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Combination Artesunate and *Tinospora crispa* Decreases Ubiquitin, HIF-1α, VEGF and iNOS Expression in Brain of Cerebral Malaria Mice Model

LE Fitri¹, Maharani D², Margareta A^{2*}, Purnomo H³, Rahayu M³ and Budiarti N⁴

¹ Department of Parasitology, University of Brawijaya, Malang, Indonesia
² Master Program in Biomedical Science, University of Brawijaya, Malang, Indonesia
³ Department of Neurology, University of Brawijaya, Malang, Indonesia
⁴ Department of Internal Medicine, University of Brawijaya, Malang, Indonesia
*Corresponding e-mail: margareta.ab@gmail.com

ABSTRACT

Introduction: Cerebral malaria is the most severe complication of Plasmodium falciparum infection. The pathophysiology of cerebral malaria is still unclear, but it is expected caused by cytoadherence, rosetting, autoagglutinations and abundant pro-inflammatory response that will induce the release of secondary molecules like ubiquitin, HIF-1a, VEGF, and iNOS. Aim: To determine the effect of Artesunate and brotowali extract (Tinospora crispa) against the expression of ubiquitin, HIF-1 α , VEGF and iNOS in the brain of cerebral malaria mice model. Methods: An experimental study of post-test only control group using CB57BL/6J mice model malaria had been done. Samples were divided into 7 groups: negative control (K-), positive control (K+), Artesunate 32 mg/BWkg/ day (P1), Tinospora crispa 70 mg/BWkg/day (P2), combination Artesunate and Tinospora crispa dose 50 mg/ kgBW (P3), combination Artesunate and Tinospora crispa dose 60 mg/kgBW (P4) and combination Artesunate and Tinospora crispa dose 70 mg/kgBW (P5). Mice model were decapitated at 7th day after infection. Expression of ubiquitin, HIF-1a, VEGF, and iNOS was measured by immunohistochemistry. Result: One Way ANOVA showed different expression of ubiquitin, HIF-1 α , VEGF and iNOS among groups. Tukey test showed, there was no significant difference in expression of ubiquitin, VEGF and iNOS among single therapy (Artesunate or Tinospora crispa) with combination therapy (p > 0.05). Expression of HIF-1 α were significantly different between single therapy (Artesunate or Tinospora crispa) with combination therapy of Artesunate and Tinospora crispa 60 mg/kgBW (p=0.019, p=0.013) and combination therapy of Artesunate and Tinospora crispa 70 mg/kgBW (p=0.034; p=0.023). Pearson correlation showed negative correlation between Tinospora crispa dose and expression of HIF-1 α (p=0.001; r=-0.832) and iNOS (p=0.001, r=-0.874). Conclusion: The combination of Artesunate and brotowali (Tinospora crispa) extract generally decreases Ubiquitin, HIF-1 α , VEGF and iNOS expression of cerebral malaria model although only brain HIF-1 α expression gives significant value.

Keywords: Cerebral malaria, Tinospora crispa, Ubiquitin, HIF-1a, VEGF, iNOS

INTRODUCTION

Malaria is one of the most severe public health problems worldwide. Malaria attacks various ages from children to adults [1]. The number of malaria cases over the last few years has increased. According to the World Health Organization, 3.3 billion people were at the risk of malaria infection in 2011 [1], there were estimated 214 million cases and 438 thousand deaths from malaria worldwide in 2015 [2]. In Indonesia, 65% endemic districts with about 45% of the district's population were at risk of contracting malaria in 2010 [3]. According to Ministry of Health Republic of Indonesia, the death rate of malaria reached 1.3% with high malaria stratification in the area of eastern Indonesia [4].

Malaria infections in humans are caused by parasites of the genus of *Plasmodium* [5]. One of the malaria complications is cerebral malaria that most often caused by *Plasmodium falciparum* [6]. Manifestations of cerebral malaria include

loss of consciousness, coma, convulsions, and neurological disorder that can affect the productivity of the population and the decline in health as well as an impact on economic and social life [7].

In its life cycle, *Plasmodium* going through the Endoplasmic Reticular Associated Degradation (ERAD) which is a system selectively targets a protein to be degraded by the Ubiquitin-Proteasome System (UPS). Ubiquitin is a part of the UPS which is required by malaria to transform the ring stage into trophozoite [8,9]. Ubiquitin-Proteasome System has an important role in protein quality control of *Plasmodium falciparum*. Ubiquitin gene is expressed during the *Plasmodium* life cycle [10].

