Annona muricata modulate brain-CXCL10 expression during cerebral malaria phase

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Abstract. Cerebral malaria (CM) contributes in malaria mortality. People in endemic region get benefices by using *A. muricata*-leaf extract (AME) before qualified for receiving standard anti-malaria, because AME restrains malaria infection and modulate immune responses. CXCL10 expressed by astrocytes limit brain inflammation. Vascular leakage was found in the brain of experimental CM. Additionally, biomarker related with vascular leakage, angiopoietin-2 (Ang-2) levels increase in CM-patients. Objectives of this study were to determine the efficacy of ethanolic-AME in regulating brain-CXCL10-expression and Ang-2 levels during CM-phase. The study was post-test-only-control-group design. Thirty Swiss-mice were randomly divided in 6 groups. C+ and C- groups were PbA-inoculated and healthy-mice, respectively. X1 and X2 groups were healthy-mice treated with AME 100 and 150 mg/Kg BW/day, respectively. X3 and X4 groups were PbA-inoculated and received either dose mentioned above. CXCL10 was stained by IHC, and determined by Allred score. Plasma-Ang-2 was measured by elisa-method. Kruskal-Wallis-test showed the difference of CXCL10-expression among the studied groups (p=0.003). CXCL10-expression of C+ group was lower than healthy-mice which were C-, X1 and X2 groups (p=0.008, p=0.045, and p=0.012). CXCL10-expression of X3 was comparable to healthy mice (C-, X1 and X2), and was higher than C+ and X4 groups (p=0.012 and p=0.028). CXCL10-expression of X4 group was lower than C- and X2 groups (p=0.011 and p=0.016). Kruskal-Wallis-test showed no difference of Ang-2-levels among 6 groups (p = 0.175). The conclusion is *A. muricata* influences brain-CXCL10 expression during CM phase, but has no association with Ang-2 levels during CM phase.

Key words: *Annona muricata*, malaria, CXCL10, Angiopoietin-2

1. Introduction

Malaria is a fatal disease in tropical and subtropical countries. WHO estimated 214 million malaria cases globally in 2015, and 438,000 of them were died [1, 2]. *Plasmodium falciparum* infection is the most common cause for severe malaria including cerebral malaria (CM). The increase risk for severe malaria might occur when the early malaria treatment for uncomplicated malaria is delayed.

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Uncomplicated *P. falciparum* malaria has clinical manifestation alike many other diseases including influenza and other common diseases related with fever. This, therefore, is difficult to do early diagnose with subsequently rapid malaria treatment of uncomplicated malaria which may avoid severe condition [3]. Malaria patients can get benefices by using traditionally malaria treatment before they are eligible for standard-malaria therapy. Anti-malaria plants increase worldwide interest, and are further studied for their capability as anti-plasmodial [4-6]. *Annonaceae* which comprises approximately of 123 genera and 2,100 species, is noticeable due to their broad traditional uses against malaria [7]. Anti-malaria of *Annona muricata* leaf extract (AME) treatment was shown *in vivo* by using imprinting control region (ICR) mice inoculated with 10^7 *P. berghei* ANKA (PbA) [8]. The CM studies showed that tumour necrosis factor-alpha (TNF-α), a pro-inflammatory cytokine, contributed to CM pathogenesis [9]. Ethanolic-AME treatment was associated with reduce TNF-α and nitric oxide (NO) produced by splenocytes of PbA-inoculated Swiss mice as a model for experimental cerebral malaria (ECM). The doses used were of 100 and 200 mg/Kg BW/day given orally before and after 10^7 PbA-inoculation. Two out of six mice was died in the group of PbA inoculated and treated with 200 mg/Kg BW/day, therefore the use of lower doses was recommended [10].

