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The Effectiveness Combination of Soursop Leaf Water Extract (*Annona muricata*) and Artemisinin Based Combine Therapy on Increased Spleen CCL22 Production

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ABSTRACT

Severe *Plasmodium falciparum* infection can result in cerebral malaria (MS). Giving herbal medicine as an adjuvant may be one solution to help the healing process due to reduce the parasitaemi-level and increase the protective-immunity. The immune response during malaria is mostly associated with the spleen, CCL22 is a chemokine produced by spleen-antigen-presenting-cells and serves to recruit Th2 cell arrivals.

This study was a laboratory experimental study with a post test only randomized control group design,used 24 Swiss-mice consisting of 4 groups namely K, T1, T2, and T3 in each group inoculated by 10^7*Plasmodium berghe* ANKA(PbA). K-group was kontrol without any treatment, T1 was given *Annona muricata* leaf-extracted by water (AME) in a preventive dose 4.68 mg/day and subsequently therapy dose 9.36 mg/day. T2 was given ACT 0.819 mg/day. T3 was given both AME and ACT in dose mention before. Statistical tests used were *Kruskal-Wallis test* followed by *Mann-Whitney U test.*

The statistical analysis showed that each treatment-group had significantly higher spleen-CCL22-production than K-groups (T1, p=0.003; T2, p=0.009; and T3, p=0.017). T3-group however had no significant difference CCL22 production than T1 or T2-groups.

The conclusion is that ADAM-ACT-combination increases spleen-CCL22-production as high as ADAM or ACT-treatment in PbA-infected-Swiss-mice.

Keywords: ADAM, CCL22, Cerebral Malaria.

INTRODUCTION

Malaria is an infectious disease caused by *Plasmodium sp* and is an important disease because it causes the death of 60% of the 3.2 billion people at risk throughout the world in 2015 [1]. Several reports on resistance to *Artemisinin Based Combine Therapy* are important to consider in malaria therapy.[2]Giving herbal medicine as an adjuvantwhich increase protective immune response, may be one solution needs to be done to help the healing process in addition to suppressing parasitemic numbers. Research on herbal medicines is also important to be carried out in the context of developing phytopharmaca.

Malaria can cause anemia partly because many blood cells are destroyed damaged by *Plasmodium sp.* Severe malaria can be fatal,

including malaria cerebral (MS). MS patients and experimental cerebral malaria (EMS) in animals prove that excessive immune responses underlie the occurrence of these fatal complications. The immune response formed during the course of malaria, is a determinant of healing or otherwise complications arise in malaria.

The immune response during malaria is mostly associated with the spleen, which is a secondary lymphoid organ where the adaptive immune response is activated after recognizing pathogenic antigens including *Plasmodium sp* [3]. CCL22 is a chemokine produced from spleen antigen presenting cells and functions to recruit the arrival of Th2 cells [4]. Several studies on CCL22 have been conducted, but there have been no studies on severe infections of *plasmodium*.

Annona muricata leaf-extracted by water (AMEW) is commercially available in the community; thereforeit is important to examine the benefits of severe malaria. The administration of *A. muricata* leaf-extracted by ethanol (AMEE) effectively reduce spleen-TNF-α-production and increase spleen-NO-production in mice that are inoculated with *Plasmodium berghei* ANKA (PbA) [5]. These immunomodulatory effects have potential roles in preventing cerebral malaria. AMEE has been proven to suppress parasitemic levels on day 3 after PbA inoculation, while this effect is no longer visible on days 5 and 7.

A. muricata extract studies have been carried out on experimental cerebral malaria (ECM) using methanol extracts that are turned out to be more toxic compared to water extracts (AMEW). Phenol levels in AMEW is higher than that extracted with methanol. Additionally, AMEE has a parasitidal effect but its immunomodulatory effect appears to play a greater role [6]. This present study therefore usedAMEW as an adjuvant therapy of standard malaria medication, *Artemisinin-based Combination Teraphy* (ACT).