The pathophysiology of cerebral malaria is caused by cytoadherence, rosetting, auto-agglutination and sequestration of erythrocytes infected with *Plasmodium falciparum*. These mechanisms will cause hypoxia and ischemic process due to the inflammatory process [11]. A post-mortem examination showed sequestration of infected erythrocytes in the blood vessels of the brain causing microvascular obstruction [12]. In addition, ruptured infected erythrocytes would release the parasite molecules named Glycosyl-Phosphatidyl-Inositol (GPI). The GPI molecule induces a pro-inflammatory cytokine, especially tumor necrosis factor- α (TNF- α) which then activates nuclear factor kappa beta (NF κ B) that triggers the expression of other mediators of inflammation. Hypoxia caused by sequestration will increase gene expression of hypoxia-inducible factor-1 alpha (HIF-1 α) in the brain tissue [13-16]. HIF-1 α is a transcription factor in activating genes that aim to improve the metabolism of cells, especially cells that are hypoxic [17]. Increased HIF-1 α induces Vascular endothelial growth factor (VEGF), which will increase the permeability of the blood-brain barrier (BBB) and inducible Nitric Oxide or iNOS [12,14,16,18]. Increased HIF-1 α and iNOS in brain tissue due to the inflammatory response will induce apoptosis [15,16].

The understanding of the pathogenesis of cerebral malaria makes it possible to search for new therapies against cerebral malaria. Expected available antimalarial drugs that work indirectly on the immune response through the ability of the drug to damage the parasite, thus decrease the amount of antigen and decrease the inflammatory process [19-21]. Because of this, do some research to find new antimalarial therapy or develop companion antimalarial drugs that already exist is very important, one of which is the study of brotowali (*Tinospora crispa*). Brotowali has effects such as antimalarial, antipyretics, anti-inflammatory, and analgesic because it has an active ingredient such as berberine, palmatin, alkaloids and flavonoids [22,23]. Although the extracted content of brotowali has been evaluated as an antimalarial, no study explained the mechanism, however, brotowali remains one choice of alternative therapy in malaria. This study was to determine the effect of the combination of Artesunate and extract brotowali on the expression of ubiquitin, HIF-1a, VEGF and iNOS in the brain of mice model of cerebral malaria.

METHODS

A post-test control group laboratory experimental design was done using female CB57BL/6J mice model of malaria. Mice were 12-16 weeks old with 20-25 grams body weight, obtained from the Eijkman Institute in Jakarta. Mice were divided randomly into 7 groups, namely the Group K- (negative control or did not inoculated by *Plasmodium berghei*), Group K+ (positive control or inoculated by *Plasmodium berghei*), Group F1 (inoculated by *Plasmodium berghei* and treated by Artesunate 32 mg/BWkg/day), a Group P2 (inoculated by *Plasmodium berghei* and treated by combination of Artesunate+brotowali 50 mg/BWkg/day), Group P4 (inoculated by *Plasmodium berghei* and treated by combination of Artesunate+brotowali 60 mg/BWkg/day) and the Group P5 (inoculated by *Plasmodium berghei* and treated by combination of Artesunate+brotowali 70 mg/BWkg/day). Surgery was performed on day 7th after being given treatment on days 4th, 5th, and 6th day. Brotowali was extracted at the Research Centre for Chemistry LIPI (Indonesian Institute of Sciences) Bandung. The research was conducted at the Laboratory of Parasitology, Faculty of Medicine, Universitas Brawijaya Malang, and Laboratory of Pathology Dr. Soetomo Hospital Surabaya. This study was conducted in January 2015 through May 2016.

Plasmodium berghei Infection

Mice CB57BL/6J inoculated with 1×10^6 *Plasmodium berghei* infected erythrocytes by intraperitoneally in all groups except the negative control group.

The Degree of Parasitaemia

Assessment of the degree of parasitemia was performed on each group except negative control from day 1st to day

7after infection through observation of blood smears.