The increase levels of many chemokines were associated with CM. The chemokines most strongly implicated in CM are those bind to (C-X-C motif) receptor 3 (CXCR3), including CXCL9, and CXCL10. CXCL10 which binds to CXCR3 with a high-affinity, is the first known chemokines which is able to direct the trafficking of activated effector CD4+ and CD8+ T lymphocytes, natural killer (NK) and natural killer T (NKT) cells [11-13]. The importance of CXCL10 had been suggested by several studies in malaria. A study of CM in Ghanaian children demonstrated that out of 36 biomarkers, only CXCL10 was serum marker independently associated with CM mortality. In addition, study of cerebral spinal fluid (CSF) of CM patients showed that CXCL10 was one of eight biomarkers significantly up regulated in the CM group. [14]. The ECM study showed that CXCL10 was highly induced in the brain especially at the neuron of PbA infected mice, and CXCL10 deficient mice were protected partially from CM mortality [11]. The recent study showed that CXCL10 provoked the apoptosis of both human brain micro vascular endothelial cells and neuroglia cells *in vitro* [15]. In contrary to those found in CM and ECM studies, an *in vitro* study on astrocyte showed a reduce CXCL10 expression during hypoglycaemia and hypoxia which both were two condition found in severe malaria [16]. Astrocytes are abundance in the brain, and this cells are important in maintaining the brain homeostasis and prevent the brain inflammation induced by T cells [17]. The aim of the study was to determine whether ethanol-AME capable of modulating CXCL10 expression in the brain of Swiss mice during CM phase. A significantly lower CXCL10 expression than normal was found during CM phase of positive control mice without AME treatment. Dose of 100 mg/Kg BW/day AME was able to normalize CXCL10 expressed in the brain.

Vascular dysfunction is one of many other CM pathogenesis, and Angiopoietin-2 (Ang-2) is biomarker contribute in it. Protective immune responses develop in malaria patients targeted to combat the infection without inducing any immunopathology that induced severe malaria including CM and severe malaria anaemia [18, 19]. CM is characterized by the present of inflammation that contributes in vascular dysfunction. Based on that point of view, it is proposed to integrate adjuvant therapy improving vascular dysfunction in CM management [20]. The vascular endothelial dysfunction in malaria indicates by many biomarkers including increase of Ang-2 due to increase vascular endothelial cell activity [21, 22]. Anti-malaria therapy affected the improve vascular endothelial function and better thrombocyte count that both were indicated by the decline of Ang-2 levels [23]. The increase of Ang-2 in CM patients is a better indicator for disease severity and mortality than Ang-1 which released by thrombocytes. This fact suggested that vascular endothelial dysfunction in CM might preferably due to the increase of Ang-2 rather than the reduced Ang-1 released by thrombocytes [24-26]. Based on those studies, therefore, Ang-2 level can be used to prove the benefice particular treatment which alleviate CM. The sequestered parasitized red blood cells and accumulation of leukocytes, both have a strong relation with CM [27]. The treatment used may traditional herbs having anti-malaria and anti-inflammatory effects. The herb used is expected to lessen Ang-2 level which indicates the ease of vascular dysfunction in CM. Natural product including traditional plants are used for malaria treatment

in malaria endemic area [28]. Natural product having both anti-malaria and immunomodulatory effects had been extensively studied in *in vitro* and *in vivo* [29-34]. The advantages of leaf of *Annona muricata* toward malaria have been reported. The AM leaf extract (AME) showed anti-plasmodial effect and low toxicity in *in vitro* studies by using *P. falciparum* and normal kidney cells, respectively [35, 36]. Methanol and water based AME showed low toxicity, and both of them have anti-inflammatory and anti-oxidant activity in *in vitro* and *in vivo* studies [37, 38]. Recent published data showed that ethanolic-AME reduce tumor necrosis factor-alpha (TNF-α) and nitric oxide (NO) produced by stimulated spleen of Swiss mice during CM phase [39]. The aim of the study was to determine whether ethanol-AME capable of reducing Ang-2 in the blood of Swiss mice during CM phase. No significant lower of Ang-2 levels in those receiving AME treatment was found during CM phase. It is conclude that AME might not influence Ang-2 levels of Swiss mice during CM phase.

2. Material and methods

2.1. Preparation of ethanol extract leaves of Annona muricata

Ethanolic extract of *A. muricata* leaf was kindly provided by experts from SidoMuncul company, Indonesia. Raw material was sorted, washed, dried and weighed, and milled coarse. The materials then further processed until the condensed-extracts was obtained.

2.2. Experimental Mice and Animals handling

This study was post test only control group design. Thirty adult female Swiss mice used were 8-10 weeks old, and 30 – 35 grams body weight. The strain and health of mice were certified by agriculture department of Indonesian Republic. They were housed in six plastic cages under hygienic conditions. The animal care room were maintain in temperature at 25 ± 4º C with 12 hours alternate exposure to light and dark. The mouse adaptation for 7 days was done before experiment. The experiment procedures done in the mouse were approved by ethical committee of health researches, Medical Faculty of Diponegoro University and Karyadi Hospital Semarang. The mice divided in 2 control and 4 treated groups. All mice received hygienic standard diet and water. C(-) group was healthy mice without AME treatment, while X_1 and X_2 groups were healthy mice treated with AME 100 and 150 mg/kg BW/ day, respectively. AME treatment was given orally for period of 14 days. C(+) was group of PbA-inoculated mice. X_3 and X_4 groups were PbA-inoculated mice which received AME either dose mention above in period of 7 days before and 7 days after PbA inoculation.

