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ARTICLE

INCREASED OF SPLEEN WHITE PULP DIAMETER POST DBL2β-PfEMP1 RECOMBINANT PROTEIN INJECTION IN WISTAR RATS: PRE-CLINICAL STUDY FOR MALARIA VACCINE DEVELOPMENT

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ABSTRACT

Malaria is a major infectious disease worldwide, and vaccination is essential for disease control. The *Plasmodium falciparum Erythrocyte Membrane Protein-1* (PfEMP1) is a potential malaria vaccine candidate due to its involvement in pathogenesis. Injection of DBL2β-PfEMP1 recombinant protein in animal models induces IgG and CD4+ production and inhibits binding with host endothelial receptors. This study aimed to analyze the spleen immune response by measuring the white pulp diameter. The experimental study used Wistar rats (*Rattus norvegicus*) that were divided into four groups, a control group and three treatment groups, which were injected with 100, 150, and 200 µg of DBL2β-PfEMP1 protein. Injection was done three times with three-week intervals (days 0, 21, and 42). On day 56, rats were euthanized, and spleens were prepared for histology examination. The white pulp diameter increased along with increasing the dose of protein. The ANOVA test showed a significant difference between groups (p=0.001). The post-hoc Bonferroni test showed a significant difference between the control and the 150 and 200 µg groups (p=0.013, p=0.002) and the 100 µg and 200 µg groups (p=0.027). In conclusion, the DBL2β-PfEMP1 recombinant protein injection increased the spleen white pulp diameter in Wistar rats, and the 200 µg dose resulted in the highest increase.

Keywords: DBL2β; Malaria; *Plasmodium falciparum*; PfEMP1; Spleen-white pulp

АБСТРАКТ

Малярия является одним из основных инфекционных заболеваний во всем мире, и вакцинация необходима для контроля заболевания. Мембранный белок эритроцитов Plasmodium falciparum Erythrocyte Membrane Protein-1 (PfEMP1) является потенциальным кандидатом в малярийные вакцины благодаря его участию в патогенезе. Инъекция рекомбинантного белка DBL2β-PfEMP1 на животных моделях индуцирует выработку IgG и CD4+ и ингибирует связывание с рецепторами эндотелия хозяина. Целью данного исследования был анализ иммунного ответа селезенки путем измерения диаметра белой пульпы. В экспериментальном исследовании использовались крысы Вистар (Rattus norvegicus), которые были разделены на четыре группы, контрольную и три лечебные группы, которым вводили 100, 150 и 200 мкг белка DBL2β-PfEMP1. Инъекции проводили три раза с трехнедельными интервалами (дни 0, 21 и 42). На 56-й день крыс умерщвляли, а селезенки готовили для гистологического исследования. Диаметр белой пульпы увеличивался по мере увеличения дозы белка. Тест ANOVA показал значительную разницу между группами (p=0,001). Пост-хок тест Бонферрони показал значительную разницу между контролем и группами 150 и 200 мкг (p=0,013, p=0,002) и группами 100 и 200 мкг (p=0,027). В заключение следует отметить, что введение рекомбинантного белка DBL2β-PfEMP1 увеличивало диаметр белой пульпы селезенки у крыс Вистар, причем доза 200 мкг приводила к наибольшему увеличению.

Ключевые слова: DBL2β; Малярия; Plasmodium falciparum; PfEMP1; Белая пульпа селезенки

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INTRODUCTION

Malaria is an infectious disease caused by *Plasmodium sp.* and a major global health problem. World Health Organization (WHO) reported 247 million malaria cases in 2021, resulting in 619,000 deaths. Indonesia is second-ranked in Asia in terms of the highest malaria cases, with a total of 399,666 cases in 2021. The number of malaria cases in Indonesia has increased by 30% compared to the previous year, rising from 304,607 cases to 400,253 cases in 2022.[1]