Extract Brotowali (Tinospora crispa)

Brotowali (*Tinospora crispa*) extract was made at the Research Centre for Chemistry Indonesian Institute of Sciences (LIPI) Bandung. Extraction results obtained and then tested for the total amount of phenol and flavonoids using phytochemical analysis and Thin-layer chromatography (TLC). Brotowali extract is given orally once daily on 4th, 5th, and 6th day with appropriate doses of the treatment group.

Sampling of the Brain

Mice CB57BL/6J sacrificed with ether per-inhalation on day 7th after infection. The surgery was performed by cutting cranium with the sagittal direction of the caudal (occipital) to rostral (frontal) right between the 2 hemispheres of the brain of mice. Furthermore, cutting was the liberation of the brain of mice in the basal region of the surrounding connective tissue. Both hemispheres of the brain were put in a bottle that has been filled in 10% formalin solution and sealed then carried out in Pathology Laboratory of Dr. Soetomo Hospital.

Parafinization and Deparaffinization

Brain tissue was inserted to tube contained with 10% formalin, made with paraffin block and slicing with rotatory microtome 4 microns thick and heated 60°C, mounting on a glass object with 5% gelatin. Next was deparaffinization, which is soaked with xylol (2×5 min) and then rehydration terraced with absolute alcohol 95%, 85%, 70%, 50%, 30% respectively 5 minutes, then 5 minutes rinse with H₂O.

Examination of Ubiquitin, HIF-1a, VEGF and iNOS Expression

The initial step was the antigen retrieval process with citrate buffer. Slides immersed in a chamber containing citrate buffer pH 6.0 and then heated in a water bath temperature of 95°C for 20 minutes. Slides were removed from the water bath, kept at room temperature (\pm 20 min) and then washed with PBS (3 × 2 min). Immunohistochemical staining process was then performed as follows: slide drip with 3% H₂O₂ in methanol and incubated for 15 minutes, then washed with PBS for 2 minutes 3 times. After that blocked with unspecific protein and dripped by background sniper, incubated 15 minutes at room temperature, then washed with PBS for 2 minutes 3 times. Primary antibody was dripped (primary antibody ubiquitin dissolved in PBS buffer with a ratio of 1: 300 and 2% BSA, the primary antibody HIF-1a were dissolved in PBS buffer with a ratio of 1: 300 and 2% BSA, antibody VEGF dissolved in PBS buffer with a ratio of 1: 300 and 2% BSA, and iNOS primary antibody dissolved in PBS buffer with a ratio of 1:50 and 2% BSA) overnight at a temperature of 4°C. Slides were then incubated with secondary antibodies for 30 minutes at room temperature and then washed with PBS for 2 minutes 3 times. After giving by the SA-HRP enzyme, slides were incubated for 20 min at room temperature, then washed with PBS for 2 minutes and rinsed 3 times with distilled water. DAB and DAB buffer were dripped with 1:50 ratio and incubated 3-10 minutes at room temperature, then washed with PBS for 2 minutes 3 times and finally washed with distilled water for 2 minutes 3 times. Furthermore, slides were dripped Mayer and aquadest with a ratio of 1:10 and incubated 5-10 minutes at room temperature, then rinsed with tap water, dried and observed under a microscope with a magnification of 400 times.

Evaluation Methods

Ubiquitin, HIF-1 α , VEGF and iNOS expression of the brain samples were observed using a 400X magnification microscope. Cells that express VEGF showed cytoplasmic brown mainly on the vascular endothelium, the cells that express ubiquitin and HIF-1 α showed the nucleus and cytoplasm of brown on all types of nucleated cell. Cells expressing iNOS showed cytoplasmic brown on all nucleated cells. Measurement was done by calculating the mean of cells expressing VEGF, HIF-1 α , Ubiquitin, and iNOS of 10 fields of view.

Statistical Analysis

The data obtained in this study were analyzed statistically using one-way ANOVA test to determine the differences in the expression of ubiquitin, HIF-1 α , VEGF and iNOS in control and treatment groups. It was statistically significant at p<0.05. The analysis was continued by Tukey test to compare differences in the treatment group. Pearson correlation test was used to determine the correlation between the dose of brotowali with ubiquitin, HIF-1 α , VEGF and iNOS expression. Linear regression was used to determine the relationship between the expression levels of ubiquitin, HIF-1 α , VEGF and iNOS expression.

1α, VEGF, and iNOS.