2.3. Malaria Parasite.

Plasmodium berghei ANKA (PbA) used for inoculation was provided by Parasitology department of Medical Faculty UGM,Yogyakarta, Indonesia. PbA was passage twice in swiss mice before experiments. The blood was collected from the second passage when the mice had parasitemia level of 15 – 20 %. Dose of 10^7 iRBC collected from the second passage was injected peritoneally to swiss mice used for the AME study.

2.4. Parasitemia levels

Parasitemia level was observed on thin blood slide stained by Giemsa. Parasitemia was determined by technician of MFDU-Parasitology Department. This was done at day 3, 5 and 7 p.i. Parasitemia level was percentage of parasitized Red Blood Cells (pRBCs) out of 1000 RBCs in each blood slide.

2.5. Immuno Histochemestry of CXCL10 and Allred score

Starr Trek Universal HRP detection system (Catalog number: STUHRP700 H, L10; Biocare Medical, Concord, California, USA) and primary antibody which was anti-CXCL10 polyclonal antibody for mouse/ rat (Catalog number: bs-1502R; Bioss Antibodies, USA) was used to stain CXCL10 expressed in the mouse brain. Parafine block of brain was cut as thick as 3 – 4 μm, and attached to the object glass. This was followed by deparaffinization by using xylene and rehydrated by using alkohol. The next step was decloaking proccess which followed by cooling at room temperature for 1 hours, and washed the section by using aquadest, and put PBS on it for 5 minutes. The normal wine serum was put on the section for 10 minutes, and then washed the section by using PBS. The IHC staining by using CXCL10 primary antibody and HRP detection system were then done on the section. The samples, then, were observed under light microscope. Allred score was used for evaluating CXCL10 expression.

2.6. Ang-2 measurement

Plasma used for Ang-2 measurement was isolated from whole blood collected from *plexus retro-orbital*. Ang-2 levels were measured by Elisa method.

2.7. Statistical analysis

Kappa test used was used to evaluate the agrreement beween two pathology anatomy expert who determine the Alred score. Variable immunohistochemestry examinations (CXCL10 levels) were measured by two (2) blindly anatomical pathologist, and test the proportion of responses to 'deal' wear kappa test. Kappa values <0.40 means poor agreement, values between 0.41 to 0.60 indicates moderate agreement, values between 0.61 to 0.80 indicates a good agreement, and values ≥0.81 means very good agreement. Kruskal-Wallis test followed by Mann-Whitney U test was used to analyses CXCL10 expressed in the brain. The Ang-2 levels then analyzed using Kruskal-Wallis test.

3. Result and Discussion

3.1. CXCL10 expression

CXCL10 expression was determined by Allred score obtained by calculating intensity and proportional score (Figure 1). Kappa test result showed Kappa value of 0.547 meaning moderate agreement between 2 experts determines the Allred score on CXCL10 expression (Table 1).

The CXCL10 expressions were normally distributed in all studied groups. Analysis of variance, however, showed there were data in the studied groups were not homogen ($p = 0.001$). Kruskal Wallis Test, a nonparametric test, therefore, was performed, and it showed significant difference among the studied groups ($p = 0.003$). C(+) group was significantly expressed lower CXCL10 than those of healthy groups without or with *A. muricata* treatment (C(-), $p = 0.008$; X_1, $p = 0.045$; or X_2, $p = 0.012$) (Figure 2A, Table 2.). CXCL10 expression of X_3 group was comparable with those of healthy mice (C(-), X_1 and X_2), meanwhile the expression was significantly higher than C(+) and X_4 groups ($p = 0.012$ and $p = 0.028$, respectively). X_4 group showed CXCL10 expression significantly lower than healthy groups which were negative control and X_2 groups ($p = 0.011$ and $p = 0.016$, respectively) (Figure 2; Table 2).

