International Journal of Allied Medical Sciences and Clinical Research (IJAMSCR)

ISSN:2347-6567

IJAMSCR |Volume 8 | Issue 2 | Apr - Jun - 2020
www.ijamscr.com

Research article *Medical research*

Combination of songga wood stem (*strychnos lucida*) and act is associated to CXCL12 and parasitemia on *plasmodium berghei* anka infections

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ABSTRACT

Introduction

Snake wood (Strychnos lucida) and Artemisinin-based-combined-therapy (ACT) possess antimalarial and immunomodulatory activity. Combination of these two therapies on parasitemia and CXCL12, which have protective properties toward Plasmodium berghei ANKA (PbA) infection, is still unknown. This research aims to prove the association between combination of ethanolic extract from songga wood stem (EESWS)-ACT treatment and decreasing parasitemia level-increasing production of CXCL12 on Swiss Webster mice that are susceptible to PbA infections.

Methods

This experimental laboratory research applied post test only control group design that distributed 25 female Swiss Webster mice into 5 groups. K1 group consisted of healthy mice, whereas K2, K3, P1, and P2 group consisted of mice infected with PbA at the dose of 107 and diagnosed as positive at the 3rd day following the infection. K2 group had no treatment; K3 group was treated with ACT (combination of dihydroartemisin (DHP) and piperaquine) at the dose of 0,819 mg/kg weight/day. Group P1 and P2 were treated with EESWS at preventive dose (0,15 mg/kg weight/day) for ten days and since day 4 of infection, the groups were treated with EESWS at therapeutic dose (0,3 mg/kg weight/day). As for P2, ACT treatment was also included. 14-days treatment was finalized with observation of parasitemia level and spleen cell culture. Difference and correlation test were performed using SPSS.

Result

One-way-ANOVA and post-hoc Bonferroni test on parasitemia level (p=0,0001) showed that P1 (mean±SD%; 4,68±1,94) and P2 (4,06±1,13) were not significally different from K3 (6,40±0,96;p=1,00 and p=0,88); as well as P2 that was not significantly different from P1 (p=1,00). Kruskal-Wallis and Mann-Whitney U test of CXCL12 level (p=0.001) showed that P1 (median(min-max))ng/mL 0,51(0,45–0,58)) and P2 (0,95(0,93-1,79)) were not significantly different from K1 (2,71(0,42-2.81);p=0,117 and p=0,116). Parasitemia was not proven to be associated with CXCL12 (Pearson test; r=0.311; p=0,131).

Conclusion

Treatment of EESWS and EESWS-ACT combination are associated with decreasing parasitemia level and normal production level of CXCXL12 in spleen.

Keywords: Strychnos lucida, Dihydroartemisinin, Piperaquine

INTRODUCTION

Severe malaria becomes one of most crucial global concerns that contributes the highest number of death toll in the world [1]. Research on effectivity of ACT in Asia reported that ACT started to become resistant, proven by extended theraphy duration and parasite clearance [2]. In Indonesia, ACT resistance is proven by increasing LC_{50} level on *in vitro* test of artemisinin sensitivity toward *P. faslciparum* strain Papua 2300 [3]. One of the effective solutions to solve the problem is by providing adjuvant treatment that combines herbal extract and standar antimalarial medications. This combination improves the effectivity and antimalarial property of natural agents. Beside that, adjuvant therapy is potential in delaying parasitic resistance upon standard antimalarial medications [4].

Based on previous researches, *in vitro* test of kayu ular extract on *P. falciparum* using organic solvents with gradual polarity (n-hexane, ethyl acetate, and ethanol) proves that ethanolic extract of kayu ular had the highest antimalarial activity (IC_{50} : 3,09 µgml $^{-1}$) [5]. Based on *in vivo* test, ethanolic extract of kayu ular stem on PbA-infected Swiss Webster mice shows the highest and stringest antimalarial activity (IC_{50} : 8,478 mg/kg weight [6]. Aside from possessing antimalarial properties, several previous researches also showed that kayu ular has several immunomodulatory activities, such as antioxidant that reacts with free radicals and inhibits α-glucosidase [7], inhibition of nitric oxide (NO) production [8], inhibition of superoxide stimulation [9], increase in leucocyte production, and also phagocytic activity [10].

