Reduction of TNFR2 Expression in Brain Endothelial Cells in Cerebral Malaria Mice Models after Hyperbaric Oxygen Exposure

Prawesty Diah Utami, Usman Hadi, Yoes Prijatna Dachlan, Guritno Suryokusumo, Loeki Enggar Fitri

Abstract: Tumour Necrosis Factor Receptors 2/ TNFR2 is the main receptor of TNF-α expressed by brain endothelial cells and contributes to Nuclear Factor Kappa Beta/ NFkB activation which induces sequestration of leucocyte cells. The role of hyperbaric oxygen/HBO as a regulator of the inflammatory process can be used as a supportive therapeutic approach in cerebral malaria infection. The research aimed to examine the impact of HBO administration on expression of TNFR-2 in brain endothelial cells of mice infected with P. berghei ANKA. P.berghei ANKA / PbA infected C57BL/6 divided into 3 groups with 13 mice per group: G1 (negative control, normal mice); G2 (positive control; mice infected with PbA without HBO); G3 (treatment group; mice infected with PbA with HBO). Observation of TNFR2 expression using immunohistochemical techniques on the last day of treatment.

Study results revealed that there has been a significant decrease in TNFR2 expression in brain vascular endothelial cells in the group that received HBO (p < 0.05). Exposure to HBO 2.4 ATA for 10 sessions can prevent cerebral malaria by decreasing TNFR2 expression in vascular endothelial cells of the brain of the mice model.

Keywords: Cerebral Malaria, HBO, Plasmodium berghei ANKA, TNFR2.

I. INTRODUCTION

Malaria infection is a vector transmitted disease caused by blood parasite, Plasmodium sp. Number of malaria infections based on WHO data shows 200 million people have been infected and can cause 400 thousand deaths. In the absence of a malaria vaccine and reports of resistance to ACT (artemisinin combination therapy) being the cause of this infectious disease are still a global health problem [1]-[2].

Clinical manifestations of malaria vary from asymptomatic - flu-like disease (in populations living in endemic areas) to clinical manifestations that are less common but fatal and can cause death. Complications of severe malaria include severe anemia, metabolic acidosis, respiratory system disorders, multi-organ disorders and cerebral malaria [3]. Cerebral malaria was a malaria complication characterized by decreased awareness or the appearance of neurological disorders (ataxia, seizures, paraplegic, etc.). The emergence of various neurological disorders and decreased awareness of cerebral malaria is associated with the occurrence of excessive inflammatory processes in response to a high degree of parasitemia [4].

Pathogenesis of cerebral malaria involves complex biological processes, one of which is the excessive activity of proinflammatory cytokines TNF-α/ Tumour Necrosis Factor Alpha. Proinflammatory cytokine TNF-α has two receptors such as TNFR1 and TNFR2, but previous studies have proven that TNFR2 takes a major part in the development of cerebral malaria in both humans and laboratory animals models. Tumour Necrosis Factor Receptor 2 / TNFR2 was one of TNF-α receptors expressed by endothelial cells, glial cells, and leukocyte cells. Previous studies have shown that human endothelial cells, as well as experimental animals with cerebral malaria show, increased expression of TNFR2. TNFR2 expression is strongly influenced by Interferon Gamma/IFN-γ released by Th Helper 1/ TH1. Activated monocytes express TNF transmembrane on the membrane surface to bind to TNFR2 in endothelial cells. These bonds will activate Nuclear Factor Kappa Beta/ NFkB to express ICAM-1 which will induce adhesion of PRBC, leukocytes, platelets which will cause obstruction and ischemia of blood vessels and endothelial cell damage [5].

Hyperbaric oxygen / HBO was a systemic action of 100% oxygen administration with a standard pressure 2 – 3 atmospheres absolute (ATA) inside the chamber. Previous studies have shown that the administration of HBO can reduce the inflammatory process, and HBO is used for adjuvant therapy of various inflammatory-related diseases such as necrotizing tissue infections, gangrene gas, burns, osteomyelitis and chronic wounds [6]. The administration of HBO can improve the function of macrophages as the main source of TNF-α, where macrophages and monocytes that are exposed to HBO will produce fewer TNF-α than those without HBO exposure. This decrease in proinflammatory cytokine production is associated with decreased NFkB activity and increased HO-1 activity[6]-[7]. Based on the above phenomenon, the goal of this study was to examine the impact of HBO administration on expression of TNFR2 in brain endothelial cells of mice infected with cerebral malaria.
administration in brain endothelial cells mice with cerebral malaria on TNFR2 expression.