3.2. Parasitemia

Parasitemia levels were low at day 3 p.i. in all PbA-inoculated groups which were C(+), X_3 and X_4 groups. A sharp increase of parasitemia levels were observed on day 7 p.i. The mean ± SD % parasitemia levels of C(+), X_3 and X_4 groups were 23.91 ± 12.89 %, 21.59 ± 10.15 %, and 20.91 ± 13.98 %, respectively. Shapiro-Wilk test showed that parasitemia levels were normally distributed in each group ($p > 0.05$). Test of homogeneity of variance revealed the homogeneity of parasitemia levels ($p > 0.05$). These test then followed by one-way anova test which showed no difference of parasitemia levels among those three groups ($p = 0.916$).

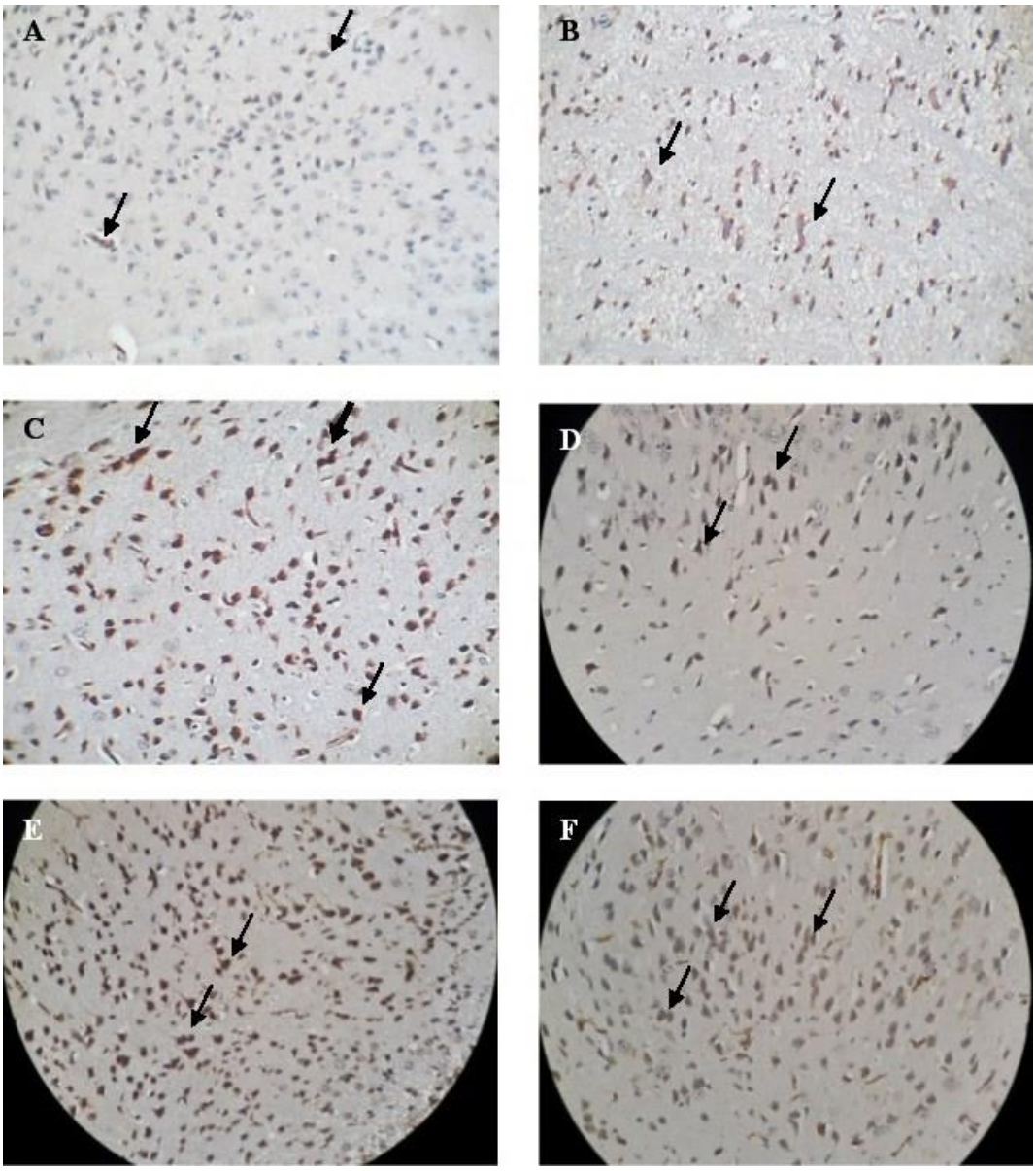

Figure 1 showed IHC of brain-CXCL10 expression used to determine the Allred score. Allred score was sum of proportion score and intensity score observed using light microscope with magnification 400 x. Fig 1.A, B and C were CXCL10 score 1, 2 and 3. The score was determined by the proportion of cells expressed CXCL10. Score 1 = one cell was positive for CXCL10; score 2 = 1 – 10% positive; score 3 = 10 - 33% positive; score 4 = 34 – 66% positive; and score 5 > 66% positive. Fig 1.D, E, F were CXCL10 intensity 1, 2 and 3.

