

# Effect of N-Acetyl Cysteine administration to the degree of parasitemia and plasma interleukin-12 level of mice infected with plasmodium berghei and treated with artemisinin

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## Abstrak

**Pendahuluan** Proteksi terhadap infeksi malaria memerlukan respon imun seluler yang dimulai dengan pelepasan interleukin-12 (IL-12) dari antigen presenting cells (APC). N-Acetyl Cysteine (NAC) merupakan precursor dari glutathione, sementara glutathione dapat meningkatkan produksi IL-12. Pemberian NAC yang dikombinasi dengan artemisinin diharapkan akan meningkatkan imunitas seluler dari mencit selama infeksi Plasmodium berghei. Tujuan dari penelitian ini adalah untuk mengetahui efek pemberian NAC terhadap derajat parasitemia dan kadar IL-12 plasma pada mencit yang diinfeksi dengan P. berghei dan diterapi dengan artemisinin.

**Metode** Desain dari penelitian ini adalah post-test-control-only design menggunakan 5 kelompok mencit yang terdiri dari kelompok A (kelompok kontrol negatif), kelompok B (kelompok kontrol positif, atau mencit yang diinfeksi dengan P.berghei tanpa terapi), kelompok C (mencit yang diinfeksi dengan P.berghei dan diterapi dengan artemisinin 0.04 mg/g BB selama 7 hari), kelompok D (mencit yang diinfeksi dengan P.berghei dan diterapi dengan artemisinin kombinasi dengan NAC 1 mg/g BB selama 7 hari) dan kelompok E (mencit yang diinfeksi dengan P.berghei dan diterapi dengan artemisinin kombinasi dengan NAC 1 mg/g BB selama 3 hari dan diturunkan atau tapering menjadi 0,5 mg/g BB selama 4 hari). Parasitemia diikuti setiap 2 hari dan setelah 6 hari pasca infeksi atau ketika derajat parasitemia mencapai  $\pm 10\%$  terapi dimulai. Pada hari ke 3, 5, dan 7 setelah terapi, mencit dari tiap kelompok diterminasi dan diukur kadar IL-12 plasma (ELISA, Bender Medsystems GmbH, Vienna, cat. BMS6004).

**Hasil** Hasil penelitian menunjukkan bahwa semua mencit yang diterapi baik dengan artemisinin mono-therapy maupun terapi kombinasi artemisinin dan NAC, derajat parasitemia turun secara bermakna ( $P=0.000$ ). Tidak ada perbedaan yang bermakna ( $P>0.05$ ) dalam menurunkan derajat parasitemia di antara kelompok kontrol maupun kelompok perlakuan. Kadar IL-12 plasma meningkat secara bermakna pada kelompok yang menerima terapi kombinasi artemisinin dan NAC baik dosis konstan maupun dosis tapering dibandingkan dengan kelompok yang menerima artemisinin mono-therapy ( $p < 0,05$ ). Kadar IL-12p70 plasma pada kelompok kombinasi artemisinin dan NAC dosis tapering lebih tinggi secara bermakna daripada kelompok lainnya pada hari ke 5 dan 7 pasca terapi.

**Kesimpulan** Kesimpulan dari penelitian ini adalah artemisinin mono-therapy menurunkan derajat parasitemia sama efektifnya dengan kombinasi artemisinin and NAC. Terapi kombinasi artemisinin dan NAC, dosis konstan maupun tapering, lebih meningkatkan kadar IL-12p70 plasma daripada artemisinin mono-therapy. Kadar IL-12p70 plasma tertinggi ditekan pada kelompok yang diterapi kombinasi artemisinin dan NAC dosis tapering pada pengobatan selama 7 hari. (Med J Indones 2009; 18: 5-9)

## Abstract

**Introduction** Protection against malaria requires a cell-mediated immune response which is initiated by releasing interleukin-12 (IL-12) from antigen presenting cells (APC). N-Acetyl Cysteine (NAC) is a precursor of glutathione, while glutathione itself increases IL-12 production. Treatment with NAC combined with artemisinin is supposed to increase cellular immunity of mice during Plasmodium berghei infection. The aim of this study was to measure the effects of NAC administration on the degree of parasitemia and plasma IL-12 level in mice infected with P. berghei and treated with artemisinin.

**Method** The research was done using post-test-control-only design using 5 groups: group A (negative control group), group B (positive control group, or mice infected with P.berghei without therapy), group C ( mice infected by P.berghei and received artemisinin 0.04 mg/g BW for 7 days), group D (mice infected with P.berghei and received artemisinin in combination with NAC 1 mg/g BW for 7 days) and group E (mice infected with P.berghei and received artemisinin

in combination with NAC 1 mg/g BW for 3 days and tapered into ½ mg/g BW for 4 days). Parasitemia was followed up every two days. Approximately six days post infection or when the degree of parasitemia reached  $\pm$  10% therapy was begun. On the 3rd, 5th, and 7th days post therapy, mice from each group were terminated and assayed for plasma IL-12 level (ELISA, Bender Medsystems GmbH, Vienna, cat. BMS6004).