Plasmodium falciparum, P. ovale, P. vivax, P. malariae, and *P. knowlesi* are the species that cause malaria infection in humans. 90% of malaria deaths in the world are caused by *Plasmodium falciparum.*[2,3] Severe malaria can occur at any age in areas with low transmission rates, as well as in individuals with a history of traveling to malaria-endemic areas. The initial symptoms of malaria are malaise, headache, muscle pain, fever, nausea, vomiting, diarrhea, and orthostatic hypotension. Malaria can cause severe clinical manifestations such as cerebral malaria, acidosis, severe anemia, acute renal failure, and hypoglycemia.[4]

The manifestation of severe *P. falciparum* malaria is due to cytoadherence and rosetting mechanism. Cytoadherence is the attachment of infected red blood cells (IRBCs) to the *Intracellular Adhesion Molecule 1* (ICAM-1) receptor in the host endothelium. While rosetting is an attachment of infected red blood cells (IRBCs) and uninfected red blood cells (URBCs), forming a flower-like structure. Cytoadherence and rosetting are mediated by the antigen protein *Plasmodium falciparum Erythrocyte Membrane Protein-1* (PfEMP1). This process leads to microvascular blockage, ischemia, and hypoxia and causes organ damage.[5]

PfEMP1 is a protein that binds to the membrane surface of host IRBCs. The outer part of the PfEMP1 consists of the N-terminal segment (NTS), the cysteine-rich interdomain region (CIDR), and the Duffy binding-like (DBL).[6] The CIDR domain binds to multiple receptors, including Cluster of Differentiation 36 (CD36) and Endothelial Protein C Receptor (EPCR), and is divided into α, β, and γ subtypes. The DBL domain is found in almost all parts of the PfEMP1 head. It has subtypes α, β, γ, δ, ε, and χ. The DBL2β domain specifically binds the ICAM-1 receptor. Injection of PfEMP1 could induce IgG and CD4+ production, which reduces the risk of severe malaria by 37% and inhibits merozoite invasion, cytoadherence, and infection.[7] It supports the evidence for the development of the DBL2β-PfEMP1 recombinant protein as a malaria vaccine.[8]

Vaccine development involves two main stages, i.e., pre-clinical and clinical studies. The pre-clinical study uses animal models to determine the immunogenicity, safety, and effectiveness of vaccine candidates in a laboratory and *in vivo* setting. Previous studies reported the immunogenicity of the DBL2β-PfEMP1 recombinant protein by inducing IgG and CD4+ production. This study evaluated further immune response by the spleen-white pulp diameter. The spleen is a secondary lymphoid organ and has a crucial role in regulating immune response.[9] The spleen has two main structures: the white pulp and the red pulp. The white pulp is the part surrounding the central artery and is constituted by three regions; the periarteriolar lymphocyte sheath (PALS, T-cell area), lymphoid follicles (B-cell area), and marginal zones (MZ, B-cell area). It consists of T and B cells, contains white blood cells, and is involved in the adaptive immune system activation response. The whole white pulp contains about a quarter of the total number of lymphocytes in the body. It allows the formation of specific antigen immune responses that protect the body from disease. Changes in the cellular composition occur in the area of the white pulp after exposure to immunomodulatory agents.[9,10] The study aimed to analyze the effect of DBL2β-PfEMP1 recombinant protein on the spleen-white pulp diameter.

MATERIAL AND METHODS

This study has received ethical approval from the Research Ethics Committee of the

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Faculty of Medicine, University of Jember, No. 1830/H25.1.11/KE/2023.

The study was a true experimental study with a post-test-only control group design. The samples were Wistar rats (*Rattus norvegicus)* aged 2-3 months, weighing 150–250 g, and divided into a control group and three treatment groups. Rats were acclimatized for two weeks in optimal condition before receiving treatment with injection of NaCl 0.9% for the control group and DBL2β-PfEMP1 recombinant protein at doses of 100, 150, and 200 µg for treatment groups.