One of chemokines that actively increases during severe malaria case is CXCL12 (CXC Ligan 12) [11]. CXCL12 treatment on EMS model is proven to increase dendritic cells in spleen and is also indicated in controlling PbA infection [12]. CXCL12 induces Th1 cells to produce IL-10 that plays a significant role in controlling nonspecific and cellular immune reaction that reduces mediator activity of proinflammatory cytokines [13-15]. Ethanolic extract of songga wood stem (EESWS) is a potential natural adjuvant that is expected to show protective properties with ACT for Swiss Webster mice, especially from severe PbA infections.

EESWS treatment is expected to act as immunomodulating agent that increase CXCL12 level in spleen that stimulates and directs regulation of T cells activity. This action is directed to produce antiinflammatory cytokines and increase parasitemia inhibitory activity. This research aims to prove that the effectivity of EESWS is not only restricted to inhibit post-severe-malaria Plasmodium growth, but also to modulate immune system prior to and during severe malaria infection. By doing so, preventive dose may be applied prior to infection and therapy dose can be applied 3 days after infection.

METHODS

This experimental laboratory research employed post test only control group design. Inclusion criteria are 8-weeks-old healthy female Swiss Webster mice weighed 30-35 gr with normal anatomy, activity, and behavior. Sampling was performed using simple random sampling method. Random grouping was conducted 7 days after adaptation in Integrated Biomedical Laboratory, Faculty of Medicine, Universitas Islam Sultan Agung Semarang.

Random grouping yields 2 treatment groups and 3 control groups with 5 mice each. Samples of this research were 25 Swiss Webster mice distributed into 5 treatment groups. Group K1 consisted of healthy Swiss Webster mice. K2 group comprised of Swiss Webster mice with no treatment, whereas group K3 were Swiss Webster mice that were treated with ACT (DHP) 0,819 mg/kg weight/day. Group P1 was treated with EESWS at the dose of 0,15 mg/kg weight/day for prevention and 0,30 mg/kg weight/day for therapy. Group P2 was administrated with EESWS at the dose of 0,15 mg/kg weight/day for prevention and 0,30 mg/kg weight/day for therapy combined with ACT 0,819/mg/kg weight/day. On the 22nd day, blood collection using tail vein sampling was conducted to observe parasitemia percentage. Observation was performed with microscope (400x). Following blood collection, mice were then terminated and spleens were collected for supernatant culture. CXCL12 level measurement was performed using the supernantant culture and ELISA. Free variable of this research was treatment of EESWS and ACT combination therapy. Dependent variable of this research is production of spleen CXCL12 and parasitemia level.

Primary data was obtained from this research. Production level of CXCL12 and parasitemia level data were analyzed in SPSS 25. Production level of CXCL12 was analyzed for normality using Shapiro-Wilk test. Since all data showed nonnormal distribution, the test was continued with nonparametric Kruskal-Wallis test and Mann-Whitney post-hoc test to analyze any significant difference between two treatment group. Normality for parasitemia level data was analyzed using Shapiro-Wilk test. Analysis was then continued with One-Way-ANOVA and Bonferroni post-hoc test due to normally distributed data.

