Hyperbaric Oxygen Exposure Reduces ICAM-1 And HIF-1α Expression in Brain Endothelial Cells from Experimental Cerebral Malaria Mice

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Abstract

This study aimed to reveal the role of ICAM-1 and HIF-1α in brain endothelial cell of cerebral malaria mice model after exposure to 2.4 absolute atmospheres (ATA) hyperbaric oxygen (HBO). Thirty-nine C57BL/6 mice were divided into three groups: control negative (normal mice without any exposure), control positive (*Plasmodium berghei* ANKA [PbA] infection without HBO exposure), and treatment (PbA infection and exposed to HBO for 10 sessions after the parasite grew). Parasitemia and clinical symptoms were observed every day. Brain tissues were isolated on day 13 post-infection for histopathological and immunohistochemical examination (observed at 400x magnification in 10 visual fields). HBO decreased HIF-1α and ICAM-1 expression in endothelial cells. There was a moderate correlation between HIF-1α and ICAM-1 expression. Ten HBO sessions prevented cerebral malaria, as denoted by the decreased expression of ICAM-1 and HIF-1α in brain vascular endothelial cells from the experimental mice.

Keywords: *Plasmodium berghei ANKA, hyperbaric oxygen/HBO, ICAM-1, HIF-1α.*

Introduction

Malaria is an infectious disease caused by the parasite *Plasmodium* sp. Based on a 2017 World Health Organization (WHO) report, there were 219 million malaria cases that caused 435,000 deaths worldwide[1]. Millions of malaria cases are caused by *P. falciparum*, and approximately 1% of cases will develop into cerebral malaria. Even with adequate antimalarial therapy and intensive therapy, malaria mortality rates still reach 15-20%, and there are long-term neurological deficits in patients who have been declared cured[2]. Cerebral malaria is characterized by neurological abnormalities, seizures, decreased consciousness, and coma. The pathogenesis of this disease is still unclear. However, previous studies showed that one of the causes of brain damage is brain vascular endothelial damage triggered by hypoxic conditions due to vascular obstruction, sequestration of parasitized red blood cells (pRBCs) using vascular endothelial receptors, and excessive inflammation[3].

The main regulator that participates in cell adaptation and survival in hypoxic conditions is hypoxia-inducible factor 1α (HIF-1α)[4]. There is cross-talk between hypoxia and inflammation, where HIF-1α activation becomes a signaling pathway that initiates transcription factors, namely nuclear factor kappa B (NF-κB), which then increases inflammation. Hypoxic conditions during malaria infection will increase HIF-1α expression, leading to NF-κB activation and increased intercellular adhesion molecule-1 (ICAM-1) expression[5]. Various studies suggest that ICAM-1 is the main endothelial receptor in the brain, and it plays a crucial role in the endothelial sequestration process by binding to *P.*

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falciparum erythrocyte membrane protein-1 (PfEMP-1), which is expressed by pRBCs[6].

HBO therapy is the systemic exposure to 100% oxygen in a closed room with a pressure greater than 1 absolute atmosphere[7]. Research on the effect of HBO on malaria infection is very limited. HBO exposure directly increases dissolved oxygen in the blood plasma, a change that can reduce HIF-1α[8] and modulate the inflammatory response, namely by reducing ICAM-1 expression[5]. The purpose of this study was to examine the effect of exposure to 10 sessions of 2.4 ATA and 100% O_2 HBO three times for 30 min on the expression of ICAM-1 and HIF-1α in C57BL/6 mice infected with *P. berghei* ANKA (PbA).

