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Annona muricata EFFECT toward NITRIC-OXIDE PRODUCTION in SEVERE and ACT-TREATED-RECOVERY-PHASE of MALARIA

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Abstract: ***Chloroquine, and artemisinin reverse partly hemozoin-induced-M2 to M1-phonotype-cells in vitro. These M1-cells however, cannot significantly increase nitric oxide (NO)-production important for protecting severe-malaria-infection. An adjuvant malaria therapy might be needed to increase NO-production. The aim of this study was to determent whether water-extracted of Annona muricata-leaves (WAM) increased spleen-cells-NO-production of Plasmodium berghei ANKA (PbA)-Swiss-mice treated with Artemisinin-based-combination-therapy (ACT), an anti-malaria recommended by WHO. This study was continuing study of a post-test-only-randomized-control-group-design used 24 PbA-infected-female-Swiss-mice, divided into control-(K)-group without any intervention, and treatment-P1, P2 and P3-groups received WAM, ACT (Dihydroartemisinin Piperaquine; DHP) and WAM-ACT-combination, respectively. The spleen-cells obtained on day-7-PbA-infection were cultured. The NO-production was then measured in the cell-culture-supernatants using Griess-reagent. Parasitaemia-percentage was also evaluated. Statistical-analysis used was Kruskal-Wallis-test followed by Mann-Whitney-U-test. The NO-productions were significantly different among those four-groups (p<0.001). The highest-NO-production was found in the P1-group with the severe-PbA-infection. Those of P1-group was significantly higher than K-group. Meanwhile, the lowest were found in the P2 and P3-groups with the PbA-infection-recovery-phase, and those of P2 and P3-groups were not different. The conclusion is WAM-treatment increases the splenic-NO-production during severe-PbA-infection, and no longer increases the production in the recovery-phase of those treated with ACT.***

***IndexTerms - Annona muricata*, ACT, Nitric Oxide, *P. berghei* ANKA**

INTRODUCTION

Plasmodium sp-infection, can develop into fatal-disease. Artemisinin-based-combination-therapy (ACT) is recommended by WHO as the main treatment for malaria cases.[1] Adjuvant therapy however is needed as a companion to anti-malarial drugs in the management of malaria cases before progressing to severe malaria, because adjuvant therapy can provide better clinical outcomes.[2] The immune response during malaria determines the development of severe malaria. This underlies the need to consider the provision of adjuvant therapy, which is a good immunomodulator to inhibit the development of severe malaria. The herb extensively studied as immunomodulator of experimental-cerebral-malaria (ECM), is *Annona muricata* (AM). The ethanol-extracted-(E)AM-leaves affects the spleen, an important organ for developing protective and pathologic immune responses in ECM. The EAM has anti-inflammatory, as indicated by a decrease splenic-tumour-necrosis-factor-alpha (TNF-α)-production and an increase splenic-Nitric-Oxide-(NO)-production of *Plasmodium berghei* ANKA (PbA)-infected-Swiss-mice, an ECM-susceptible-mice.[3] NO involves in suppressing the parasitaemia-level of malaria-mouse-model.[4] The treatment used is an oral-supplementation of arginine, an amino-acid involved in NO synthesis, in combination with lysine and valine, both of which are arginase-inhibitors). The treatment reduces parasitemia level which associated with increase NO-production and innate-immune-respond-dependent-MyD88-pathway. NO also protects ECM-mice from cerebrovascular-constriction.[5] The water-extracted-AM-leaves (WAM) which is commonly consumed by the public has not been studied in detail regarding its role as an adjuvant-therapy capable of modulating splenic-NO production in severe PbA infections. In addition, ACT has immunomodulatory effect in several diseases.[6, 7] The study-aim was to determine whether WAM modulate splenic-NO-production in those ACT-treated-malaria.

MATERIALS AND METHODS

This was continuing study done before used a post-test-only-randomized with control-group-design, using 24 female-Swiss-mice which were divided into 3 treatment-groups and 1 positive-control-group.[8] Health Research Ethical Committee Faculty of Medicine Universitas Diponegoro approved this study (Ethical Clearance No. 55/EC/H/FK-RSDK/V/2018). The sample inclusion-criteria were female-mice, weighing 20-25 grams, 6-8 weeks old, healthy, and active. The died-mice during the study, were drop-out. All samples were adapted for 6 days and randomized to K(+)-group given standard feed and distilled water, P1-group given a WAM-preventive-dose (4.68mg/ 30g mice-weight) for 7-days before and 3-days after PbA-inoculation. This then followed by a WAM-

therapeutic-dose (9.36mg/ 30g mouse-weight) until day-6-PbA-infection. P2-group was treated with ACT (0.819mg/ 30g mouse-weight) since day-4 until day-6 PbA-infection. P3-group was given both WAM and ACT. The parasitaemia-level was also observed. The mice were terminated on day-7-PbA-infection and the spleen were collected and processed for cell culture described elsewhere.[9] The culture-supernatant of LPS-stimulated-spleen-cells was measured for NO-levels by using the Griess-reagent kit (Catalog number: 30100, Biotium, Fremont, CA, USA). Statistical analysis used was Kruskal-Wallis-test followed by Mann-Whitney-U-test with a significance degree of $p<0.05$.