RESULTS

The content of total phenol and flavonoid from the phytochemical test obtained $43.34 \pm 1.92\%$ per dry weight of total phenolic and 74.26 ± 1.32 per dry weight of total flavonoid of the extract brotowali (*Tinospora crispa*).

This study showed the highest rates of ubiquitin expression, which was obtained in the positive control group with a mean 29.00 ± 8.19 , while the lowest rates expression was in the P5 with mean 11.00 ± 1.00 . Tukey test showed no significant differences between the groups K- to K+ (p=0.281), P1 to P2 (p=0.871), P1 to P3 (p=1.000), P1 to P4 (p=0.958), P1 to P5 (p=0.830), P2 with P3 (p=0.830), P2 to P4 (p = 0.365), P2 to P5 (p=0.212), P3 to P4 (p=0.974), P3 to P5 (p=0.871) and P4 to P5 (1.000). There were significant differences between the groups of K+ with P1 (p=0.026), K+ with P3 (p=0.022), K+ with P4 (p=0.005) and K+ with P5 (p=0.002). All treatment groups did not differ significantly with K- (p>0.05) (Figures 1 and 2).



Figure 1 Expression of ubiquitin with immunohistochemistry magnified 400x; (K-) Negative control, (K+) Positive control (P1) Artesunate dose 32 mg/BWkg/day, (P2) *Tinospora crispa* dose 70 mg/BWkg/day, (P3) Combination Artesunate and *Tinospora crispa* dose 50 mg/BWkg/day (P4) Combination Artesunate and *Tinospora crispa* dose 60 mg/BWkg/day (P5) Combination Artesunate and *Tinospora crispa* dose 70 mg/BWkg/day. Ubiquitin showed by arrow



Figure 2 Graphic of ubiquitin expression in all groups. (K-) Negative control, (K+) Positive control (P1) Artesunate dose 32 mg/BWkg/day, (P2) *Tinospora crispa* dose 70 mg/BWkg/day, (P3) Combination Artesunate and *Tinospora crispa* dose 50 mg/BWkg/day (P4) Combination Artesunate and *Tinospora crispa* dose 60 mg/BWkg/day (P5) Combination Artesunate and *Tinospora crispa* dose 70 mg/BWkg/day

The highest rates of HIF-1 α expression was obtained in the positive control group with mean 44.67 ± 3.51 and lowest rates was in negative control group with mean 9.67 ± 0.58. There were significant differences between K+ with K-(p=0.000), P1 (p=0.000), P2 (p=0.000), P3 (p=0.001), P4 (p=0.003), P5 (p=0.005), There were significant differences between K- with P1 (p=0.000), P2 (p=0.000), P3 (p=0.000), P4 (p=0.000), P5 (p=0.000). There were no significant differences between treatment groups P3 with P4 (p=0.953), P3 with P5 (p=0.889) And P4 with P5 (p=1.000). There were a significant difference between P1 with P4 (p=0.019), P1 with P5 (p=0.013), P2 with P4 (p=0.034) and P2 with P5 (p=0.023) (Figures 3 and 4).



Figure 3 Expression of HIF-1a with immunohistochemistry magnified 400x. (K-) Negative control, (K+) Positive control (P1) Artesunate dose 32 mg/BWkg/day, (P2) *Tinospora crispa* dose 70 mg/BWkg/day, (P3) Combination Artesunate and *Tinospora crispa* dose 50 mg/BWkg/day (P4) Combination Artesunate and *Tinospora crispa* dose 60 mg/BWkg/day (P5) Combination Artesunate and *Tinospora crispa* dose 70 mg/BWkg/day



Figure 4 Graphic of HIF-1α expression in all groups. (K-) Negative control, (K+) Positive control (P1) Artesunate dose 32 mg/BWkg/day, (P2) *Tinospora crispa* dose 70 mg/BWkg/day, (P3) Combination Artesunate and *Tinospora crispa* dose 50 mg/BWkg/day (P4) Combination of Artesunate and *Tinospora crispa* dose 60 mg/BWkg/day (P5) Combination of artesunate and *Tinospora crispa* dose 70 mg/BWkg/day

The highest rates of VEGF expression obtained in the positive control group with a mean 14.00 ± 1.00 , while the lowest rates in P5 group with a mean of 5.67 ± 1.53 . On the Tukey test showed no significant differences between the groups K- with K+ (p=0.154), the group P1 with P2 (p=1.000), P1 with P3 (p=0.999), P1 with P4 (p=0.954), P1 with P5 (p=0.154), P2 with P3 (p=1.000), P2 to P4 (p=0.978), P2 with P5 (p=0.192), P3 with P4 (p=0.997), P3 with P5 (p=0.290) and P4 with P5 (p=0.572). There are significant differences between the groups of K+ with P5 (p=0.047) and all treatment groups did not differ significantly with K- (p>0.05) (Figures 5 and 6).