Table 1. *Kappa* test for examination CXCL10 level
Observer 1 * observer 2 Cross tabulation

Count

		Observer 2							Total
		2	3	4	5	6	7	8	
Observer 1	2	3	2	0	0	0	0	0	5
	3	0	13	2	1	0	0	0	16
	4	1	3	8	5	1	0	0	18
	5	1	0	11	28	22	2	0	64
	6	0	1	2	8	83	12	3	109
	7	0	0	0	1	6	36	4	47
	8	0	0	0	0	0	1	5	6
Total		5	19	23	43	112	51	12	265

Kappa = 0.547 (*moderate agreement)*

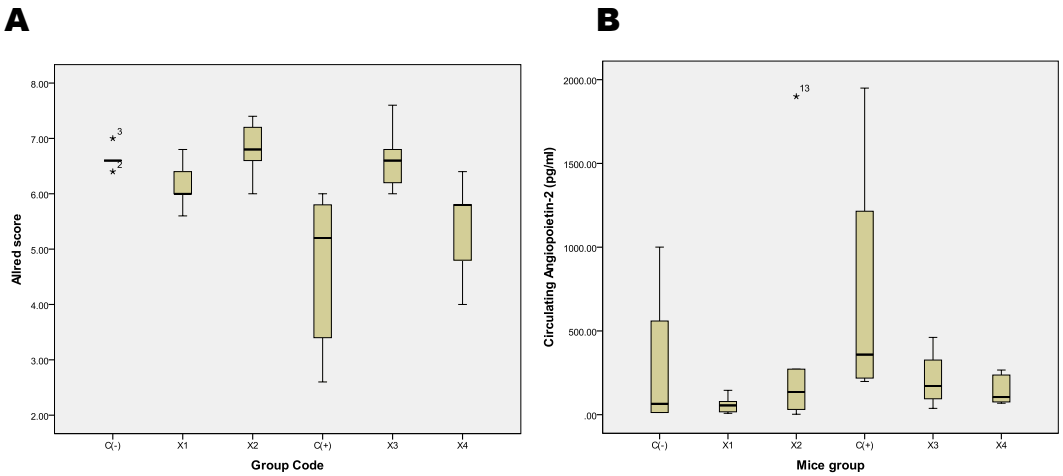

Figure 2. was box plot of Swiss mice in ethanolic-AME study. Fig 2 A. was CXCL10-expression in the brain. **Fig 2 B.** was circulating angiopoietin-2 levels.
These groups were C(-) group which was healthy mice without AME treatment; $X_1$and X_2 groups were healthy mice which received 100 and 150 mg AME/kg BW/day, respectively; C(+) group was group of PbA-inoculated mice; X_3 and X_4 groups were groups of PbA-inoculated swiss mice which received 100 and 150 mg AME/kg BW/day, respectively.

Table 2. Statistical analysis of CXCL10 expressed in the brain of healthy or PbA-inoculated mice with or without *A. muricata* treatment

Groups	C(-)	X_1	X_2	Control (+)	X_3	X_4
C(-)	(-)	(p=0.089)	(p=0.389)	(p=0.008)*	(p=0.829)	(p=0.011)*
X_1		(-)	(p=0.090)	(p=0.045)*	(p = 0.203)	(p=0.140)
X_2			(-)	(p=0.012)*	(p=0.673)	(p=0.016)*
C(+)				(-)	(p=0.012)*	(p=0.459)
X_3					(-)	(p=0.028)*
X_4						(-)

Mann-Whitney U test with significant difference ($p < 0.05$)