RESULTS

The Saphiro-Wilk-normality-test of NO-level showed that the data were not normally distributed. The Kruskal-Wallis showed significant different of the NO-levels among the group ($p < 0.05$), and Mann-Whitney-U-test were then carried out to assess the differences between two groups as shown in the table. The NO-production observed in this study was done on those K(+) and P1-groups with severe PbA-infection, and those P2 and P3-gropups with PbA-infection-recovery-phase. The P1-group-NO-production was significantly higher than K-group. These suggested that WAM-treatment associated with an increase spleen-cell-NO-production during severe-PbA-infection. Meanwhile, those produced by P2 and P3-groups was significantly lower than K-group. The finding demonstrated that those in malaria-recovery-phase associated with a reduce-NO-production. Additionally, P2 and P3-groups showed no different of NO-production. These indicated that WAM-treatment was no longer increase NO-production in those entering the recovery-phase of those treated with ACT.

Table Mann-Whitney-U-Test of NO levels produced by spleen-cells of PbA-infected Swiss mice

Groups	**NO Level Median (min – max) µmol**	***p value***		
		P1	**P2**	**P3**
K (+)	40.09 (11.03 – 58.30)	0.005	0.002	0.002
P1	59.05 (44.41 – 86.07)		<0.001	<0.001
P2	11.02 (5.43 – 21.39)			0.427
P3	11.15 (8.47 – 13.96)			

DISCUSSION

WAM-treatment associated with higher spleen-NO-production of severely-PbA-infected-mice than those without treatment (Table). The culture supernatant used in this study was collected from previous study which showed WAM-treated and untreated-groups were in the severe-PbA-infection.[8] This study therefore suggested that WAM contributed in the increase-spleen-NO-production of severe-PbA-infected-Swiss-mice. This in agreement with the findings in EAM-study using the same mouse-model.[3] This also supported by the fact that phytotherapy used can lead to M1 macrophage-development.[10] The lower of NO-production is observed in *P. falciparum* and PbA-infection.[3, 11] The decrease-NO-production is due to M2-macrophage-monocyte activation in malaria-falciparum.[11] Monocytes become M2-type after phagocytizing the pigment *Plasmodium*-hemozoin (Hz).[12] A study using NO donors demonstrates that NO inhibits the CM-manifestation by suppressing the pathological-CD4+Th-cell and CD8+CTL of PbA-infected-mice, without influencing parasitaemia-level.[13] Severely-PbA-infected-untreated-mice associates with a higher-brain-Granzyme-B (GzmB)-expression above normal-healthy-mice, while EAM-treatment assossiates with a normalize-brain-GzmB-expression in severely-PbA-infected-Swiss-mice.[8] Whether the increase-spleen-NO-production mediated by WAM, is associated with the increase of brain-non-pathogenic CD8+T-cell-accumulation needs to be elucidated in PbA-infected-mice.

Both ACT and WAM-ACT-treated associated with lower spleen-NO-production than untreated-severe-PbA-infected-mice (Table). The spleen-NO-production difference was not observed between the two-treated-groups. The samples used in this study originated from previous study which showed those treated groups were in the recovery phase of PbA-infection with no different of parasitaemia-levels between those groups.[8] WAM-treatment therefore, had no obvious additional effect toward spleen-NO-production in the recovery-phase of those received ACT-treatment. The findings of this study were aligned with serum-NO-levels observed in the Sago-caterpillar-(*Rhyinchophorus ferrugineus*)-flour-supplementation-study in ACT-treated-PbA-infected-Swiss-mice.[14] Antimalarial *in vitro* study shows that chloroquine, and artemisinin can partially reverse Hz-phagocytizing-M2-macrophages to M1-type, indicated by no effect of those antimalaria toward arginase-activity and NO-production.[12] NO-production is induced by malaria-toxin including combination of Hz-malaria-DNA, and glycosylphosphatidylinositols (GPI).[15] This therefore, the influence of both ACT and malaria toxin on NO-production needs to be further studied. The conclusion is that WAM-treatment increases NO-spleen-production in severe-PbA-infection, meanwhile the WAM limits the increase of NO-spleen-production in the recovery phase of ACT-treated-PbA-infection.

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