The DBL2β-PfEMP1 recombinant protein was produced in *Escherichia coli* BL21(DE3). It started by culturing the clone in Luria-Bertani media at 37 °C with a shaking incubator until reaching an optical density of 0.6-0.8 at 595 nm wavelength. The culture was inducted using isopropyl β-d-1-thiogalactopyranoside (IPTG), and recombinant proteins were extracted using a sonicator by adding lysozyme. Then, they were purified using NI-NTA resin with elution buffers containing imidazole 60 and 100 mM. The purified protein was then quantitatively analyzed using Bradford protein assay and qualitatively by SDS-PAGE.

The treatment was done by subcutaneous injection on days 0, 21, and 42. The DBL2β-PfEMP1 recombinant protein was mixed with the *complete Freund's adjuvant* (CFA) in the primary injection (day 0) and the *incomplete Freund's adjuvant* (IFA) in the secondary injections (day 21 and 42) in a 1:1 ratio. On day 56, the rats were euthanized with *ketamine-xylazine*, and spleens were taken for histopathology preparation. The slides were prepared using a microtome and stained using hematoxyline-eosin (HE).

The white pulp diameter was observed using the *Olympus CX23LED* microscope with a 100× magnification. Five visual fields were selected using the zig-zag method and documented using the Optilab camera. Diameter measurements were done using the *ImageJ* application. The white pulp diameter was calculated by adding the longest diameter (transversal diameter) and the perpendicular diameter, then divided into two. Two observers performed the examination.

The reliability of data was determined by the Cronbach alpha test.

The data were analyzed using SPSS. Statistical analysis was performed using the Saphiro-Wilk normality test, Levene homogeneity test, and One-way ANOVA test followed by the posthoc Bonferroni test. This study uses a confidence interval of 95%.

RESULT

The features of spleen histology are presented in Figure 1.

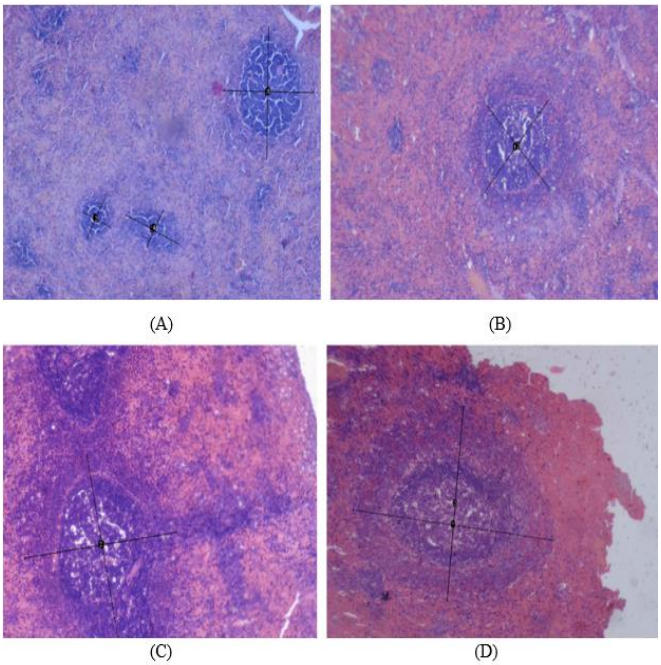

Figure 1. Histology appearance of the spleen with white pulp in all groups stained with hematoxylin-eosin (HE) and observed by microscope at 100x magnification. (A) control group, (B) treatment group 100 µg (C) treatment group 150 µg, and (D) treatment group 200 µg.

In Figure 2, the average number of spleen-white pulp diameters was displayed for your consumption. The fact that the data were reliable was demonstrated by the fact that the Cronbach alpha test yielded a value of α=0.985 (Sig.>0.7). The observation was carried out by two persons.