II. MATERIALS AND METHODS

Research methodology was true experimental design in which the research units are assigned randomly to an experimental and control group and the measurement of parameters is done once in the final phase of treatment. The experimental unit of this study was C57BL/6 female mice aged 7-10 weeks, an average weight of 15-20 grams obtained from PT Indoanilab Bogor which has been declared free of pathogens. The experimental unit will be divided into 3 groups as follows:

1. Group 1 (G1/negative control): 13 mice infected with PbA infection and no HBO administration.
2. Group 2 (G2/positive control): 13 mice infected with PbA without HBO administration.
3. Group 3 (G3/treatment group): 13 mice infected with PbA and administration of HBO 2.4 ATA exposure 3 times 30 minutes 100% O₂ for 10 consecutive days.

The research includes the process of PbA infection carried out in the clinical parasitology laboratory of the Faculty of Medicine, Universitas Brawijaya Malang; the presentation of HBO in the Hyperbaric Laboratory of Medical Faculty Hang Tuah University, Surabaya and the reading of the immunohistochemistry / IHC preparations was carried out in the Anatomical Pathology Department of Medical Faculty Airlangga University, Surabaya. The research was also supported by Faculty of Veterinary Medicine’s ethics committee at Airlangga University, Surabaya.

A. Mice Models

The C57BL/6 mice used in this study was the most commonly used cerebral malaria mice model because it has a high susceptibility to P.berghei ANKA infection. Clinical manifestation outcomes were similar to human cerebral malaria in terms of neurological symptoms appearance, increased proinflammatory cytokines, increased lactate production, increased endothelial receptors, the occurrence of vasculopathy such as platelet activation, vascular leakage, edema and micro hemorrhage in the brain [8].

The experimental animals were euthanized on day-13 after infection by an anesthetic injection of 1 ml ketamine and 0.5 ml xylazine (dissolved in 0.9 percent normal saline at 8.5 ml). This lethal dose is injected intraperitoneally and three times the anesthetic dose (0.3 ml/10 g body weight)[9]. Brain tissue was drained and soaked in 10% neutral formalin (BNF) buffered (pH 6.5-7.5) immediately. The formalin-to-tissue ratio was 10:1.

B. Plasmodium berghei ANKA Infection Procedure

The acclimatization phase is carried out for 1 week in a room, food, and drink that has been sterilized by ultraviolet light. Maintenance of blood-stage parasites PbA stored nitrogen solution and carried out the serial passage on donor mice. The stage of PbA infection begins by infecting donor mice with PbA and observing the level of parasitemia up to 15%. Then intracardiac blood samples were taken and diluted with PBS solution twice. Mice were infected with 1 x 10⁶ erythrocytes infected with PbA by intraperitoneal injection. The growth of parasites is observed serially from day 1 to day 12 post-infection. Observation of the level of parasitemia was done by counting the number of infected erythrocytes divided by 1000 erythrocytes multiplied by 100% using a binocular microscope and Giemsa staining[10].

C. Hyperbaric Oxygen Administration

One session of HBO is 100 percent oxygen administration at 2.4 atmosphere absolute level pressure for 3 times 30 minutes at 5 minute intervals of air (20-21%) compression[11]. The interval between one session and the next session is 24 hours, and 10 sessions exposure are completed within 10 days.

D. Immunohistochemical Examination of TNFR2 Expression

With a 4 μm thick incision, brain tissue is cut in the corona plane and stained with hematoxylin-eosin. Stages of immunohistochemical staining are started by washing slides with phosphate saline solution (PBS, pH 7.4) for 5 minutes, 3% H₂O₂ for 20 minutes, and PBS for 3x 5 minutes. Using 5% fetal bovine serum (FBS) and 0.25% TritonX-100, non-specific protein is blocked and washed with PBS for 5 minutes. Monoclonal antibody TNFR2 incubation (Santa Cruz Biotechnology, catalog number: sc-80411) for 1 hour, accompanied by PBS 3x 5 min washing. The sections are incubated at 25 °C for 10 minutes with chromogenic diaminobenzidine (DMB), followed by H&E solution and finally rinsed with tap water.

