ORIGINAL ARTICLE

Effect of High Dose DBL2β-PfEMP1 Recombinant Protein on Wistar Rats Liver's Structure and Function as Malaria Vaccine Candidate

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ABSTRACT

Introduction: One prominent candidate for malaria vaccine is the Duffy-binding-like domain of *Plasmodium falciparum* erythrocyte membrane protein-1 (DBL2β-PfEMP1) because of its role in malaria pathogenesis. Studies showed that DBL2β-PfEMP1 recombinant protein induces specific IgG and CD4+ production. This study aimed to determine the effect of high dose DBL2β-PfEMP1 recombinant protein injection on livers' histological structure and function in Wistar rats. **Methods:** This was a true experimental study with a post-test-only control group design. Twelve Wistar rats weighing 150-250g were randomly divided into control and treatment groups. The control group was injected with 0.9% NaCl, and the treatment group was injected with 750µg DBL2β-PfEMP1 recombinant protein. Injection was performed once on day 0, and blood samples were taken on days 5, 7, and 14 for SGOT and SGPT measurement. Termination was conducted on day 14, and the liver was prepared for histological examination using Manja Roenigk's scoring. **Results:** The average of SGOT and SGPT levels on days 5, 7, and 14 in the control group is higher than in the treatment group, but statistical analysis using the Mann-Whitney test showed p=0.67, p=0.48, and p=1.00 for SGOT and p=0.39, p=0.24 and p=1.00 for SGPT. The Manja Roenigk score for hepatocytes revealed that the treatment group had a lower score compared to the control group with the Mann-Whitney test p=0.08. However, both groups presented normal liver histopathological features. **Conclusion:** The high-dose DBL2β-PfEMP1 recombinant protein injection does not affect liver structure and function, indicating the protein's safety for further malaria vaccine development.
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INTRODUCTION

Malaria is one of the global health problems, with an estimated 249 million cases in 85 endemic countries in 2022. The number was 5 million increased compared to 2021. Malaria deaths increased by 10% in 2020 compared to 2019, to an estimated 631.000 globally (1). The percentage of total malaria deaths in under 5 years has constantly decreased to 76% over the last 23 years. In the WHO-South-East region, malaria deaths decreased by 77% in 2022, where India and Indonesia accounted for approximately 94% of all malaria deaths in this region (1). WHO recommended the malaria vaccine as one of the strategies to eradicate and control malaria. However, the development of a malaria vaccine is challenging due to the complexity of the parasite-causing malaria, including its life cycle, biology, genome, and parasite's evasion of the human immune system (2, 3).

Five Plasmodium parasite-causing malaria in humans are *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale*, and *Plasmodium knowlesi* (4). Among five, *P. falciparum* was responsible for more than 50% of malaria cases worldwide, and it resulted in more severe clinical manifestations leading to death. Thus, research has been conducted to develop a vaccine against P. falciparum for malaria control and possible eradication (1).

After almost 60 years of research on malaria vaccine development, there are only two malaria vaccines approved by WHO to use in endemic countries (5). The

first is RTS,S/AS01 to use in moderate to high malaria transmission, especially in African countries. This vaccine was approved in October 2021 (6). The second is R21/Matrix-M to prevent malaria in children living in areas where malaria is a major health problem (7).

Malaria vaccines are classified based on the parasite's targeted developmental stage, i.e., pre-erythrocytic vaccines, commonly known as anti-infection vaccines, erythrocytic vaccines, are also referred to as blood-stage vaccines, and transmission-blocking vaccines, which are targeted to block the transmission from human to mosquito, so prevent malaria spreading (5). The two approved malaria vaccines were developed based on the circum-sporozoite (CSP) protein. The ultimate goal is to activate the immune system against the parasite as soon as it enters the human body, preventing the parasite from invading hepatocytes and impairing the parasite's development, and invading erythrocytes. The RTS,S/AS01 has 35-56% efficacy with a 4-dose regimen, while the R21/Matrix-M showed 67-75% efficacy (6–8).

Studies on the development of erythrocytic vaccines are continuously performed, and the efforts focus on antigens or constructs that induce cross-reactive immune responses (9)(10). Several blood-stage antigens have already been studied, one of them is *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP1), which has a vital role in the pathogenesis of severe malaria. PfEMP1 is a complex protein that is secreted during the erythrocytic cycle and deposited on the surface of the infected erythrocyte (11, 12). It is composed of extracellular and intracellular parts. The extracellular part has two major domains, i.e., cysteine-rich interdomain regions (CIDRs), which are classified into five main classes: α, β, γ, δ, and pam, and Duffy-binding-like (DBL) domains, which are divided into seven main classes: α, β, γ, δ, ε, ξ, χ. Each class of domain has the capacity to bind specific receptors on the host cells. The CIDR1α has the capacity to bind Cluster of Differentiation (CD) 36, Endothelial Protein C Receptor (EPCR). and immunoglobulin (IgM), DBL1βcan bind complement receptor (CR)-1, DBL2β binds intracellular adhesion molecule-1 (ICAM1) (13–16). Studies demonstrated that specific antibodies to PfEMP1 have been linked to a reduction in severe malaria (11, 17, 18).

