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Evaluation of Kidney Proximal Tubule Following Immunization with Plasmodium falciparum CIDR1α-PfEMP1 Recombinant Protein in Rats

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Abstract

Malaria vaccines are continuously explored as an approach to eradicate malaria. The cysteine-rich interdomain region 1α-Plasmodium falciparum erythrocyte membrane protein 1 (CIDR1α-PfEMP1) is an antigenic protein that can bind to the endothelial protein C receptor (EPCR) and CD36, resulting in microvascular obstruction. The PfEMP1-induced antibody can induce antibodies, reducing the severity of malaria risk by impeding cytoadherence and destructing rosette formation. Preclinical safety testing is an important step of vaccine development,including safety testing of the kidney as the main excretory organ. The proximal tubule has the most mitochondria to support its main role in reabsorption and excretion, making it prone to oxidative stress caused by foreign substances. This study aimed to evaluate kidney proximal tubule cells after CIDR1α-PfEMP1 immunization in rats. This study was conducted at the Laboratory of Biology Molecular and Biotechnology, Faculty of Medicine, University of Jember. Eight rats were injected subcutaneously with 150 µg of the protein and four rats were injected with 0.9% NaCl on days 0, 21, and 42. The rats were euthanized on day 56. The kidney histopathological slides were stained using Hematoxylin-Eosin (HE) and the necrotic proximal tubule cells were counted at five (5) visual fields (100 cells/visual fields). The average number of necrotic cells of the control and the treatment groups were 0.125±0.25 and 2.438±2.5972 while the Mann-Whitney test showed a significance value of p=0.12, indicating no significant difference between the control and treatment groups. In conclusion, there is no change in the kidney histopathology based on the proximal tubule necrotic cell count after CIDR1α-PfEMP1 immunization in rats.

Keywords: CIDR1α, histopathology, kidney, malaria, PfEMP1

Introduction

Malaria remains a significant health concern with high mortality rates, particularly in tropical and subtropical regions such as Africa, Asia, and South America. Based on data from the 2022 World Malaria Report, there were approximately 241 million cases of malaria worldwide, resulting in 619,000 deaths globally.[1] The majority of malaria-related deaths are caused by severe malaria complications stemming from *Plasmodium falciparum* infections.[2] The pathogenesis of severe malaria involves cytoadherence and rosetting processes mediated by *Plasmodium falciparum Erythrocyte Membrane Protein-1* (PfEMP1), an adhesion molecule encoded by the *var* gene, which consists of 2 exons, i.e., exon 1 and exon 2, and separated by an intron. The molecule is encoded mostly by exon 1, which is polymorphic, 3.5–9 kb in size, and is expressed on the surface of *P. falciparum*-infected erythrocytes in the knob protrusion region. Exon 1 encodes an extracellular domain consisting of an N-terminal segment (NTS), several Duffy-binding-like (DBL) domains, several cysteine-rich interdomain region (CIDR) domains, and a transmembrane segment (TMS). The CIDR domain flanked by two DBL domains is consistently found in the head structure of the *var* gene as a conserved head structure. Exon 2 is relatively short at 1–1.3 kb and encodes the intracellular acidic terminal segment (ATS) domain.[3]

The CIDR1α-PfEMP1 specifically binds

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to *endothelial protein C receptor* (EPCR) and CD36 receptors, playing key roles in severe malaria pathogenesis. Infected erythrocytes can adhere to endothelium, platelets, or uninfected erythrocytes resulting in microvascular obstruction in vital organs, ultimately leading to organ failure.[4] Protein C activation will regulate coagulation, blood vessel inflammation, and increase blood vessel permeability so that PfEMP1 binding to EPCR triggers inflammation and coagulation of cerebral blood vessels. Pathological processes in the brain will result in brain edema which then causes clinical manifestations of cerebral malaria. A previous study showed that the proportion of the *var* gene encoding the CIDR1α bound to EPCR was higher in severe malaria patients than in uncomplicated malaria patients, with a median percentage of 54.1% vs. 7.4%.[5]