Materials and Methods

This study used a randomized post-test-only control group design. This study was performed at the Clinical Parasitology Laboratory, Faculty of Medicine, Universitas Brawijaya (for infecting the experimental mice), the Hyperbaric Laboratory, Faculty of Medicine, the University of Hang Tuah (for the HBO treatment and euthanasia), and the Anatomic Pathology Department, Faculty of Medicine, Universitas Airlangga (for hematoxylin & eosin [H&E] and immunohistochemistry [IHC] preparation, staining, and observation). The experimental subjects were 39 pathogen-free 7-to-10-week-old C57BL/6 female mice. They were divided into three groups: Group 1 (G1) or control negative (mice were not infected with PbA and did not receive HBO treatment), Group 2 (G2) or control positive (mice were infected with PbA without receiving HBO exposure), and Group 3 (G3) or treatment (mice were infected with PbA and subjected to HBO exposure). PbA isolates were obtained from the Clinical Parasitology Laboratory, Faculty of Medicine, Universitas Brawijaya.

The experimental mice (15-20 g) were obtained from Indoanilab, Bogor, Indonesia. The adaptation and acclimation phases were performed for 1 week in a room that was sterilized one day before conducting the research using ultraviolet light for 2 h. The mice were given sterilized mineral water and food pellets *ad libitum*. The study was approved by the Ethics Committee, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya (No.2KE.070.04.2018).

HBO exposure

G3 mice were subjected to HBO exposure with a pressure of 2.4 ATA and 100% O_2 three times for 30 min with a 5 min resting phase between each exposure[9]. HBO exposure was performed in an animal chamber every morning before examination of parasitemia and the clinical manifestations of cerebral malaria. The temperature during HBO exposure was adjusted to 25ºC. Each G3 mouse was subjected to 10 exposure sessions, beginning on day 3 post-infection (when parasitemia was apparent) until day 12.

Parasitemia examination

Observation of the parasitemia level was performed serially from day 1 to day 12 post-infection by microscopic examination of a blood smear aseptically taken from the tail and stained with Giemsa. Observations and calculations were repeated twice each day and performed by two different observers. The percentage of parasitemia was calculated using the following formula: pRBC/1000 RBC x 100%[10].

Brain tissue Immunohistochemical Stainning

Brain tissues were incised with a 4-µm-thick incision, with the direction of the incision in the coronal field, and stained with hematoxylin-eosin[12]. The immunohistochemical staining process commenced by washing slides with phosphate-buffered saline (PBS, pH 7.4) for 5 min, 3% H_2O_2 for 20 min (endogenous peroxide block), and PBS 3 x 5 min. Non-specific protein binding was blocked using 5% fetal bovine serum (FBS) and 0.25% Triton X-100, and sections were washed with PBS for 5 min. Sections were incubated with monoclonal HIF-1a antibody (Santa Cruz Biotechnology, catalog number: sc-53546) and ICAM-1 (Santa Cruz Biotechnology, catalog number: sc-107) for 60 min, and then washed with PBS 3 x 5 min. Sections were incubated with the chromogen diaminobenzidine (DMB) at 25 °C for 10 min, followed by H&E solution for 3 min, and finally rinsed in running water. HIF-1a and ICAM-1 expression in the brain vascular endothelium was observed in the cerebrum and cerebellum at 400x magnification.

Statistical Analysis

Average values and standard deviations were

determined for all data, and normality and homogeneity were examined using the Shapiro-Wilk and Levene tests, respectively. Multivariate analysis of variance (MANOVA), with post-hoc analysis, was used to determine differences between groups (for normally distributed data). Pearson's correlation tests were conducted to determine the correlation between two variables of normally distributed data. Data that were not normally distributed or homogeneous were analyzed using the Kruskal-Wallis or Mann-Whitney U test to determine differences among groups. All *p*-values <0.05 were considered to be significant, and there was a correlation between the studied variables.

Results and Discussion

Parasitemia appeared on day 2 post-PbA infection. The peak parasitemia level occurred on day 7 post-infection, and it decreased on days 8–12. This phenomenon is probably due to the increased pRBC circulation in the tissue due to the sequestration process or pRBC trapping in capillaries[14]. G3 mice, which received HBO treatment, showed negative parasitemia on days 10-12, while G2 (control positive mice) showed a positive parasitemia level until the last day of observation.