Figure 5 Expression of VEGF with immunohistochemistry magnified 400X. (K-) Negative control, (K+) Positive control (P1) Artesunate dose 32 mg/BWkg/day, (P2) *Tinospora crispa* dose 70 mg/BWkg/day, (P3) Combination Artesunate and *Tinospora crispa* dose 50 mg/BWkg/day (P4) Combination Artesunate and *Tinospora crispa* dose 60 mg/BWkg/day (P5) Combination Artesunate and *Tinospora crispa* dose 70 mg/BWkg/day



Figure 6 Graphic of VEGF expression in all groups. (K-) Negative control, (K+) Positive control (P1) Artesunate dose 32 mg/BWkg/day, (P2) *Tinospora crispa* dose 70 mg/BWkg/day, (P3) Combination Artesunate and *Tinospora crispa* dose 50 mg/BWkg/day (P4) Combination Artesunate and *Tinospora crispa* dose 60 mg/BWkg/day (P5) Combination Artesunate and *Tinospora crispa* dose 70 mg/BWkg/day

The highest rates of iNOS expression obtained in the positive control group with a mean of 46.33 ± 4.16 , while the lowest rates in negative control group with a mean 11.67 ± 4.51 . On Tukey test showed that there are no significant differences between groups P1 with P3 (p=0.59), P1 with P4 (p=0.06) and P1 with P5 (p=0.05). There are significant differences between P2 with P4 (p=0.04), P2 with P5 (p=0.05). There was no difference between K- with P4 and P5 (Figures 7 and 8).



Figure 7 Expression of iNOS with immunohistochemistry magnified 400X. (K-) Negative control, (K+) Positive control (P1) Artesunate dose 32 mg/BWkg/day, (P2) *Tinospora crispa* dose 70 mg/BWkg/day, (P3) Combination Artesunate and *Tinospora crispa* dose 50 mg/BWkg/day (P4) Combination Artesunate and *Tinospora crispa* dose 60 mg/BWkg/day (P5) Combination Artesunate and *Tinospora crispa* dose 70 mg/BWkg/day



Figure 8 Graphic of iNOS expression in all groups. (K-) Negative control, (K+) Positive control (P1) Artesunate dose 32 mg/BWkg/day, (P2) *Tinospora crispa* dose 70 mg/BWkg/day, (P3) Combination Artesunate and *Tinospora crispa* dose 50 mg/BWkg/day (P4) Combination Artesunate and *Tinospora crispa* dose 60 mg/BWkg/day (P5) Combination Artesunate and *Tinospora crispa* dose 70 mg/BWkg/day

From the calculation of ubiquitin expression, HIF-1 α , VEGF, and iNOS were observed showed in Table 1.

Groups	Average ± SD Ubiquitin	Average ± SD HIF-1α	Average ± SD VEGF	Average ± SD iNOS
Negative Control	$20.67\pm5.03ab$	9.67 ± 0.58 a	7.33 ± 3.21 ab	11.67 ± 4.51 a
Positive Control	$29.00\pm8.19b$	$44.67 \pm 3.51 \text{ d}$	$14.00\pm1.00b$	$46.33 \pm 4.16 \text{ d}$
Artesunate (P1)	$15.67 \pm 0.58a$	23.67 ± 3.05 c	$12.33\pm4.04ab$	25.67 ± 1.15 bc
Tinospora crispa (P2)	$20.00\pm2.00ab$	$23.00 \pm 2.00 \text{ c}$	$12.00 \pm 3.61ab$	26.67 ± 2.08 c
Combination I (P3)	$15.33 \pm 4.04a$	18.33 ± 3.06 bc	$11.33 \pm 4.16ab$	21.00 ± 2.64 bc
Combination II (P4)	$12.33 \pm 4.04a$	16.67 ± 1.15 b	$10.00 \pm 1.00 ab$	17.33 ± 2.08 ab
Combination III (P5)	$11.00 \pm 1.00a$	16.33 ± 1.52 b	$5.67 \pm 1.53a$	17.00 ± 2.00 ab

