023). *P. falciparum* is the most common cause of severe malaria because this Plasmodium species can infect all stages of erythrocytes and has the capacity to bind normal erythrocytes to form rosettes binding to several host receptors on endothelial cells called cytoadherence, the closure of blood microvasculature, hypoperfusion and further resulted in multiple organ failure (Rowe et al., 2009; Wiser, 2023). This process is mediated by a protein secreted by Plasmodium, called *Plasmodium falciparum* Erythrocyte Membrane Protein-1 (PfEMP-1) (McQuaid & Rowe, 2020; Rowe et al., 2009; Turner et al., 2013).

PfEMP1 is an antigenic protein and is encoded by the *var* gene. PfEMP1 is expressed on the surface of infected erythrocytes during the trophozoite stage, where a large antigenic variable extracellular region consisting of several domains, namely Duffy-binding like (DBL) domain and Cysteine-rich interdomain (CIDR) protein mediates adhesion to host cell receptors such as CD36, Intercellular adhesion molecule-1 (ICAM1), endothelial protein C receptor (EPCR) and chondroitin sulfate A (CSA) (Hviid, 2010; Olsen et al., 2019). The DBL domain is further classified with subclasses α, β, γ, δ, ε and λ (Gullingsrud et al., 2013). In the brain vasculature of cerebral malaria patients, several DBL domains were found to bind to ICAM-1 (Chan et al., 2014).

One important pathology of severe malaria is the binding of ICAM-1 receptor and DBL2β, and study reported that the antibody against the domain would inhibit these binding (Ndam & Moussiliou, 2017),(Tessema et al., 2019). Thus, this DBL2β domain could be a potential vaccine candidate (Sulistyaningsih et al., 2018). Studies also reported the immunogenicity, T cells, and B cell epitope prediction of the DBL2β-PfEMP1. At a dose of 150 µg, the Indonesian group isolated DBL2β-PfEMP1 recombinant protein is immunogenic because it can induce the production of specific IgG antibodies and CD4+T cells. The DBL2β-PfEMP1 has two conservative T cell epitope sequences recognized by almost 90% of the population in malaria-endemic areas and two B cell epitope sequences bind to the ICAM-1 receptor (Rachmania et al., 2021),(Rachmania et al., 2020).

The development of a vaccine is a long road. One crucial step is to analyze the safety of the vaccine candidate. This study was conducted to analyze the acute toxicity effect of the DBL2β-PFEMP1 recombinant protein as a peptide-based malaria vaccine candidate by analyzing body weight, toxicity symptoms, and IL-6 concentration. Interleukin-6 (IL-6) is a pro-inflammatory cytokine. It is a vital cytokine in the innate immune response, and the increased IL-6 is associated with mortality in severe malaria (Hamilton et al., 2023; Wilairatana et al., 2022).

MATERIALS AND METHODS

The Study site and Ethical Clearance

The study was conducted at the Laboratory of Biocehmistry and Molecular Biology Faculty of Medicine, the Animal Laboratory of Faculty of Dentistry, and the laboratory of Center for Development of Advanced Science and Technology, University of Jember in October 2021-June 2022. All procedures have been approved by the Ethical Committee of Health Research of Faculty of Medicine, University of Jember No. 1536/UN25.8/KEPK/DL/2021.

Recombinant protein production

The construct of DBL2β-PFEMP1 recombinant protein was described previously (Sulistyaningsih et al., 2018). *E. coli* BL21(DE3), which had been transformed with the DBL2β-PfEMP1 domain, was grown in LB media and incubated at room temperature with 190 rpm until the OD_{600} of the culture reached 0.8. Protein expression was induced using IPTG 0.1 mM at room temperature for 6 hours. The culture was then centrifuged at 6,000 rpm at 4^{o}C for 10 minutes. The pellet was extracted with extraction buffer

containing NaCl 300 mM, Imidazole 5 mM, and Tris HCl 50 mM with a ratio of 1:2 for the pellet: extraction buffer. The pellet was then treated with 1 mg/ml lysozyme, sonicated, and further centrifuged at 12,000 rpm at 4^{o}C for 20 minutes. The soluble fraction of protein was measured using the Bradford Protein Assay Kit (Sigma-Aldrich, USA) as a previous study (Rachmania et al., 2021).