This recent IHC study was comparing the CXCL10 expression in the brain of PbA-infected mice with and without AME treatment (Fig 2 A). No significant difference was found among positive control and those received AME 150 mg/kg BW/day (Table 2). The difference however, was observed between positive control and X_3 ($p = 0.013$). CXCL10-expressions of X_3 group were comparable to those of negative control. AME treatment might normalize CXCL10 expression in the brain during CM phase of Swiss mice. CXCL10 is expressed by astrocytes, the abundant cells in the brain which function to maintain CNS homeostasis [9]. Astrocytes are able to induce T-cell apoptosis and subsequently avoid inflammation [17, 40]. Since CXCL10 is chemokine which attract Th1 cells, thus the reduce CXCL10 expressed by astrocytes is speculated to reduce the Th1-cell apoptosis in the brain of PbA-inoculated swiss mice. This then, maintains inflammation in the brain of PbA-infected Swiss mice. More recent study found that inflammatory activated astrocytes had no role in Th1 polarization [41]. Whether this occure in PbA-infecd swiss mice has not been studied. All together support futher studies including the use of CXCL10-deficient Swiss mice in order to strengthen the notion that CXCL10 expressed by astrocytes has important role in ECM. The CXCL10 generation is induced by IFN-γ, a cytokine signature of Th1 cells. Interestingly, IFN-γ is also needed for the release of IL-10, an anti-inflammatory cytokine, by microglia/ macrophage and Th1 cells [42, 43]. The relation between those cytokines and astrocyte-CXCL10 expression necessitates to be clarified in PbA-infected mice treated with AME.

Preventing neuronal and endothelial apoptosis alone may not sufficient to improve survival of those suffer from CM, it need immune modulator treatment [44, 45]. This therefore, AME treatment is remain possible providing any protection on CM. Study on C57BL/6 mice inoculated with 5×10^5 PbA-pRBC showed that two distinct anti-apoptosis treatment successfully prolonged survival in sepsis animal model, were not successful prevent neuronal apoptosis and improve survival of PbA-inoculated mice [44]. C57BL/6 mice inoculated with 10^6 pRBC was used to evaluate the protective effect of Neuregulin-1. Serum CXCL10 levels of PbA-inoculated C57BL/6 mice with and without any Neuregulin-1 and arthemeter treatment were compared. Neuregulin-1 significantly reduce serum CXCL10 and other inflammatory cytokines (TNF-α, IL-1β, IL-6 and G-CSF) while significantly increase anti-inflammatory cytokines including IL-5 and IL-13 at day 11 than positive control group, PbA-inoculated mice without treatment. Neuregulin-1 prevented mortality of ECM mice, and other beneficial effects including preserved BBB stability and reduced leukocyte accumulation in the brain [45]. Whether any of these occur in PbA-inoculated mice treated with AME need to be elucidated.

Brain CXCL10 expression increased in most brain infection [46, 47]. CXCL10 produced by monocytes and netrophils was responsible for CM developed in PbA-infected mice [48]. CXCL10 KO mice or CXCL10 neutralization reduced inflammation in the brain of ECM mice model [49]. Swiss mice used in the recent study are CM susceptible mice, therefore it is expected that CXCL10 expression increases in those PbA-inoculated mice particularly on day 7 p.i. which is CM phase. The CXCL10 expressions were statistically different among the six-studied groups ($p = 0.003$). The CXCL10 expression of PbA-infected Swiss mice (the positive control group) was significantly lower than healthy groups which were without or with AME treatment (negative control, $p = 0.008$; X_1, $p = 0.045$; or X_2,

$p = 0.012$). The difference was also observed by comparing X_4 and X_2 groups ($p = 0.016$). This study was the first observed CXCL10 in the brain of Swiss mice during CM phase. The recent finding demonstrated that Swiss mice might modulate the immune response to ameliorate inflammation in the brain during CM phase. The increase number of large astrocyte clusters and astrocyte dilatation were found in PbA-infected CBA/CaH mice [50]. Neuronal and astrocyte apoptosis was found in PbA-inoculated C57BL/6 mice [51]. Astrocyte dilatation and apoptosis may modulate the astrocyte-CXCL10 expression in PbA-inoculated Swiss mice and explained the reduce CXCL10 expression in PbA-inoculated Swiss mice. Hipoxia and hypoglicemia is possibly reduce astrocyte-CXCL10 expression during malaria. *In vitro* study showed that the hypoxia and hypoglycemia result in reduce astrocyte-CXCL10 expression [16]. Whether all of these influencing brain-CXCL10 expression during CM phase of Swiss mice need to be elucidated.