One potential strategy in malaria control is the malaria vaccine. In 2015, the Malaria Vaccine Initiative (MVI) launched the first-generation malaria vaccine based on the *Circumsporozoite Protein* (CSP), named RTS,S/AS01, with a vaccine efficacy of 34.8% against severe malaria.[6] However, due to the complexity of *P. falciparum* antigen proteins throughout its life cycle, malaria vaccine development encounters significant challenges. Further research utilizing other *P. falciparum* antigen proteins is necessary. We have selected CIDR1α-PfEMP1 as a protein target for peptide-based malaria vaccine development. The previous study demonstrated that injection of 150 µg CIDR1a-PfEMP1 in rats increased the IgM level after primary injection ($p<0.05$), IgG level after primary and secondary 1 and 2 injections ($p<0.05$), and CD4+ level after secondary 2 injections.[7] The production of these antibodies is expected to prevent the binding of CIDR1α domain to EPCR and CD36 receptors. Another previous study revealed that antibodies induced by DBL2β-PfEMP1 reduced the risk of severe malaria by 37% through blockade of cytoadherence and disruption of rosette formation.[8]

Vaccine development is a long way process, from laboratory, preclinical and further clinical steps. After choosing the immunogenic candidate, the next step is the safety assessment. Safety assessments of vaccine candidates are necessary to ensure that immune responses and pharmacodynamic effects post-vaccination do not endanger vital organs, such as the kidneys. The kidney is an essential organ responsible for blood filtration and the excretion of toxins and metabolic waste, making it vulnerable to exposure to foreign substances and the risk of pathological changes. The renal proximal tubules are particularly susceptible to free radicals resulting from toxic substances, as they have a high level of cytochrome P450.[9]

Histopathology is recognized as the single most appropriate screen for evidence of drug-induced kidney injury. Three of the most common renal tubular changes associated with drug-induced kidney injury are vacuolation, degeneration, and necrosis.[10] Previous studies have reported cellular damage and narrowing of the proximal tubules following herbal compound administration, as well as proximal tubule cell necrosis after oral administration of *Archidendron pauciflorum*.[11] To date, no safety evaluations have been conducted on the kidneys in relation to the CIDR domain of PfEMP1, a potential malaria vaccine candidate. Accordingly, this study was designed to evaluate histopathological changes in renal proximal tubule cells following immunization with CIDR1α-PfEMP1 in rats.

Methods

This study was conducted at the Laboratory of Molecular Biology and Biotechnology, Faculty of Medicine, University of Jember, from June to November 2023. Ethical approval was obtained from the Ethical Committee of the Faculty of Medicine, University of Jember (Ref. No. 1826/H25.1.11/KE/2023). The recombinant CIDR1α-PfEMP1 protein was produced and purified prior to administration. The protein was injected into experimental animals, and histopathological assessment of proximal tubule kidney cell nucleus necrosis was performed following animal termination.

The CIDR1α-PfEMP1 domain amplification was conducted before the ligation of the target gene into the pET-30a (EMD Biosciences, catalog number 69909-3) plasmid and subsequently transformed into *Escherichia coli* BL21 (DE3) cells (Thermo Fisher Scientific Inc, catalog number C600003). All steps were conducted based on the previous studies.[12] *Escherichia coli* BL21 (DE3) cells were cultured in Luria–Bertani (LiofilchemS.r.l., Teramo, Italy, cat. number 610245) containing 50 µg/mL kanamycin (Thermo Fisher Scientific Inc., Waltham, MA, USA, cat. number 11815024) at 37°C by shaking at 190 rpm until reaching OD_{600} 0.6-0.8. Induction of recombinant protein production was done using 0.3 M isopropyl-D-1-thiogalactopyranoside (IPTG) (Promega

Co., Madison, WI, USA, cat. number V3955) by shaking at 190 rpm at room temperature (RT) for eight hours and was harvested through centrifugation afterward. The harvested pellet was solubilized using an extraction buffer (300 mM NaCl, 50 mM NaH_2PO_4, and 5 mM imidazole in a pH of 8.0). The lysate was incubated using 1 mg/mL lysozyme (VWR International LLC, Radnor, PA, USA, cat. number 470301-618) for 30 min at 4°C prior to sonication.[7]