Table 1 Expression of ubiquitin, HIF-1α, VEGF, and iNOS

Correlation test between dose of brotowali with ubiquitin was not significant (p=0.070, r=-0.540) as well as VEGF (p=0.094, r=-0.505), however, dose of brotowali correlated significantly with iNOS (p=0.001, r=-0.874) and HIF-1 α (p=0.001, r=-0.832), with a negative correlation. This indicated that the larger the dose brotowali, the smaller expression of HIF-1 α , iNOS. In the processing of data using multiple linear regression analysis, obtained brotowali dose relationship to HIF-1 α as Y=23.638-0.109X and against iNOS as Y=25.914-0.126X, with Y was HIF-1 α or iNOS and X was a dose brotowali.

We described the correlation test between marker expression in detail, ubiquitin against HIF-1 was α (p=0.000, r=0.793), ubiquitin against VEGF was (r=0.581; p=0.000), ubiquitin against iNOS was (p=0.000, r=0.769), VEGF against HIF-1 α was (p=0.016, r=0.557), VEGF against iNOS was (p=0.036, r=0.564), HIF-1 α against iNOS was (p=0.000, r=0,905). All correlation test results has a significant positive value (p<0.05). Multiple linear regression analysis was performed, the obtained regression model of the relationship of ubiquitin against HIF-1 α with the model was Y=1, 4,002 148X, R2=79.3%, p=0.000, Ubiquitin regression against iNOS model Y=6.144-1.134X, R2=76.9%, p=0.000, ubiquitin regression against VEGF with the model Y=16.195-0.058X, R2=38.8%, p=0,011, HIF-1 α with the model Y=6.678-1.570X, R2=55.7%, p=0.016, regression model against VEGF iNOS 10.1211, Y=428X, R2=49.7%, p=0.036. All the regression test results have a significant value (p<0.05) (Figure 9).



Figure 9 Graphic of regression test. (A) *Tinospora crispa* dose toward HIF-1α (B) *Tinospora crispa* dose toward iNOS (C) HIF-1α toward iNOS (D) Ubiquitin toward VEGF (E) VEGF toward HIF-1α (F) VEGF toward iNOS (G) Ubiquitin toward HIF-1α (H) Ubiquitin toward iNOS

DISCUSSION AND CONCLUSION

Groups that received therapy of brotowali extract and combination group of Artesunate and brotowali extract could decrease the degree of parasitemia (data not shown), this is due to the content of berberine, palmatin and flavonoids that have anti-inflammatory, antioxidant and antimalarials known from previous research. Methanol extracts of *Tinospora crispa* at a dose of 100 mg/kg and 200 mg/kg has inhibitory effects on the growth of the parasite 35% and 50% [22]. The effect increased to 100% in brotowali extract and pyrimethamine combinations showed the aqueous extract dose of 500 mg/kg can work as an antimalarial and can extend the lifetime of infected mice [24]. Brotowali extract containing berberine which is a quinoline compound. These compounds are known to kill parasites with working mechanisms in the parasite food vacuole to prevent heme polymerization so hemozoin is not formed [25].

Research on CB57BL/6J mice strain showed the characteristics of genetic factors associated with cerebral malaria. In these strains, parasitemia appeared after 3-9 days post-infection and mice died early on days 7-9 post-infection with neurological symptoms such as seizures resemble the symptoms in people who have cerebral malaria [26]. Infected erythrocyte accumulation in the brain increased in these mice showed that this model is suitable for experimental cerebral malaria compared to BALB/c mice [27].

In this study, the expression of Ubiquitin, HIF-1 α , VEGF, and iNOS increased in the positive control group who were not given any treatment. Infected erythrocytes will rupture and release toxins parasites Glycosyl-Phosphatidyl-Inositol (GPI). GPI molecule was able to induce mononuclear cells to produce pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF- α), interleukin-1 alpha (IL-1 α), IL-6. IL-10, IL-1 β , IFN- κ , and others through activation of the transcription factor NF- κ B. These pro-inflammatory cytokines subsequently induce the activation of NF- κ B further that cause hypoxia and ischemia. Hypoxia and activation of NF- κ B would lead to an increase in HIF-1 α , VEGF and iNOS and other pro-inflammatory cytokines such as COX-2, TNF- α , IL-1 β , IL-6, etc [28,29].