One of the well-studied erythrocytic vaccine candidates is the DBL2β domain of PfEMP1 (DBL2β-PfEMP1), which has an essential role in severe malaria pathogenesis. Previous study showed the capacity of the DBL2β-PfEMP1 protein to induce humoral and cellular immunity (19). The following step is to investigate the safety of the protein to the vital organs, particularly the liver, as the main metabolic organ (20)(21). Currently, no preclinical safety studies involving the liver have been carried out in relation to the DBL2β-PfEMP1 protein as a potential malaria vaccine. Therefore, this study aimed to determine the effect of high-dose DBL2β-PfEMP1 recombinant protein injection on the liver's histological structure and function in Wistar rats.

MATERIALS AND METHODS

Sample and Ethical Clearance

The study was conducted at the Laboratory of Biology and Biotechnology of the Center for Development of Advanced Science and Technology, Laboratory of Biochemistry and Laboratory of Histology, Faculty of Medicine, and Animal Laboratory of the Faculty of Dentistry, University of Jember. The study has been approved by the Ethical Committee for Health Research of the Faculty of Medicine, University of Jember, no. 1934/UN25.8/KEPK/DL/2023.

This study used Wistar rats (Rattus norvegicus) aged 6-8 weeks and weighing 150-250g as the animal samples. The sample number was calculated using the resource equation. Wistar rats were acclimatized to the laboratory conditions for two weeks.

Production and Injection of DBL2β-PfEMP1 Recombinant Protein

The DBL2β-PfEMP1 recombinant was produced in Escherichia coli BL-21(DE3) cells, as previous study (19). The protein was purified using NiNTA resin based on manual procedures. The purified protein was calculated using the Bradford protein assay at 595 nm. It was 72 kDa protein (19). The purified protein was calculated by entering the absorbance value based on the formula y=0.0078x-0.0703 (R2=0,9962). The concentration of purified protein was 2 µg/µL.

As many as 12 rats were divided into control and treatment groups. Each rat in the control group was injected with physiological saline NaCl 0.9%, while each rat in the treatment group was injected with 750µg/200gBW purified DBL2β-PfEMP1 recombinant protein and Complete Freund Adjuvant (CFA) with a ratio 1:1. The injection was performed subcutaneously on day 0. Blood was collected on days 5, 7, and 14 for SGOT and SGPT examination using the ELISA method. On day 14, rats were euthanized using ketamine and xylazine, and liver slides were prepared for histological examination using Haematoxyline-eosin staining.

Histological examination

Histological sections were taken from the liver of each rat at the median lobe of approximately 0.2 cm x 0.2 cm. They were fixated using formaldehyde solution, dehydrated in absolute alcohol, cleared in xylene twice, and infiltrated in 2 changes of paraffin wax in the automatic tissue processor, embedded in molten paraffin wax, and then sliced using a semi-automatic rotary microtome. The sections were stained using Haematoxyline-Eosin.

Slides were examined using an Olympus microscope

CX21LED, while each part was subjected to observation by previously documented using the Optilab 3.0 camera with 400x magnification. Five visual fields in zone-3, around the central vein, and 20 hepatocytes per visual field area were documented for each slide. So, a total of 100 cells was chosen (22). The hepatocyte score was classified into 4 grades: Grade 1: normal hepatocytes with the characteristics of polygonal cells, homogeneous red cytoplasm, and cell membranes with clear boundaries; Grade 2: parenchymal degeneration with the characteristics of steatosis condition with swollen cells, cloudy cytoplasm, and the appearance of granules in the cytoplasm; Grade 3: hydropic degeneration with the characteristics of swollen cells and pale cytoplasm with vacuolization; and Grade 4: necrosis hepatocyte with the characteristics of shrunken cell nuclei with a darker color (pyknotic), destroyed cell nuclei to form fragments (karyorrhexis), or chromatin and dissolved cell nuclei (karyolysis) (23, 24). The examination was performed by two observers and supervised by a competent medical doctor. The average score from 2 observers was presented as the result.