The crude protein was purified by Ni-NTA resin (Qiagen, Hilden, Germany, cat. number 30210) based on the affinity chromatography method, washed with 1 mL of wash buffer I (300 mM NaCl, 50 mM NaH_2PO_4 and 20 mM imidazole; pH 8.0) and wash buffer II (300 mM NaCl, 50 mM NaH_2PO_4, and 50 mM imidazole; pH 8.0). A stepwise manner was used to elute the protein with elution buffer (300 mM NaCl, 50 mM NaH_2PO_4; pH 8.0) containing 80 mM, 100 mM, or 120 mM imidazole (Sigma-Aldrich Co., St.Louis, MO, USA, cat. number I5513-5G). To determine the purified protein, the elution was visualized using SDS-PAGE (Bio-Rad Laboratories Inc., Hercules, CA, USA), and the concentration was measured using the Bradford protein assay (HiMedia Laboratories, Maharashtra, India, cat. number ML106-500 ML) at 595 nm.[7]

Wistar rats (Rattus norvegicus) were used in this study. Sample size determination was carried out using the resource equation method.[13] This method calculates the value "E," representing the degrees of freedom for the analysis of variance (ANOVA). An E-value between 10 and 20 is generally considered adequate. An E-value below 10 suggests the need for more animals to increase the likelihood of obtaining statistically significant results, whereas an E-value above 20 indicates that additional animals are unlikely to enhance statistical power. In the present study, two experimental groups were used, with eight rats per group. The E-value was calculated using the following formula:

E=(number of groups x number of experimental animals)-number of groups

E=(2x8)-2

E=16-2

E=14

An E-value of 14 was obtained, which falls within the acceptable range for adequate sample size. However, in alignment with the 3Rs principle—particularly the reduction principle—the number of animals in the control group was limited to four, representing half the number used in the treatment group. This adjustment was made to minimize animal use while still maintaining sufficient statistical power. Reducing the number of animals in the control group is supported by ethical, practical, and research efficiency considerations.[13]

Twelve rats were divided into two groups, i.e. a treatment group consisting of 8 rats injected with recombinant CIDR1α-PfEMP1 protein at a dose of 150 µg and a control group consisting of 4 rats injected with 0.9% NaCl solution subcutaneously on days 0, 21, and 42.[14] Subcutaneous administration was chosen to increase the possibility of encountering Langerhans cells whose role as an antigen-presenting cell thus increases the generated immune response. In addition, the recombinant protein has a molecular weight >20 kDa, causing limited transport into the capillaries and predominantly crossing into the circulation system via the lymphatic system accessed through subcutaneous injection.[15]

Recombinant CIDR1α-PfEMP1 protein was mixed with Complete Freund's Adjuvant (Santa Cruz, sc-3727) for the primary injection and Incomplete Freund's Adjuvant (Santa Cruz, sc-24019) for the secondary injection at a 1:1 ratio, respectively. The Freund's Adjuvant was employed to ensure a continuous release of antigens, essential for inducing a robust and enduring immune response. The Complete Freund's Adjuvant (CFA) was administered on the initial injection due to its heat-killed *Mycobacterium tuberculosis* to attract macrophages and other immune cells to the injection site. It will maximize the exposure of the recombinant protein to the antigen-presenting cell to enhance the immune response. The Incomplete Freund's Adjuvant (IFA) has the same compound as CFA without the mycobacteria, and was used for booster immunization without the side effects of CFA.[16]

Rats were euthanized on day 56, and kidney slides were prepared and stained using Hematoxylin-Eosin (HE) for histopathology examination. Kidney slides were observed using an Olympus CX23LED light microscope at a magnification of 400X connected to an Optilab 3.0 camera. Two observers examined by counting 100 proximal tubule cells in the kidney cortex from 5 visual fields using the Fiji ImageJ application with a double-blind technique. The observers have the same interpretation of the characteristics of necrotic proximal tubule cells used as the parameters to count, i.e. pyknosis (shrunken/condensed cell nucleus, denser and darker, loss of chromatin), karyorrhexis (cell nucleus divides into several fragments),