Purify proteins by affinity chromatography

Protein purification was carried out based on affinity chromatography using the QIAexpressionist Ni-NTA resin kit, according to the manufacturer's procedure (Qiagen, UK). The purified protein was then visualized using SDS-PAGE. The gel was stained using Coomassie brilliant blue (CBB) solution and visualized based on the standard method.

Immunization of experimental animals

As many as 12 rats weighing 200-250 g were included in the study, they consisted of 5 males and 5 females in the treatment group and 2 in the control group. Each rat in the treatment group was injected subcutaneously with 750 µg DBL2β-PfEMP1 recombinant protein, which was mixed with Freund Complete Adjuvant. Any indications of clinical toxicity were closely monitored within 4 hours of injection up to 24 hours. Observations were made, including body weight and toxicity symptoms such as rising fur, tremors, salivation, diarrhea, weakness, draping, excitability, twitching, and deathfor14 days. Body weight was measured before treatment and every two days after injection (day 3, 5, 7, 9, 11, 14). Blood was collected on days 5, 7, and 14 for IL-6 measurement, and all rats were euthanized on day 14.

Measurement of IL-6 concentrations using ELISA

IL-6 concentration was measured using the ELISA method based on the manufacturer's protocol (BT-Lab, China). The plate has been coated with IL-6 monoclonal antibodies. Calculation of IL-6 concentration is carried out using a standard curve with the regression formula of:

$y = 0.0671x + 0.167$

$R2 = 0.971$

Data analysis

Data were processed using the Statistical Package for Social Sciences (SPSS 26) for Windows computer program. Data were tested for normality using the Shapiro-Wilk test and homogeneity using the Levene test. A difference between groups was analyzed using a t-test for body weight and the One Way Anova test for IL-6 concentration with a p-value of <0.05, considered statistically significant.

RESULTS AND DISCUSSION

Recombinant Protein Visualization

The purified protein was visualized using SDS-PAGE. The DBL2β-PFEMP1 recombinant protein was visualized as a 72 kDa band (Sulistyaningsih et al., 2018), as shown in Figure 1. The protein concentration determined using the Bradford protein assay was 2 µg/µl.

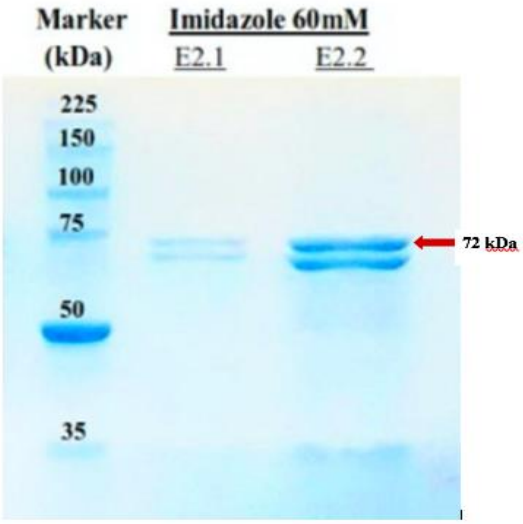

Figure 1. Visualization of the DBL2β-PfEMP1 recombinant protein by SDS-PAGE. The observed blue band is 72 kDa DBL2β-PfEMP1 recombinant protein (red arrow).

Observation of body weight and toxicity symptom

Rats were observed for their body weight and toxicity symptoms, as well as death. Data on body weight is presented in Figure 2. The baseline data before treatment showed the average body weight of rats from the male treatment group (176 g) was higher than the female (143.4 g) and control (130 g) groups. And there is a trend of body weight increased by day in all groups. Data were further statistically analyzed. The normality test using the Shapiro-Wilk test resulted in $p>0.05$ (range 0.188-0.964), meaning the data have a normal distribution. The dependent t-test to differentiate the pre-treatment and post-treatment showed $p= 0.665$, indicating that there is no significant difference in body weight between the pre-and post-treatment of all groups. Furthermore, we analyzed the difference between the control and treatment groups, as well as the difference between the male and female treatment groups, all resulting in $p>0.05$, meaning there is no statistical difference between groups. The weight gain of rats could be due to the increased age (Ghasem et al., 2021).