SGOT and SGPT examination

The blood of rats was collected from the tail and put in the EDTA tube. Blood sera were proceeded by centrifugation at 3.000 rpm for 15 min. The biochemical parameters like serum enzymes Serum Glutamic Oxaloacetic Transaminase (SGOT, IU/L) and Serum Glutamic Pyruvic Transaminase (SGPT, IU/L) were examined using a DIALAB kit. The value was determined by its absorbance measured using a spectrophotometer with a wavelength of 340 nm.

Data analysis

Data reliability was tested using Cronbach's alpha. It resulted in p=0.834 (p>0.7), indicating that the data were reliable. Data were presented univariate and analyzed using the Mann-Whitney test with a significance of p<0.05.

RESULTS

Liver structure

The observation of liver structure was performed around the central vein, which was potentially affected by any substances. The histological appearance of the liver in control and treatment groups was presented in Figure 1. Majority of hepatocytes had a normal structure with a polygonal shape, red and homogeneous cytoplasm, clear boundary cell membranes, and no enlargement. A tiny portion of hepatocytes in the control group showed characteristics of parenchymatous and hydropic degeneration with swollen, cloudy, and granular cytoplasm. There were no necrotic hepatocytes in both groups.

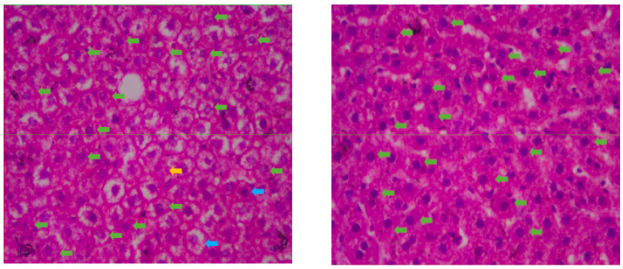

Figure 1: Histological examination of the rat's liver using 400x magnification. Parenchymatous and hydrophic degeneration were observed in the control group. A. control group; B: treatment group. green arrow: normal; yellow arrow: parenchymatous degeneration; blue arrow: hydrophic degeneration; black arrow: necrosis.

The average number of total liver structural damage scores is presented in Table I. It was calculated by two examiners with the Cronbach alpha test was 0.83 (α>0.7), indicating the data reliability. The Mann-Whitney test showed a significance value of 0.08, which indicated no significant difference between the control and treatment groups (Table 1).

Liver Function

The liver function was measured biochemically using serum Glutamic Oxaloacetic Transaminase (SGOT) and Serum Glutamic Pyruvic Transaminase (SGPT) for days 5, 7, and 14 after protein injection. There was a slight increase in SGOT level on day 7 in the control group but no increase in the treatment group. The SGPT level increased along with the day in both groups. However, the SGPT in the control group is higher than in the treatment group (Table I). And the value is higher than the normal range of 8-12 weeks-rat liver enzymes, i.e., 87.00-114.00 IU/l for aspartate aminotransferase (AST) or SGOT, and 28.00-40.00 IU/l for alanine aminotransferase (ALT) or SGPT (25). And the statistical analysis revealed no significant differences between groups with p>0.05, i.e., p=0.67, p=0.48, and p=1.00 for SGOT, and p=0.37, p=0.24, and p=1,00 for SGPT, respectively, for days 5, 7, and 14.

Table I: The Manja Roenigk Score and liver function test

Group	Manja Roenigk Score ± SD	SGOT level (IU/L)			SGPT level (IU/L)		
		Day 5	Day 7	Day 14	Day 5	Day 7	Day 14
Control	1.09 ± 0.03	128.25±38.40	153.80±27.15	133.18±39.96	57.89±6.63	70.46±2.14	84.77±17.68
Treatment	1.03 ± 0.33	116.08±33.21	116.05±52.30	114.68±40.94	43.74±23.02	46.06±25.28	74.52±32.04
p-value	0.08	0.67	0.48	1.00	0.39	0.24	1.00

DISCUSSION

This study analyzed the histological features and biochemical parameters of the liver post-injection of the purified DBL2β-PfEMP1 recombinant protein as part of the acute toxicity test of the protein as a malaria vaccine candidate. As a metabolic organ, the liver will metabolize all substances that enter the body (26). Hepatocytes comprise approximately 85% of the liver mass and play an essential role in diverse biochemical and metabolic functions (27). Histological features were observed at the median lobe, which is the biggest lobe and has many branches of hepatic vein and portal vein. The median lobe received blood supply from two prominent portal veins, i.e., left and right portal veins, and drainage by three hepatic veins, i.e., medial left hepatic vein, media middle hepatic vein, and medial right hepatic vein, implying its sensitivity to any possible changes (28). The examination was performed at the centrilobular or the third zone of the liver, where the hepatocytes were the closest to the central vein and have lower oxygen and nutrients but higher metabolites, and have higher cytochrome P450 enzyme to metabolize various substances, thus, are at the high risk of damage (28).