The C57BL/6 mice study showed that astrocytes expressed CRG2 (CXCL10) mRNA [52]. By comparing C57BL/6 and Balb/c mice, it was found that genetic factors had strongly influence on intracranial chemokines. Astrocyte-CXCL10 expression in the brain of C57BL/6 mice was reaching peak earlier than Balb/c mice during *Toxoplasma* encephalitis [53]. Most CXCL10 studies on PbA used C57BL/6 mice, while this study used Swiss mice inoculated with 10^7 PbA-pRBC. C57BL/6 mice inoculated with 10^6 pRBC showed that CXCL10 mRNA in the brain was higher in PbA-inoculated C57BL/6 mice than those inoculated mice received vitamin D. The treated group showed a reduce adhesion molecules result in reduce pathologic T cell accumulation in the brain. Spleens of treated mice were also showed reduce differentiation, activation and maturation of DC, while increase T regulatory cell and IL-10 expression. Survival of PbA-inoculated mice was significantly longer in those received vitamin D [54]. Additionally, other study using C57BL/6 mice inoculated with 10^6 PbA-pRBC showed the increase CXCL10 mRNA in the brain and parasite biomass in PbA-inoculated mice with reticulocytosis than those without reticulocytosis [55]. The two studies compared CXCL10 expression between PbA-inoculated groups with and without particular treatment, while the trend of reduce brain-CXCL10 expression in our study was found by comparing PbA-inoculated than healthy Swiss mice. Additionally, similar finding was also observed by comparing healthy and PbA-inoculated group which both of them received similar treatment dose.

The similar degree of CXCL10 expression were found in those negative control, healthy Swiss mice receiving 100 or 150 mg/kg BW/day for period of 14 days. CXCL10 expressed by those healthy groups in our study has not yet been explained, therefore further studies warrant to be performed to confirm and find mechanism involve. Astrocytes cultured without any stimulation result in detectable level of CXCL10 in the culture supernatant [56]. This might in contribute in explaining that astrocyte of healthy Swiss mice expressed CXCL10 in our study.

Parasitemia levels were not different among positive control and two groups receiving AME treatment. Parasitemia might be not a sensitive marker for evaluating the benefice of AME treatment in this recent study. The pRBCs was sequestered in the brain blood vessels and other tissues of PbA-infected mice [57-59]. This may cause no association between parasitaemia levels and the development of CM. However, pRBCs sequestered in the brain blood vessels during ECM developed during PbA infection is remain a matter of debate, and even if there seems to be happening to a small extent [60]. PbA-infected mouse used and treatment used resulted in an obvious pRBC sequestered in the brain. CXCL10 KO mice reconstituted with bone marrow of wild type mice showed an obvious pRBCs and leukocytes sequestered in the brain [48].

Ang-2 levels were not normally distributed, and Kruskal-Wallis test showed no significant different among six studied group ($p = 0.175$). The interesting finding showed that the median value of Ang-2 levels of positive control group tended to be higher than negative control (Fig 2B). This study was the first showing circulating Ang-2 levels of Swiss mice during CM phase. A trend of higher Ang-2 levels found in positive control group during CM phase was not entirely supported by previous finding in CM patients. CM patients showed significantly higher circulating Ang-2 levels [24]. The study compared CM patients with retinopathy (CM-R) and CM without retinopathy (CM-N). The study recruited CM patients based on WHO criteria. Retinopathy considered positive when one or more of retinal finding including hemorrhage, vessel change, and papilloedema. Ang-2 levels of CM-R patients were

significantly higher than CM-N patients. Vascular dysfunction, indicated by the retinopathy, was obviously present in CM-R. The study defined CM-R patients as confirmed CM patients. The study, however, remained needed to be validated. It is open possibility that CM occur without obvious vascular dysfunction. Increase mRNA Ang-2 expression in the brain was observed in day 5 after PbA-inoculation of C57/BL/6 mice [21]. Circulating Ang-2 levels had been observed in PbA inoculated C57BL/6 mice as a model for placental malaria [61]. This recent study done, therefore, was the first study proved that circulating Ang-2 levels is not associated with CM phase of Swiss mice. The use of circulating Ang-2 level as biomarker in CM phase of Swiss mice should be taken with caution. No different found among the six studied group indicates that ethanolic-AME might have no relation with circulating Ang-2 levels of Swiss mice during CM phase. The finding, however warrant to be further confirmed by evaluating Ang-2 expression in the brain of Swiss mice during CM phase.

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