There was a significant difference in HIF-1a expression in the brain endothelial cells between G3 and G2 mice (p = 0.001), but there was no significant difference between G1 and G3 mice (p = 0.258; Figure 1). The average ICAM-1 expression in vascular brain endothelial cells in G3 mice was significantly lower than in G2 mice (p = 0.005). ICAM-1 expression in G3 brain was higher than in G1 brain, but this difference was not statistically significant (p = 0.059; Figure 2). Representative micrographs of HIF-1α and ICAM-1 staining appear in Figures 3 and 4, respectively. Correlation analysis showed a moderate correlation between HIF-1a and ICAM-1 expression in brain endothelial cells (r = 0.507; p = 0.001).

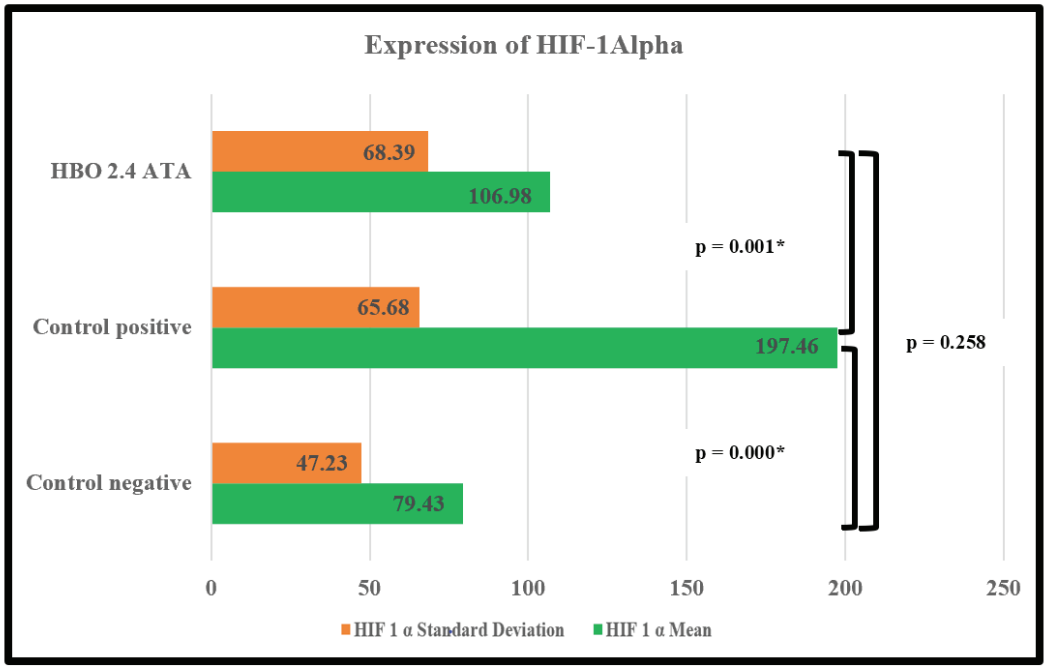

Figure 1. The avarage HIF-1α expression in the control negative (G1), control positive (G2), and HBO treatment (G3) groups. *MANOVA test and post-hoc least significant difference (LSD) test showed a significant difference; $p < 0.05$.