Result of this study showed decreased expression of Ubiquitin, HIF-1 α , VEGF, and iNOS in all treatment groups. This might be due to the effects of anti-inflammatory, antioxidant and anti-parasitic contained in brotowali. Brotowali extract has been tested by phytochemical analysis which performed at LIPI Bandung indicating the phenol content of Gallic acid equivalents amounted to 43.34% and the lavonoid content of the equivalent of 74.26%.

Tukey test analysis on ubiquitin and VEGF expression showed no significant difference among the treatment groups. There was no significant difference of ubiquitin and VEGF expression among treatment and negative control groups. These results indicate that administration of Artesunate and extract brotowali able to decrease the expression of ubiquitin and VEGF as a normal state. In the pathogenesis of cerebral malaria, ubiquitin is required by *Plasmodium* to change its ring form stage into a mature trophozoite and schizont stage in order to make the process of adhesion to the endothelium [8,9]. Increases VEGF in hypoxic conditions includes in the pathophysiology of cerebral malaria, an increase in VEGF will reduce BBB permeability and lead to sequestration as well as cerebral edema got worse [30].

Combination therapy of Artesunate with brotowali could decrease ubiquitin, VEGF and iNOS expression, although did not significantly different from the single therapy group. However the expression of HIF-1 α showed significant differences between single group and the group that given the combination of Artesunate and brotowali at doses of 60 mg/KgBW and 70 mg/KgBW. In cerebral malaria, cytoadherence and rosetting will lead to hypoxia in the brain [16]. Hypoxia is a condition where there is a decline in the availability of oxygen (O₂) and the functions of O₂ homeostasis regulator that require HIF-1 α [17]. Increased activity of HIF-1 α also increases the NF- κ B, which is a transcription factor for the expression of many genes associated with inflammation, including iNOS, COX-2, TNF- α , IL-1 β , and IL-6 [28,29]. In the study conducted by Lin, et al., and Fu, et al., explained that berberine, which is one of substance active in brotowali can increase the protein degradation of HIF-1 α and was down-regulated the expression of HIF-1 α and VEGF in cancer cells [31-33]. Based on this existing research, mechanisms of a combination of Artesunate and berberine that contained on brotowali is by reducing HIF-1 [34]. It is revealed that the combination of Artesunate and brotowali can reduce HIF-1 α better than single therapy alone.

Pearson correlation test toward brotowali dose with Ubiquitin and VEGF expression showed no significant results, but the expression of HIF-1 α and iNOS showed a significant negative correlation. This indicates that the larger dose of brotowali correlated with the lower expression of HIF-1 α and iNOS. It is known that berberine can reduce the activity of HIF-1 α expression and NFkB which will then decrease the expression of VEGF and iNOS [32,33]. A previous study showed that brotowali dose of 70 mg can reduce the degree of parasitemia, the lower degree of parasitemia then the lower expression of NFkB and can be concluded also can decrease the expression of HIF-1 α [23].

Pearson correlation test between Ubiquitin and VEGF showed a significant correlation (p=0.011; r=0.581), the expression of iNOS and HIF-1 α also showed a significant correlation (p=0.000; r=0.905). This positive correlation means that the higher of ubiquitin expression will be followed by the higher of VEGF expression, moreover, the higher of HIF-1 α expression continued by the higher of iNOS expression [35,36].

Pearson correlation test showed a significant relationship between the expression of Ubiquitin and HIF-1, Ubiquitin and VEGF, Ubiquitin and iNOS, VEGF and HIF-1 α , VEGF and iNOS, HIF-1 α and iNOS has a positive value that means the higher expression of one parameter will increase the expression of the others. Multiple linear regression analysis was done, all the regression test results have significant value (p<0.05) [37,38].

The limitations of this study are the use crude extract of brotowali so it is still not known the active substances that were most responsible in effecting the decreasing levels of ubiquitin, HIF-1 α , VEGF and iNOS in the brain of mice models of malaria that has been infected by *Plasmodium berghei*. The use of crude extracts brotowali also gave possible interactions between the active ingredients or with Artesunate which can induce or inhibit the activity of other active substances.

DECLARATIONS

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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