RESULTS

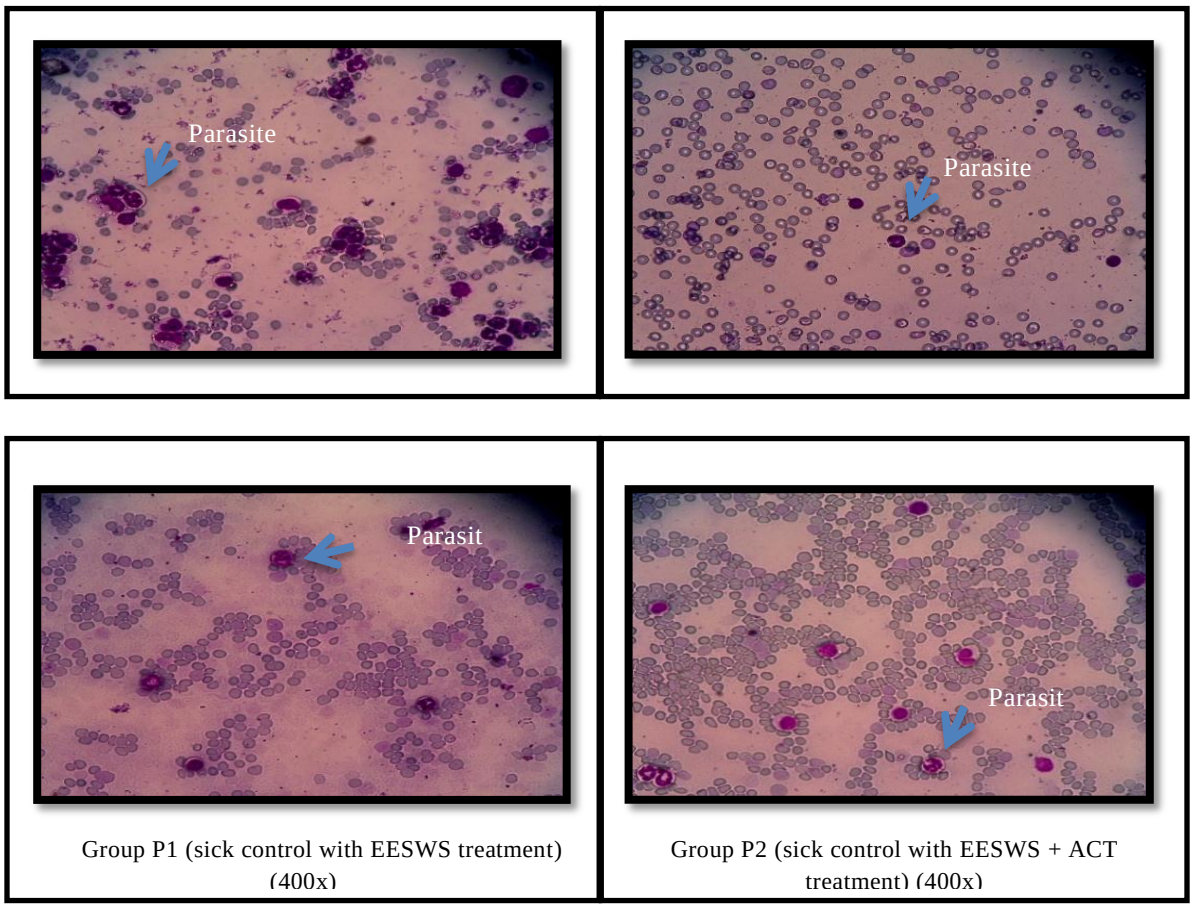

Fig.1. Results of blood observation sampled from tail vein of group K2, K3, P1, and P2 on day 22 under light microscope (400x).

Analysis of Parasitemia Level

Based on blood observation on day 22 from 4 groups of 20 Swiss Webster mice, the result is presented on Table 1 below.

Table 1. Bonferroni test result for parasitemia level.

Group	Average of parasitemia level (Mean ±SD)	*P* value		
		K3	P1	P2
K2	13.20 ± 4.18	0.03*	0,001*	0.001*
K3	6.40 ± 0.96	(-)	1.00	0.88
P1	4.68 ± 1.94		(-)	1.00
P2	4.06 ± 1.13			(-)

*significant difference ($p < 0,05$)

Results from descriptive analysis shows that highest average of parasitemia percentage is found in group K2 (13.20%), whereas the group that has the lowest average of parasitemia percentage group P2 (4.06%) as shown in Table 1. All data on parasitemia level from each group is normally distributed based on normality test. Levene test for homogeneity also yield the result that all four groups have homogenous parasitemia level data (p = 0.054). Difference test on parasitemia level from all 4 groups was conducted using One-Way-ANOVA. The result shows that all groups have significant difference ofn parasitemia level (p = 0.0001). Difference of parasitemia level between the groups was then continued with post-hoc Bonferroni test. This test was employed since all data met the criteria, which was normally and homogenously distributed. The result for post-hoc Bonferroni test is shown on Table 1, which explains the essential result from parasitemia level; group P1 and P2 are not significantly different from group K3.