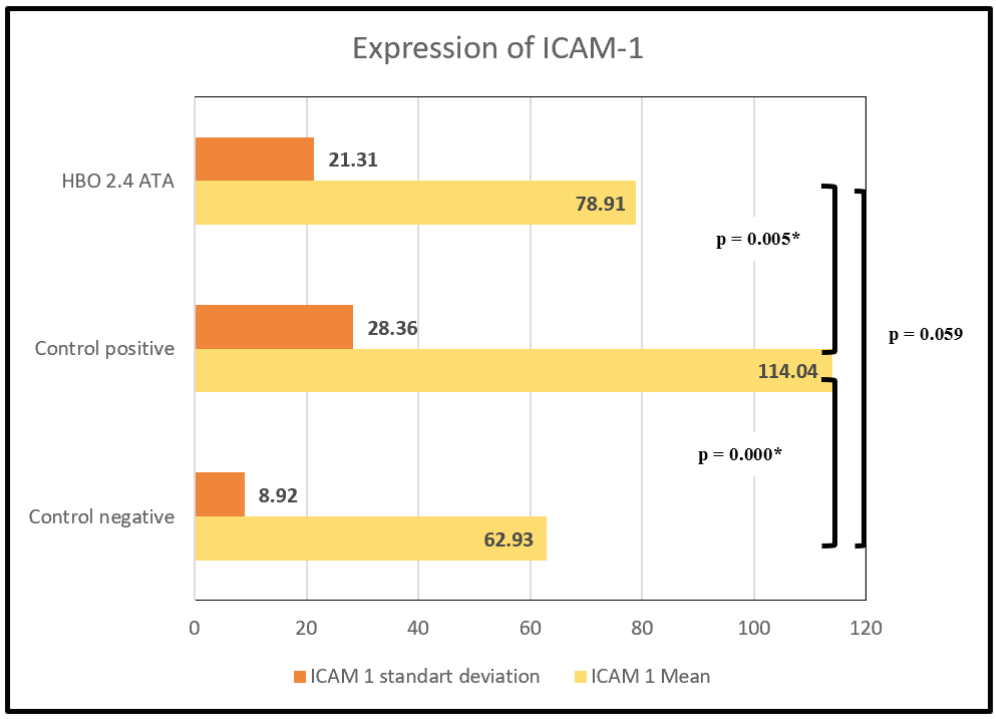

Figure 2. The avarage ICAM-1 expression in the control negative (G1), control positive (G2), and HBO treatment (G3) groups. *MANOVA test and post-hoc Games Howell test showed significant differences; $p < 0.05$.

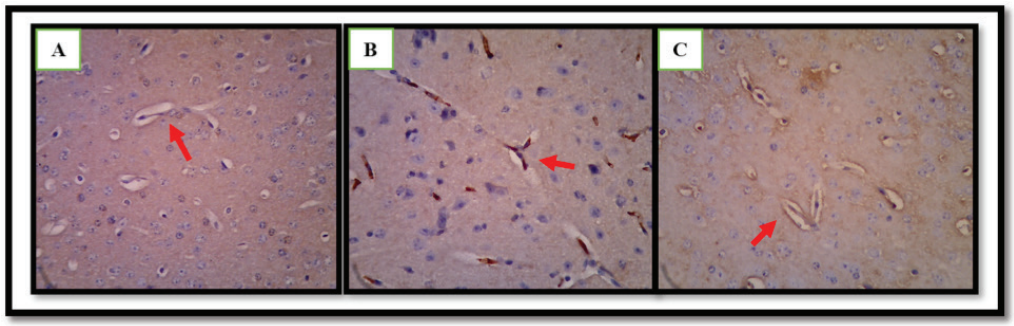

Figure 3. HIF-1α expression in the (A) control negative (G1), (B) control positive (G2), and (C) HBO treatment (G3) groups. 400x magnification.