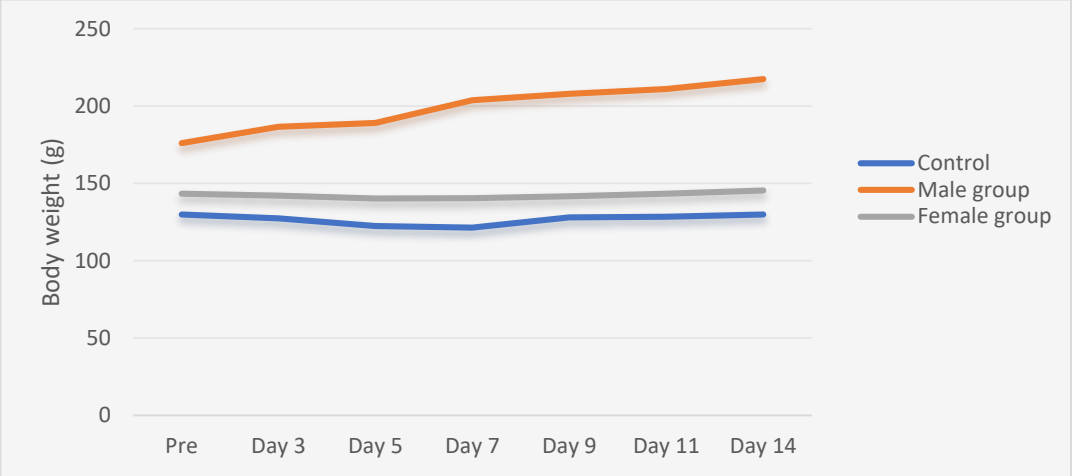

Figure 2. The averages of body weight (g) in all groups before and after treatment

Observation of toxicity symptoms found no toxicity appeared in all animal samples (Table 1); however, one rat of the male treatment group died on day 6 (Table 2), but there was no symptom of toxicity or infection observed.

Table 1. Observation of toxicity symptoms

Group	rising fur	tremors	salivation	diarrhea	weakness	draping	excitability	twitching
Control	0	0	0	0	0	0	0	0
Male rat group	0	0	0	0	0	0	0	0
Female rat group	0	0	0	0	0	0	0	0

Remarks: 0: no toxicity symptom; 1: toxicity symptom available

Table 2. The number dead animals after the treatment

Group	Day													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Male rat group	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Female rat group	0	0	0	0	0	0	0	0	0	0	0	0	0	0