Result of Spleen CXCL12 Production

Measurement of CXCL12 level from spleen cell culture of Swiss Webster mice was conducted using ELISA reader at the wavelength of 450 nm. The result of descriptive analysis tested using post-hoc Mann-Whitney test is shown below in Table 2.

Table 2. Mann-Whitney test results for CXCL12 production level.

Group	Median CXCL12 (max- min) ng/ml	*P* value			
		K2	K3	P1	P2
K1	2.71 (0.42-2.80)	0.347	0.009*	0.117	0.116
K2	4.78 (0.88-5.24)	(-)	0.009*	0.009*	0.249
K3	5.59 (5.55-6.09)		(-)	0.009*	0.009*
P1	0.50 (0.44-0.58)			(-)	0.009*
P2	0.96 (0.92-1.79)				(-)

*significant difference (p < 0,05)

Result of descriptive analysis shows that group K3 has the highest average of CXCL12 production level (5.84 ng/mL) and group P1 has the lowest average (0.50 ng/mL). Normality test shows that the data of CXCL12 production level is not normally distributed at p value < 0.05 on group K1 (p = 0.021), K2 (p = 0.024), and P2 (p = 0.009). Nonparametric Kruskal-Wallis test was applied for analysis which showed p value of 0.001 (p<0.05). Based on the result, there was a significant difference among two groups. Post-hoc Mann-Whitney test was then applied to observe which group that was different significantly for the CXCL12 production level data. The result is shown in Table 2. Post-hoc test shows that group P1 and P2 are not significantly different from group K1.

Result for Correlation Test between Parasitemia and Spleen CXCL12 Level

Based on research hypothesis, there is correlation between parasitemia and CXCL12 production level. Correlation test was conducted to observe the correlation between the increase and decrease of parasitemia level towards CXCL12 production level and vice versa. Pearson correlation test shows p = 0.131 with R value of 0.331. According to this result, parasitemia level does not correlate with CXCL12 production level.

DISCUSSION

Results from this research demonstrates association between ACT, EESWS, and EESWS-ACT combination treatment with controlled PbA infection, indicated by lower parasitemia level in group K3, P1, and P2 compared to nontreatment control (group K2) (Table 1). However, parasitemia level from groups that receive ACT and EESWS treatment are not different from each other. Treatment of EESWS-ACT combination shows lowest parasitemia level compared to each ACT or EESWS treatment. In the other hand, no significant difference is found between the three treamtent groups. These important results indicate that the capability of EESWS is no less than ACT in controlling PbA infection on Swiss Webster mice. However, EESWS is not proven to enhance ACT capability in controlling PbA infection.