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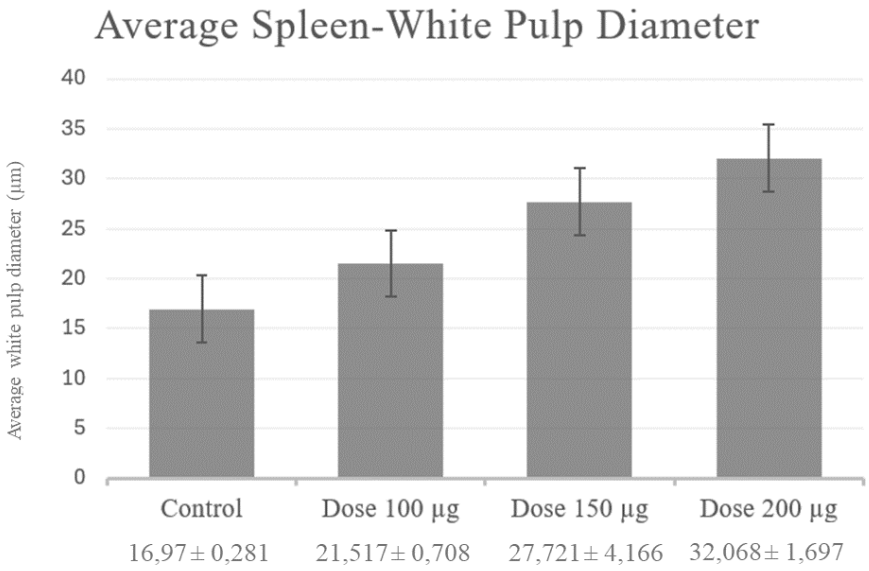

Figure 2. The average number of spleen-white pulp diameter in all groups. The white pulp diameter is increased with the increasing DBL2β-PfEMP1 recombinant protein dose.

The Saphiro-Wilk and Levene tests for each group resulted in p>0.05, meaning all data were normally distributed and homogenous. The one-way ANOVA test showed p=0.001, indicating a significant difference between groups. Further analysis using a posthoc Bonferroni test resulted in a significant difference between a control group and treatment group 150 µg and 200 µg, and a group 100 µg and 200 µg (Table 1).

Table 1. The post hoc Bonferroni test results

	Control	Dose 100 µg	Dose 150 µg	Dose 200 µg
Control		0.306	0.013*	0.002*
Dose 100 µg	0.306		0.114	0.027*
Dose 150 µg	0.013*	0.114		0.527
Dose 200 µg	0.002*	0.027*	0.527	

*= significantly different groups (p<0.05)

DISCUSSION

The study aimed to determine the effect of DBL2β-PfEMP1 recombinant protein injection on the spleen-white pulp diameter. An established approach for the manufacture of the recombinant protein DBL2β-PfEMP1 has been established through previous studies. The use of recombinant protein has some advantages regarding the process and cost. Furthermore, studies have reported the immunogenicity of the protein by inducing IgG and CD4+.[16]

There was an increase in white pulp diameter post-protein injection, along with the increasing protein dose, and the widest diameter was observed in a dose of 200 µg. The results were supported by *a posthoc Bonferroni* test, which showed significant differences between this group and control and a group dose of 100 µg.

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The DBL2β-PfEMP1 recombinant protein is a protein with a molecular weight of 72 kDa[8], while a protein with a molecular weight of more than 8 kDa is potentially antigenic and immunogenic. In this study, the DBL2β-PfEMP1 recombinant protein is subcutaneously injected, which further reaches the spleen through the circulatory system and induces the immune system. The increase in spleen-white pulp diameter indicated an induction of immune response. An acute immune response to antigens may increase cellularity in the B-cell areas and increase secondary follicles with prominent germinal centers. Immature B cells, or immunoblasts, will proliferate in response to antigenic stimuli. They will mature into immunoglobulin-producing plasma cells and migrate into the red pulp.[11,12] The proliferation of B cells will be observed as the increased white pulp diameter. Furthermore, the DBL2β-PfEMP1 recombinant protein could activate central memory T cell and T-dependent B cell germinal center resulting in antibody production in response to serial recombinant protein injection.