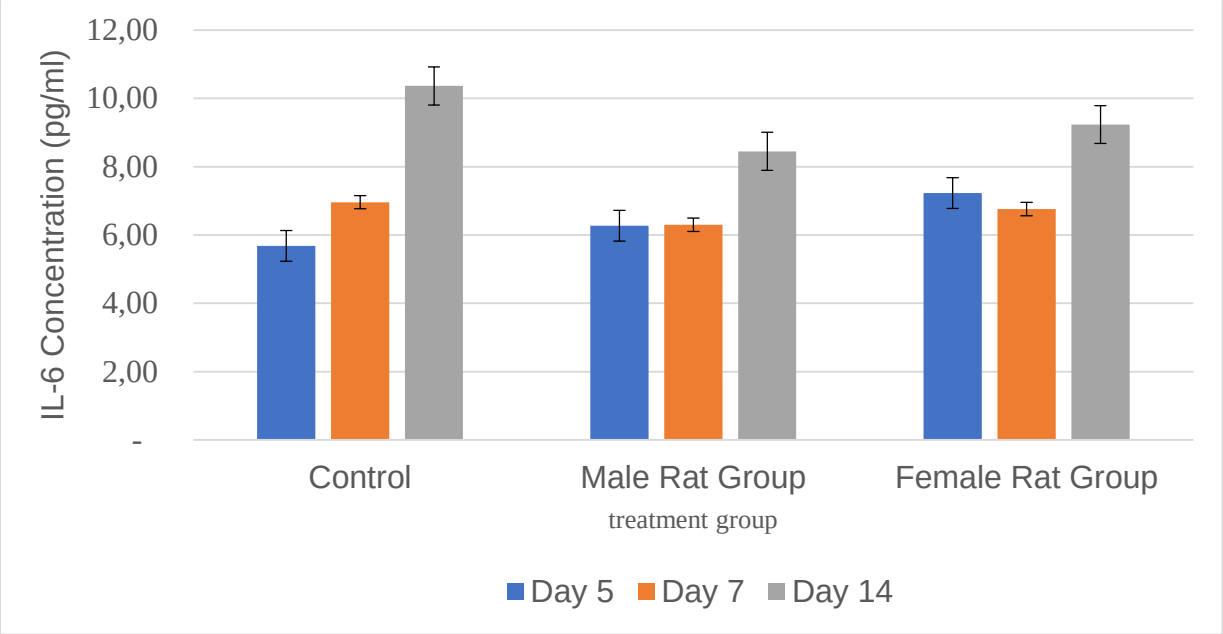

Figure 3. IL-6 Concentration (pg/ml) in all groups after treatment with DBL2β-PfEMP1 recombinant protein. Examination was performed on days 5, 7, and 14.

IL-6 concentration

The study examined the IL-6 concentration on days 5, 7, and 14, and the result is shown in Figure 3. There was a trend of increasing IL-6 concentration by day in control and treatment groups, and the highest IL-6 concentration was observed on day 14.

The normality test using the Shapiro-Wilk test showed p>0.05 (range p=0.278-0.771), and the homogeneity test using the Levene test showed p>0.05 (range p=0.098-0.155). Further analysis using the ANOVA test for the difference in IL-6 concentration between groups showed no significant difference with p=0.399, 0.681, and 0.663, respectively, for IL-6 concentration on days 5, 7, and 14.

IL-6 is a pro-inflammatory cytokine, and the study found that high levels of IL-6 were associated with severe malaria (Perera et al., 2013). Lower IL-6 level was found in children with hyperparasitemia than those with lower parasitemia and high IL-6 was found in children with cerebral malaria than those with non-cerebral malaria (Lyke et al., 2004; Mandala et al., 2017). However, a significant increase in IL-6 was found in severe malaria patients than in non-severe malaria patients, indicating its potency as a severe malaria marker (Wilairatana et al., 2022). Furthermore, IL-6 is generated at sites of infection and inflammation by immune cells, adipocytes, and endothelial cells. It promotes the differentiation of naive CD4+ T cells, which is proposed to have a vital role in the development of adaptive immunity. IL-6 also plays an important role in the acquired immune response by stimulating antibody production and effector T-cell development. Because it has a pleiotropic activity, the high production of IL-6 leads to the development of severe pathogenesis (Tanaka et al., 2014). In severe malaria, parasite-derived toxins induce cytokine production, resulting in increased levels of cytokines locally and in vital organ tissues that will upregulate endothelial receptor expression. This situation causes increased parasite adhesion and microvascular obstruction, decreasing oxygen delivery from the endothelium and ultimately contributing to severe pathogenesis (Perera et al., 2013).

In this study, we observed an increased level of IL-6 on day 14. However, the increase was not significantly different between the control and treatment groups, as well as between the male and female treatment groups. These results indicated that the DBL2β-PfEMP1 recombinant protein could induce the inflammatory response at the level to produce an immune response to develop adaptive immunity, as in a previous study (Rachmania et al., 2021), but it is safe as a vaccine candidate.

CONCLUSIONS AND SUGGESTION

The injection of high dose DBL2β-PfEMP1 recombinant protein did not affect the body weight and did not cause toxicity symptoms such as rising fur, tremors, salivation, diarrhea, weakness, draping, excitability, and twitching and increase IL-6 concentration of the animal model significantly. These results implied the immunogenicity and safety of the recombinant protein for further use as a malaria vaccine candidate.

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