In this study, the control group was injected with 0.9% NaCl as an isotonic saline or a physiological solution, while the treatment group was injected with the purified DBL2β-PfEMP1 recombinant protein and Complete Freund Adjuvant (CFA). CFA is the most widely used and most effective adjuvant for polyclonal antibody production, which consists of heat-killed Mycobacterium tuberculosis, nonbiodegradable mineral oils, and emulsifier to increase the immunogenicity responses. The antigen, which is emulsified in the oil, is released slowly to give a prolonged exposure to the immune system, and Mycobacterium tuberculosis can cause an inflammation which further attracts macrophages and other cells to the injection site. Study in animal model, 0.1 ml CFA can increase SGOT and SGPT level due to inflammation and potentially liver damage (29).

The study showed that there is no significant difference in the liver's histological appearance between the control and treatment groups. The average Manja Roenigk's score was 1.09 for the control group and 1.03 for the treatment group (Table 1), indicating normal histological appearance, as confirmed by the statistical analysis, which showed no significant difference between control and treatment groups. It reflected that the high dose of DBL2-PfEMP1 recombinant protein did not affect the hepatic structure. A very small portion of parenchymatous and hydropic degeneration was observed in the control group, and no hepatocyte necrosis was observed in either group. Parenchymal degeneration is associated with ATP depletion because of exposure to foreign substances, which results in protein transport failure. The further level of damage is hydropic degeneration, which is associated with ATP depletion and changes in the Na+ and K+ ions channel to absorb more water into the cell, so cells are unable to eliminate water and cell organelles, resulting in water accumulation (30, 31). The last stage of cell damage is necrosis; the process is classified into three stages, i.e., pyknosis, karyorrhexis, and karyolysis (32, 33). Necrosis is associated with ATP depletion with the increasing anaerobic glycolysis as the compensation mechanism, causing a decrease of glycogen and an increase of lactic acid and further changes in pH and cell nucleus resulting in nuclear chromatin compaction, nucleus rupture, and fade (30, 31). A previous study on circum-sporozoite protein (FMP013) as a vaccine candidate showed that the protein is a harmless substance for animal use (34). Another study on the SARS-Cov-2 vaccine using recombinant protein had similar results, with no significant histopathological changes observed in the liver of the animal model (35).

Hepatocyte damage occurs due to various causes, including drugs, infections, oxidative stress, or autoimmune, causing phenotype changes, microenvironment adaptation, and altering the surrounding cell population (27). The DBL2-PfEMP1 recombinant protein is a foreign substance that could induce hepatocyte changes. However, this study demonstrated that the protein did not cause the hepatocyte changes.

The biochemical examination to determine the liver function test was performed using SGOT and SGPT levels. The liver function test can determine the vaccine safety. The elevated liver enzymes indicated potential hepatotoxicity. As the malaria infection itself can cause an increase of the liver enzymes such as SGOT and SGPT, possibly due to the host immune response to malaria infection., assessing the liver function test could also be essential to determine the vaccine efficacy or potential drug-induced liver injury (DILI). The study showed a minimal SGOT elevation on day 7 and a decline on day 14 in the control group but no elevation in the treatment group. The SGPT level elevated along with the day in both groups, with overall SGPT in the control group being higher than in the treatment group (Table I). Although the values of SGOT and SGPT were higher than the normal range for rats 8-16 weeks, which is 74-143 IU/L with a mean of 105+20 IU/L for SGOT and 18-45 IU/L with a mean of 28+7 IU/L for SGPT, but those normal range was observed from the Crl:WI(Han rat. Furthermore, there is a general agreement among toxicologists and clinical pathologists that the concurrent control data for the indicators are the best for comparing and for determining a conclusive findings and interpretations (25). Overall, the differences between groups for SGOT and SGPT on days 5, 7, and 14 are not significant ($p>0.05$). Thus, implied the safety of the DBL2β-PfEMP1 recombinant protein to the liver cells. The SGPT is considered more sensitive and specific than

SGOT as the liver function indicator because SGOT can be found in other organs, such as the heart and muscles. The increase in SGPT is more suggestive of liver damage because it is primarily found in the liver.

CONCLUSION

This study found that injection of dose 750µg/200gBW DBL2β-PfEMP1 recombinant protein in rats does not affect liver structure and function as displayed by normal histological appearance and no significant difference of SGOT and SGPT level between control and treatment groups. Therefore, it implies the protein's safety for further malaria vaccine development.

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