METHOD

This research was a laboratory experimental study with a *Post Test Only Randomized Control Group Design* that used Swiss strain mice as research objects. The mice were randomly selected into groups that were treated differently and observed afterwards. The treatment used was AMEW, ACT and combination of AMEW-ACT. AMEW doses used were4.68 mg/day as preventive dose for period of 10 days (7 days before PbA-inoculation and 3 days after PbA-infection)followed by 9.36 mg/day as therapy dose (since day 4 of PbA-infection when parasitaemia had been confirmed). ACT (0.819 mg/day) was given since day 4 of PbA-infection. Spleens were isolated, processed and cultured on day 7 of PbA-infection. Splenocyte-CCL22 -production was then measured from supernatant of splenocytes culturedwith lipopolysaccharide (LPS). Elisa-method by utilizing elisa-CCL22-kit was used to measure CCL22 concentration.

The animal used in this study was healthy female Swiss-mice aged 6-8 weeks. The number of mice in each group was six which determined based onthe regulations of World Health Organization(WHO) with additional one mice to anticipate drop out. Therefore, the total number of 24 mice was used. The independent variable is the combination of soursop leaf (*A. muricata*) and ACT. Dependent variable is CCL22 level. Confounding variables (age, sex, weight, food, health and environment) had been controlled.

Data Analysis

CCL22 data is processed and presented in tables and boxplots. Normality was tested using *Saphiro Wilk*. CCL22 data that were normally distributed in each group proved to be inhomogeneous. CCL22 different data test was then performed with the *Kruskal-Wallis test* to see the median differences in the five study groups. The different test was continued with the *Mann-Whitney U test*, to find out the median size between the two groups. All statistical analyzes were carried out using the SPSS program. The significance value in this study is if the analyzed variable has a p value <0.05.

RESULTS

The normality test for each group showed that CCL22 data for each group was normally distributed (p= 0.374 for K; p = 0.707 for T1; p = 0.653 for T2 and p = 0.686 for T3), the homogeneity test showed that CCL22 data were not homogeneous (p= 0.004).Therefore

nonparametric-statistical analyzes was used. *Kruskal-Wallis* test showed significant difference in CCL22 levels in all studied-groups (p = 0.013). This then followed by *Mann-Whitney U* test, to find out the differences between groups (Table 1). All treatment groups (T1, T2 and T3) showed CCL22 median values higher than K-group, and *Mann-Whitney U* test showed that these deference were significant (T1, p= 0.003;T2, p = 0.009 and T3, p = 0.017). By comparing T3-group with T1 and T2-groups, it was found that there were no significant differences (p= 0.117 and p= 0.295, respectively).

Table 1. Mann-Whitney U Test Result of CCL22 Levels

Group	Median (min – maks) pg/mL	T1	T2	T3
K	899.85(557.28-999.36)	0.003	0.009	0.017
T1	969.54(898.69-1021.80)		0.016	0.117
T2	1006.00(995.65-1011.80)			0.295
T3	1011.40(1001.70-1023.00)			

*sig < 0,05

DISCUSSION

This study is the first to prove the association between AMEW-ACTcombination treatment with increased CCL22 production of Swiss mice spleen cells and PbA-infection (Table 4.1). This evidence was based on the finding that AMEW-ACT group produced significantly higher CCL22-level than controls with PbA-infection. The research was carried out by giving AMEW in preventive and therapy dose in addition to ACT, a standard malaria therapy.The findings of this study therefore, add to the knowledge of herbal (AMEW) administration as a preventive and adjuvant therapy for severe malaria whichincrease the production of CCL22.