Either EESWS or EESWS-ACT combination treatment produce CXCL12 level that is not

different from healthy control group, eventhough those two groups are associated with decreasing parasitemia level. This finding is different from previous researches that showed the importance of CXCL12 in controlling parasitemia level. Inhibition of CXCL12 by giving CXCR4-block affect the increasing parasitemia level of *P. ch. Chabaudi* in C57BL/6J mice [12]. Supplementation of CXCL12 escalates the PbA infection control on BALB/C mice. This treatment also increases the number of dendritic cells CD11c+ in spleen [16]. Previous researches also demonstrate that exogenous CXCL12 supplementation may be resulted in significant decrease of parasitemia level. ACT treatment turns out to be in line with previous researches. ACT treatment stimulates controlled PbA infection along with higher CXCL12 production level compared to healthy control (K1, p = 0,009*) or sick control (K2, p = 0,009*; (Table 4.2). It indicates that ACT is associated to increasing CXCL12 production above normal level in Swiss Webster mice accompanied with controlled PbA infection. Immunoprotective property from CXCL12 has been researched by administrating exogenous CXCL12 on PbA-infected, on *P. ch. chabaudi*, and *P. Yoelii* mice [16, 12, 17]. CXCL12 production level is higher in sick control group with the highest parasitemia level (K2, 4.78 (0.59 – 6.23) ng/mL) compared to healthy control (K1, 2.71 (0.91 – 3.42) ng/mL) (Table 2). Difference in CXCL12 production level between two groups is not significant (p = 0.347). This finding is not in line with previous research that shows significant difference in CXCL12 production level from both groups[18]. This difference is presumably cause by different method in spleen cell isolation.

Protective effects and mechanisms of CXCL12 on *Plasmodium* and non-*Plasmodium* infections have been widely researched. *In vitro* and *in vivo* studies demonstrate CXCL12 role in B cell and centrum germinativum formation under *P. yoelii* infection in spleen of mice [17]. B cell is a type of differentiated cell from Sca-1+ c-Kit$^-$ (LSK$^-$) lineage in spleen during *P. yoelii* infection. LSK cell generates IL-17 during the *P. yoelii* infection. IL-17R signal is involved in B cell differentiation process. This signal also includes other cell types aside from LSK cell. IL-17 triggers CXCL12 production by stromal cell. Inhibition of CXCL12 production will decrease B cell differentiation rate. This finding indicates that CXCL12 is directly invloved in B cell differentiation. CXCL12 is mostly produced by stromal cell on red pulps inside the spleen of C57BL6 mice during *P. yoelii* infection. CXCL12 and LSK cells contribute in building specific humoral immunity *P.yoelii*-infected mice [19]. Spleen observation, which is related to centrum germinativum, mature B cell, and IL-17 production, will provide an explanation in protective mechanism of ACT treatment mediated by increasing spleen CXCL12 production in PbA-infected Swiss Webster mice. Stable analog CXCL12 supplementation (CTCE-0214D) inside the plasm of mice, which is then administered with LPS through subcutaneous injection, will yield lucrative results [20]. Analog CXCL12 treatment also stimulates antiinflammatory, antioxidant, and protective effects toward cells in mice.

CXCL12, along with CXCR4 as the receptor, exhibits proinflammatory effects. CXCR4-knockdown obstructs production of proinflammatory cytokines by macrophage through mechanism of activation suppression on MAPK and NF-kB [21]. However, previous researches have also proven that CXCL12/CXCR4 also yields antiinflammatory effects. CXCL12 modulate monocyte differentiation that acts as immunosuppressant in autocrine pathway [22]. Monocyte from peripheral blood also produces CXCL12 and expresses CXCR4 and CXCR7. CXCL12, through autocrine and paracrine pathwya, affects monocyte differentiation into macrophage by expressing CD163+high or becoming dendritic cell that stimulates antigen-specific T-cell. This finding is also strengthen by following research that state CXCL12/CXCR4 play a role in stimulating monocyte polarization into M2 CD163+ or resident macrophage IL-10+ [23, 24]. Other findings also show that CXCL12 from platelet product is associated to CXCR7 from monocyte that will be continued by prolonged monocyte survival [23]. CXCL12 triggers CD14+ monocyte transmigration. However, CXCL12 selectively suppress CXCR4 expression, but not with T cell [25]. Stable analog CXCL12 supplementation (CTCE-0214D) inside the plasm of mice, which is then administered with LPS through subcutaneous injection, will yield lucrative results [20]. Analog CXCL12 treatment triggers antiinflammatory, antioxidant, and cellular protective responses.