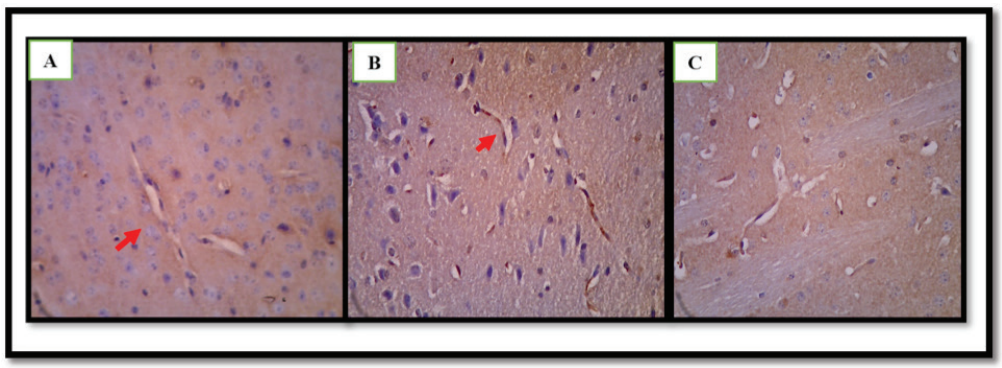

Figure 4. ICAM-1 expression in the (A) control negative (G1), (B) control positive (G2), and (C) HBO treatment (G3) groups. 400x magnification.

pRBC cytoadherence and sequestration play an important role in the pathogenesis of cerebral malaria in humans; both damage capillary blood vessel walls, inhibit blood flow to capillary blood vessels and cause hypoxia and dysfunction in some vital organs, especially the brain. Infected erythrocytes will express PfEMP-1 protein in knobs on the membrane surface with vascular endothelial receptors such as CD-36, ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), platelet endothelial cell adhesion molecule-1 (PECAM-1), E selectin, and thrombospondin. There are differences in the histopathological profile and cerebral malaria manifestations between human and mouse brains, where erythrocyte sequestration in vascular endothelium is relatively infrequent in mice. Nevertheless, in PbA-infected mice, leukocytes especially T lymphocyte cells accumulate in the vascular endothelium like what is observed in humans with cerebral malaria[15]. Various endothelial receptors that play a role in the process of sequestration of PRBC in humans are not all expressed in mice. ICAM-1 is considered the most important role in the pathogenesis of cerebral malaria[16]. High expression of ICAM-1 facilitate sequestration of T lymphocyte cells through LFA 1 integrin protein, causing obstruction and circulatory disorders in the tissues, especially the brain. ICAM-1 expression is enhanced when the body experiences inflammation[17]. ICAM-1 expression in G3 brain endothelial cells was significantly lower than in G2 ($p = 0.005$) and no different from G1 ($p=0.059$). This result showed that administration of HBO was able to regulate inflammatory response accompanied by decreased ICAM 1 expression.

Hypoxia in malaria, especially cerebral malaria, is caused by O_2 transport deficiency, a condition that leads to hemolysis, pRBC sequestration, and infiltration and activation of various immune cells that can obstruct the brain micro-vascularity[18]. Hypoxia occurs when the amount of oxygen is insufficient for cellular processes. HIF senses low oxygen concentrations; it is a transcription factor that serves as a key regulator in cell adaptation and survival in hypoxic conditions through increased angiogenesis and erythropoietin production and cell proliferation. On the other hand, high HIF-1α expression stimulates inflammatory activity through crosstalk with NF-κB. Indeed, elevated HIF-1α causes lung injury in severe malaria[4], so its expression must be suppressed to reduce excessive inflammatory processes and tissue damage. In this study, HIF-1α expression in G3 brain endothelial cells was significantly lower than in G2 brain ($p = 0.001$) and no different from G1 mice ($p = 0.258$). This finding indicates that HBO significantly reduced HIF-1α expression in brain vascular endothelial cells, likely because HBO above 1 ATA increases the O_2 concentration and solubility in blood plasma. The O_2 enters cells directly through diffusion, and so it is more quickly used by the cells compared to O_2 bound by hemoglobin. Increased tissue oxygenation will attenuate ubiquitous HIF-1α transcription via von Hippel Lindau protein, and so HIF-1α will be degraded in proteasomes[19].

Conclusion

In summary, our study showed a moderate correlation between HIF-1α and ICAM-1 expression in brain endothelial cells, between ICAM-1 expression and the histopathological profile, and between HIF-1α expression and the brain histopathological profile.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Ethical Approval: This study was approved by the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia.

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