Observation of TNFR2 expression in brain endothelial cells of the brain was observed using an Olympus BX53 microscope connected to an Olympus DP27 camera and computer. 400 x magnification on a 10 field of view, the observations were made by two different people and conducted separately. Interpretation result was made by summing all endothelial cells expressing TNFR2 compared to the total number of endothelial cells observed and multiplied by 100. The intensity of staining is measured semi-quantitatively by giving a score (0 - 3) based on cell color gradations as follows:(1)Rating 0: negative;(2) Rating 1: poor staining; (3)Rating 2: moderate staining;(4)Rating 3: heavy staining. Total score = percentage of brown colored cells x intensity (maximum value of 300); the data obtained in the form of ratio data scale[12].

E. Statistical Analysis

The data in the study were analyzed using multivariate parametric analysis Manova and continued by the post hoc LSD analysis with p value about 5 percent.

III. RESULTS AND DISCUSSION

Development of parasitemia level starts at day-2 post-PbA infection followed by administration 10 sessions of HBO exposure from day-3 to day-12 post-PbA infection. On the 13th day, the mice were terminated and their brain tissue was taken to make IHC preparations and TNFR2 expression was observed. The picture of observations of TNFR2 expression in the three groups is as follows:
Differences in expression of TNFR2 in (G1) Negative Control (G2) Positive Control (G3) Treatment Group. 400 X magnification, Olympus BX53 microscope, 5-megapixel DP27 camera.

In the picture above, the arrow shows TNFR-2 expression marked in brown on the cytoplasm of brain endothelial cells. The results showed the expression of TNFR-2 in the treatment group was lower with a lower intensity than the positive control, but its expression was more numerous and stronger than the negative control group. The results of descriptive analysis and MANOVA test on the immunohistochemical examination of TNFR2 as follows:

The bar diagram showed that TNFR2 expression in the positive control group had the highest mean and standard deviation; the negative control group had the lowest mean and standard deviation. Statistical analysis showed that TNFR2 expression distinguished significantly between positive and negative controls; positive control with the treatment group as well as negative control with the treatment group. (*: indicates a significant post hoc LSD test).

In accordance with the Wah et al findings, (2016), which concluded that cerebral malaria increases the expression of TNFR2 associated with the sequestration of immune cells (leukocyte cells) that express TNF-α transmembrane to form TNFR2 bonds and induce ICAM-1 expression [5].

In comparison, TNFR1 has a stronger bond in circulation with soluble TNF-α, so the rise in circulation TNF-α was not related to the incidence of cerebral malaria [5]. The finding of this research also presented that TNFR2 expression was affected by the rate of parasitemia, increased expression of TNFR2 was followed by increased parasitemia levels. These findings are the same as Yunga et al. study, a significant increase in TNFRI and TNFR2 and associated with a higher level of parasitemia compared to non-malaria pregnant women [12].

The negative control group showed the lowest expression of TNFR2 and differed significantly from the other groups. This is due to the absence of infection or inflammatory process in this population which causes low TNFR2 expression. The treatment group showed a significant decrease in the expression of TNFR2 relative to positive controls. HBO's mechanism to reduce TNFR2 expression is likely to occur via redox signaling theory, ROS / Reactive Oxygen Species as a second messenger that controls the immune response by reducing proinflammatory cytokine output to prevent severe damage. In the treatment group that received HBO, it would produce more ROS molecules than the control groups. Previous research has shown that intracellular ROS increased after HBO administration, ROS also acts as a second messenger affecting gene expression. ROS may regulate NFkB activity, the relationship between the two is still being debated because some say that intracellular ROS can inhibit NFkB activation, but some argue otherwise[14]. The results of this study prove that ROS decreases NFkB activity which is characterized by a decrease in TNFR2 expression. Decreased rates of parasitemia may also decrease the expression of TNFR2 as a result of decreasing parasite in the circulatory effect on decreasing effector immune cell function, resulting in decreased development of TNF-α cytokine and accompanied by decreased receptor expression, TNFR2[15].

### IV. CONCLUSION

Research results have shown that exposure to HBO in brain endothelial cells will significantly reduce the expression of TNFR2. HBO’s mechanism to reduce the expression of TNFR2 may associated with immune response regulation and decreased parasitemia rates.

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