High CXCL12 level acts as chemorepellant on Th1 cell [26]. Protective effects from ACT will be supported by the proof of ACT treatment that inhibits Th1 cell migration toward blood brain barrier (BBB) that yields CXCL12. CXCL12 will stimulate IL-10 production by Th1 cell[27]. CXCL12 is required to prolong naive CD4+ cell survival, eventhough the effect from CXCL12 is less efficient toward the cells. CXCL12 improves Bcl2 and BclXL production that prolongs naive T CD4+ cell survival[29]. Naive T CD4+ cell survival and IL-10 will exhibit protective effect toward EMS. Further research is needed to examine in-depth mechanism by ACT to inhibit EMS development.

Either EESWS or EESWS-ACT combination treatment will provide different protective effect from low production of CXCL12 that is accompanied by decreasing parasitemia level. Several researches bring through possibilities that both treatment is related to improvement in phagocytic activity and decreasing amount of Treg cells. Bacterial infection researches show that *in vivo* EESWS treatment increases leucocyte production and also elevate phagocytic activity [10]. CXCR4 as CXCR12 receptor is also proven to increase the number of Treg cells [30].

Parasitemia and CXCL12 level is proven to be not correlated. This finding also suggest that there are other factors involved in controlling parasitemia level and also CXCL12 level. Either EESWS-ACT treatment or single treatment (ACT or EESWS) significantly decreases parasitemia level compared to nontreatment control. Parasitemia level resulted from EESWS-ACT treatment is not significantly different from the single treatment (Table 4.1). Another interesting finding is that both EESWS-ACT and EESWS treatment are related to lower CXCL12 level resulted from ACT treatment (Table 4.2). The lowest CXCL12 level is found in EESWS treatment, eventhough it is not significantly different from EESWS-ACT treatment. CXCL12 level from these two groups are not significantly different from healthy control. Protective effects from both EESWS and EESWS-ACT treatment will be better examined after observation and survival test of parasitemia level after day 7 of PbA infection. Another considerable possibility is the toxic level of *Plasmodium* as an involved in this research. Parasitic erythrocyte phagocyted by macrophage will be resulted in M2 polarization [31, 32]. In the other hand, both malaria toxins (GPI and Hz) stimulates proinflammatory immune response [33, 34].

Further research is neede to examine the correlation between parasitemia-malarial toxin level combination and CXCL12 production level on either EESWS-ACT combination or single treatment. Different trigges for immune response induces antiinflammatory effect from CXCL12 through different receptors, which are CXCR4 and CXCR7 [35, 36]. CXCL12 modulates differentiation of various immune cells, such as monocyte. CXCL12 induces polarization of IL-10+ resident macrophage [24]. CXCL12 effects on monocyte, T, and B cell has been widely known. Inhibition of response on CXCL12 or antagonistic CXCR4 treatment will decrease T cells amount that migrate through high endothelial cell [37]. In the other hand, high CXCL12 level will have chemorepellant effect toward lymphocyte [26]. CXCL4 as CXCL12 receptor hampers cytotoxic T cell infiltration (Tc cell) [30], which is proven through increase in tissue Tc cell infiltrates by antagonistic CXCR4. Immunomodulatory effect from active component of EESWS will suppress CXCL12 production in mice during healing phase after PbA infection.

ACT, EESWS, and EESWS-ACT combination treatment control PbA infections. Both EESWS and EESWS-ACT combination normalize CXCL12 production that prepares immune response in controlling recurring PbA infection. ACT, which is able to increase CXCL12 production, enables immune response to provide protective effect from EMS. Further research is required to examine the protective effect upon reinfection and survival.

CONCLUSION

Both EESWS and EESWS-ACT treatment produce CXCL12 level that are not significantly different from healthy control. These two treatments are associated to decreasing parasitemia level.

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How to cite this article: Edwin A Septory, Kis Djamiatun, Endang Mahati. Combination of songga wood stem (*strychnos lucida*) and act is associated to CXCL12 and parasitemia on *plasmodium berghei* anka infections. Int J of Allied Med Sci and Clin Res 2020; 8(2): 215-222.

Source of Support: Nil. **Conflict of Interest:** None declared.