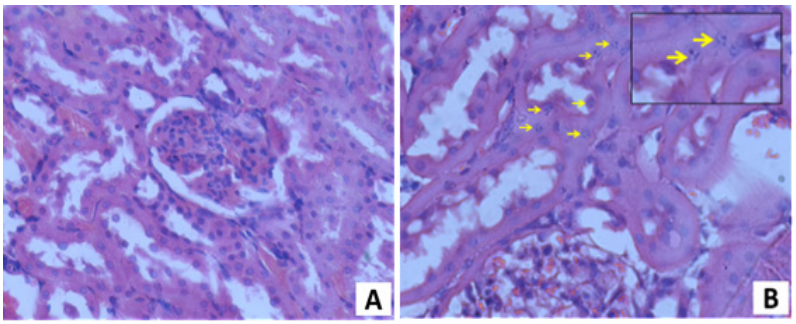

Figure 1 Kidney Histopathological Damage with H.E. Staining (400X). A: control group, B: treatment group with 150 µg CIDR1α-PfEMP1 recombinant protein. Yellow arrow: necrosis of renal proximal tubule cell nuclei, stage of karyorrhexis

and karyolysis (cell nucleus disappears).[11] To measure the consistency among the observers, the Cronbach alpha reliability test was applied. It is a test developed to measure whether the same interpretation and concept were used toward the item within a research test or questions, estimating the reliability to ensure the test validity. This statistical test has been used to assess the reliability and consistency among histopathology observers.[17] The Cronbach alpha is expressed as a number between 0 and 1, and α>0.7 is considered acceptable in most research.

Data analysis was performed using SPSS software. The Shapiro–Wilk test and Levene's test were employed to assess the normality and homogeneity of the data, respectively. As the data on proximal tubule cell nucleus necrosis did not meet parametric assumptions, the non-parametric Mann–Whitney U test was applied for statistical comparison between groups.

Results

Qualitative observations of kidney histopathology showed normal features which was simple cuboidal epithelial with intact nucleus, without any vacuolization or granules in the cytoplasm, and no cell enlargement. In addition, some karyorrhexis cells were also observed with the characteristics of fragmented cell nuclei (Figure 1). Those two histopathological characteristics

Table 1 Mean Number of Proximal Tubule Kidney Cells Undergoing Necrosis

Groups	Rats	Necrosis Cells Count		Mean/rat	Mean/group ± SD
		1st Observer	2nd Observer		
Control	1	0	0	0	0.125±0.25
	2	0	0	0	
	3	0	0	0	
	4	1	0	0.5	
Treatment	1	4	3	3.5	2.438±2.5972
(150 µg)	2	8	7	7.5	
	3	4	4	4	
	4	2	2	2	
	5	3	2	2.5	
	6	0	0	0	
	7	0	0	0	
	8	0	0	0	

were observed in both, the normal group and the treatment group, but the karyorrhexis cells were observed more in the treatment group.

The Cronbach alpha test showed a value of α=0.991, indicating that the result of proximal tubule necrotic cell counting was consistent between two observers and that the data obtained were reliable. The average number of proximal tubule kidney cells undergoing necrosis in both groups can be seen in Table 1.

Shapiro-Wilk test results indicated abnormally distributed data ($p<0.05$) and the Levene test showed data inhomogeneity ($p<0.05$). Subsequently, a non-parametric Mann-Whitney test was conducted with a significance level of 0.12 ($p>0.05$). This indicates that there is no significant difference between the control group and the treatment group.