The CCL22 chemokine produced by APC plays the role of recruiting Th2 cells [4]. IL-10, a Th2-cytokineprevents severe malaria including CM [7]. Observations in populations that are frequently exposed to *P. falciparum* with specific IgG serological indicators of *P. falciparum* show CCL22 production is detected in *neonates*, while CCL22 is not detected in mothers and children over 10 years [8]. The arrival of CD4 + Th2 cells is expected to prevent the development of severe malaria immunopathology in severe PbA infections. The superiority of AMEW-ACT combination treatment over its single treatment has not been proven in this study. Significant increases in CCL22 production by splenic cells were observed in all treatment groups (T1, T2 and T3) compared to controls with PbA infection (K-group) (Table 1). Further analysis showed that no significant differences of CCL22 production were found between T3 compared to T1 or T2. The ability to increase the production of CCL22 mice's spleen cells with PbA infection in the AMEW-ACT combination treatment did not differ significantly from each of the single treatments AMEW or ACT alone. The AMEW-ACT treatment was associated with the healing phase of PbA infection (data has not been published), while AMEW single treatment and control with infection were associated with severe PbA infection. The evidence in this study and previous research thus shows that the combination treatment of AMEW-ACT and single treatment ACT is associated with increased production of splenic cell CCL22 in the healing phase of PbA infection in Swiss mice. The single treatment of AMEW is associated with increased production of splenic cell CCL22 in severe PbA infection, and this evidence was supported by research on *Annona muricata* leaf extracted by ethanol (AMEE). AMEE increases the IL-10-production of splenic cells in severe PbA infections of Swiss mice. The increase IL-10 production of those with AMEE treatment is nonethelesslower and significantly different than the production of healthy control [9]. Previous research also found quite interesting results that CCL22 also recruited Treg cells [10]. All of these evidences and the fact that IL-10 can be produced by Treg cells that play a role in inhibiting cell poliferation, this CCL22 research needs to be supported by evidence that increased CCL22 production increases the number of splenic Treg cells.

The AMEW treatment significantly increased the spleen-CCL22 levels of PbA-infected-Swiss mice when compared to the negative control group (Table 1, p = 0.003).This proves that various active ingredients including *flavonoids* in AMEWhave a role in increasing the production of CCL22 in the phase of severe PbA infection. A significant differencein spleen CCL22 production was notfound in the AMEW-ACT combination treatment group compared to the single ACT treatment (Table 1, p= 0.295). The AMEW treatment thus did not increase the production of splenic CCL22 in the healing phase of severe PbA infection and the healing phase of Swiss mice.

The statistical analysis of CCL22 in the combined treatment of AMEW and ACT compared with the administration of AMEW or ACT alone were not significantly different (respectively p= 0.117 and p= 0.295) although the two single treatments increased CCL22 production significantly. This evidence and the findings of previous studies indicate that administration of AMEW in preventive dosefollowed by treatment dose can increase CCL22 production, although it has no effect on the level of parasitemia of severe PbA infection, while AMEW has no proven effect on CCL22 production nor the level of parasitemia in the healing phase of Swiss mice receiving ACT. The incubation period of PbA causes the number of PbA to increase and causes a large number of pRBC and components of Hz, GPI, free hemetherefore it will affect the amount of mature DCs and CCL22 production in the spleen.

The *A.muricata* leaf extract (AME)in this study used a water solvent that might not guarantee sufficient quantities of the active substance dissolved in the solvent. Additionally, the solvents might affect the dose given to the experimental animals. Another limitation in this study is that it does not measure Swiss Treg levels in Swiss yang inoculated with PbA. The latest findings showed the protective role of Treg cells in malaria [11, 12]. This opens up opportunities for further research to look at the role of Treg in cerebralmalaria inmice *Swiss*.

CONCLUSION

Based on the results of this study the combination of AMEW-ACT increase levels of CCL22 in Swiss mice infected with PbA in detail:

1. A combination of AMEW-ACT increases spleen CCL22 production of PbA-infected-Swiss significantly higher than PbA-infected-controls.
2. A combination of AMEW-ACT increases spleen CCL22 production of PbA-infected-Swiss as high as single treatment of AMEW or ACT.

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