Spleen is a remarkable lymphoid organ that integrates innate and adaptive immunity by facilitating the interaction between *antigen-presenting cells* (APCs) and lymphocytes and is responsible for removing blood-borne pathogens and eliminating old and abnormal erythrocytes from circulation.[11,12] The white pulp, which contains nodular and diffused B and T lymphocytes, macrophages, and dendritic cells, allows the formation of specific immune responses to protect the body from disease, making it a vital role in the normal immune response to infection. Changes in the cellular composition in this area after exposure to immunomodulatory agents such as the recombinant protein DBL2β-PfEMP1, which causes activation of T lymphocytes and, in turn, will activate B lymphocytes in the follicle, converting them to plasma cells, which then produce IgM and further IgG antibodies. The activation also implied T and B cell proliferation. This prolifération can increase the diameter of the white pulp.[13]

The *antigen-presenting cell* (APC) can recognize the recombinant protein DBL2β-PfEMP1 acting as an antigen. APC will present antigen in *major histocompatibility class I* (MHC I) and *major histocompatibility class II* (MHC II) and stimulate cytokines to call other immune cells. Th1 produces cytokines like *interferon-gamma* (IFN-γ) and *tumor necrosis factor-alpha* (TNF-α) that activate macrophages, monocytes, and NK cells in the cellular immune response. Th2 produces cytokines such as IL-4, IL-5, IL-6, and IL-10 that activate the humoral immunity response. IL-4 and IL-5 stimulate B cell proliferation and differentiation into plasma cells to produce specific antibodies to the PfEMP1 antigen. When an immune response occurs, APC releases various pro-inflammatory and anti-inflammatory cytokines that can trigger effects on various organs.[14,15] Antibodies to the DBL2β-PfEMP1 recombinant protein have a crucial role in preventing cytoadherence, which is a factor causing severe malaria. Previous research has shown that the recombinant proteins DBLβ2-PfEMP1 have immunogenic properties that can stimulate the production of IgG-specific antibodies and CD4+ T lymphocytes in Wistar rats.[16]

The finding of the study reinforces the immunogenicity of the DBL2β-PfEMP1 recombinant protein as the key requirement of vaccine candidates. Previous studies showed the outcome of an increase of IgG and CD4+ cells after recombinant protein injection, and this study indicated the possible mechanism of recombinant protein to induce immune response by increasing the spleen-white pulp diameter. However, the study has a limitation in its examination scope, as it only covers a morphological parameter, particularly splenic-white pulp, and does not incorporate histochemical markers. Therefore, future studies on the effect of DBL2β-PfEMP1 recombinant protein on the spleen could include more comprehensive morphological parameters and biochemical markers.

Vaccine development is a long way to go, especially for malaria vaccines with the complexity of *Plasmodium spp* life cycle as the

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causative agent. This study result is part of the pre-clinical stage of malaria vaccine development by emphasizing the immunogenicity of the protein. Further pre-clinical studies such as the safety, optimal dose, toxicity effect, formulation, and development of scalable, efficient, and reproducible manufacturing processes should be conducted before moving to a clinical study to find out the safety and efficacy in humans and further regulatory review and approval.

CONCLUSION

The present study concluded that the injection of 100, 150, and 200 µg DBLβ2-PfEMP1 recombinant protein increased the spleen-white pulp diameter in Wistar rats, and a dose of 200 µg resulted in the highest increase in the white pulp diameter. The results emphasize the DBLβ2-PfEMP1 recombinant protein is immunogenic, so it is a potential malaria vaccine. Further studies for the development of vaccines is needed.

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DECLARATIONS

Author contribution. ES: conceptualization, study design, data analysis, funding, manuscript preparation, and final draft. NAS: laboratory work, data analysis, initial draft. IFK: data analysis, manuscript final draft. RD: laboratory work, data analysis, manuscript final draft. SR: laboratory work, statistical analysis, manuscript final draft

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Conflict of interest. The authors declare no conflict of interest.

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