Discussion

This study selected the kidney as the organ to be assessed following immunization with recombinant CIDR1α-PfEMP1 protein. The kidney plays a crucial role in the filtration and elimination of foreign compounds entering the body through urine. Each kidney comprises approximately 1 million nephrons located in the renal cortex. Kidney damage can be caused by toxic compounds, which can be identified through histological structure observations, including tubular cell nucleus necrosis.[10,18]

The renal proximal tubule functions to reabsorb substances still needed by the body, such as glucose, amino acids, and electrolytes. The proximal tubule is the first segment of the nephron which eliminates protein-bound molecules including substances from the glomerulus and systemic circulation and makes up for more than 70% of all absorption and secretion activities.[19] This role requires a high rate of oxidative metabolism, hence making the proximal tubule prone to the toxic effect of foreign substances. Furthermore, the tubular cells have relatively high cytochrome P450 level, which can result in the formation of reactive metabolites.[10] It causes tubular toxicity, prohibiting tubular transport processes, reducing mitochondrial function, and diminishing ATP production – which in turn will increase free radicals, apoptosis, and cell necrosis on the proximal tubule epithelial.[20,21]

Changes in kidney histological structure can be influenced by the type and amount of compounds entering the body and their concentration within tubular cells. Foreign substances initially accumulate in the proximal tubule, and if reabsorbed, these substances pass through tubular epithelial cells at high concentrations, potentially causing structural and functional changes in the kidney.[20]

Qualitative observations of kidney histological preparations in all samples showed normal proximal tubule cells and karyorrhexis characteristics. Microscopic features of cell necrosis include shrunken or wrinkled nuclei, denser and darker appearance, and loss of chromatin known as pyknosis; nuclei divided into several fragments known as karyorrhexis; and disappearing nuclei known as karyolysis. Based on the sequence of damage stages, necrosis begins with pyknosis, followed by karyorrhexis, and finally, karyolysis. Cell death can be caused by various factors that induce abnormal stress, including exposure to necrosis-causing agents such as chemicals, biological agents like viruses, and metabolic disorders.[22] Necrosis is also associated with ATP depletion due to exposure to foreign substances, which subsequently triggers compensatory mechanisms by increasing anaerobic glycolysis. This process leads to decreased glycogen levels and increased lactic acid, resulting in pH changes that further cause chromatin condensation, nuclear fragmentation, and nuclear loss.[23] A previous study showed that proximal tubular cells necrosis indicated a kidney injury that is extensive enough to disrupt the normal function of the kidney and usually accompanied by changes in kidney BUN and creatinine levels.

The results of proximal tubule cell necrosis counting in the control group were 0.125±0.25, and in the treatment group were 2.438±2.5972. Statistical analysis using the Mann-Whitney test showed no significant difference in the number of necrotic cells between the control and treatment groups ($p>0.05$), likely due to the high standard deviation in the treatment group. The high standard deviation in the data results can be attributed to several factors. Firstly, inter-individual variations in immune response among rats contribute significantly to the variability.[24] Secondly, technical differences in determining the visual field chosen and the counted cells for calculations introduce additional inconsistencies. These factors collectively contribute to the observed variability in the data. The insignificant difference between the control and treatment groups indicates that immunization with recombinant CIDR1α-PfEMP1 protein did not result in histopathological changes, specifically

necrosis, in renal proximal tubules. A similar finding of a previous study on *Circum-sporozoite Protein* (FMP013) for malaria vaccine candidates suggested that the compound was safe in animals.[25]

In conclusion, this study found no change in the kidney histopathology based on the counting of proximal tubule necrotic cells after CIDR1α-PfEMP1 immunization in rats *(Rattus norvegicus)*. However, histopathology evaluation cannot be used as a screening method as the diminished kidney function happens before any histopathological alteration. Hence, it is necessary to measure kidney function through serum creatinine examination, identify necrotic renal tubule cells using immunohistochemistry methods, and observe other microscopic kidney structures, such as glomeruli, considering the limitations of this study. To address more specific proximal tubule function, biomarkers such as Kim-1, CysC, β2M levels, α-glutathione S-transferase (α-GST), γ-glutamyl transferase (GGT), and alkaline phosphatase (ALP) are important to measure.

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