**Results** All mice treated with artemisinin mono-therapy and combined therapy had significantly decreased parasitemia ( $P=0.000$ ). There was no significant difference ( $P>0.05$ ) in decreasing parasitemia among treatment groups. The plasma IL-12 level increased significantly in both groups that received the combination of artemisinin and NAC constant dose and tapering dose compared with the group that received artemisinin mono-therapy ( $p < 0,05$ ). Plasma IL-12p70 level in the combination of artemisinin and NAC tapering dose therapy group was higher than other groups on the 5th and 7th days post therapy.

**Conclusion** The conclusion of this research is that artemisinin mono-therapy decreased parasitemia effectively as well as the combination therapy of artemisinin and NAC. Artemisinin and NAC therapy, constant and tapering dose, increase plasma IL-12p70 level more than artemisinin mono-therapy does. The highest plasma IL-12p70 level was found in the artemisinin plus NAC tapering dose therapy group with seven days duration of therapy. (**Med J Indones 2009; 18: 5-9**)

**Keywords:** N-acetyl Cysteine, Parasitemia, Interleukin-12, Plasmodium berghei

Malaria remains a major public health problem in most tropical countries, including Indonesia. It has been estimated that 300 to 500 million individuals become infected annually and more than one million people die from malaria every year.<sup>1</sup> Approximately 60% of Indonesian people inhabit malaria endemic areas, with various levels of endemicity.<sup>2</sup>

Malaria is caused by Plasmodium, an intracellular protozoa with *Plasmodium falciparum* as the predominant species in humans. *Plasmodium berghei* (*P. berghei*), one of the species infecting rodents, has been widely used as a model for malaria research because of its molecular similarity with *P. falciparum*.<sup>3</sup>

Protection against malaria requires a cell-mediated immune response initiated by the release of interleukin-12 (IL-12) by monocytes, macrophages, and other cells.<sup>4</sup> Down regulation of IL-12 was observed in non-immune African children with severe *P. falciparum* malaria.<sup>5</sup>

Interleukin-12 (IL-12) is a hetero-dimeric cytokine composed of 35-kD (p35) and 40-kD (p40) subunits.

The biologically active form of IL-12 is a protein with 70-kD molecular weight (p70).<sup>6</sup> Blood stage malaria suppresses systemic IL-12 activity because monocytes ingest hemozoin and promote the overproduction of TNF- $\alpha$  and IL-10.<sup>7</sup>

*N-Acetyl Cysteine* (NAC) was estimated to be a potential antimalaria agent because its cysteine proteinase inhibitor can stabilize the erythrocyte membrane.<sup>8</sup> NAC is a precursor of glutathione, and glutathione increases the production of IL-12 from antigen presenting cells.<sup>9</sup>

Hydrogen peroxide ( $H_2O_2$ ), a major component of reactive oxygen species (ROSs), inhibits induction of IL-12 p70 in murine macrophages.<sup>10</sup> Another study reported that there was a decrease of free radicals and an increase of macrophage activity in mice infected with *P. berghei* and treated with a combination of chloroquine and NAC.<sup>8</sup>

Artemisinin is the new future of antimalaria drugs. In order to avoid the occurrence of resistance and to reduce adverse effects of free radicals produced by arthemisinin, it is recommended that Artemisinin is used not as a single therapy, but in combination with other anti malaria or supportive drugs.

A study using artemisinin combined with NAC in severe falciparum malaria showed that there was no adverse reaction to NAC and no mortality in severe malaria patients.<sup>11</sup> Patients treated with NAC showed accelerating normal serum lactate level, but the mechanism of action is still unclear. The aim of this study was to measure the effects of NAC administration to the degree of parasitemia and plasma IL-12 level in mice infected with *P. berghei* and treated with arthemisin.

## METHOD

**Mice.** All experiments were carried out with procedures authorized by The Ethics Committee of The University of Brawijaya. Female mice strain BALB/c from Pusat Veterinaria Farma Surabaya were used as samples. Mice were housed according to treatment, there were A, B, C, D, and E groups. Normal mice group (A), non therapy of infected mice group (B) artemisinin therapy

group (C), Combined artemisinin and NAC constant dose therapy group (D), and Combined artemisinin and NAC tapering dose therapy group (E).

**Parasite.** *P. berghei* ANKA strain from Laboratory of Parasitology, Medical Faculty Gajah Mada University was used. Before inoculation, *P. berghei* was thawed from liquid nitrogen and washed with RPMI in the laminary flow. Inoculation was done by intraperitoneal (i.p) injection of  $10^6$  parasite in 0.2 mL blood/mice.

Previously, the number of erythrocytes per mL of blood and parasitemia of mice donor were counted. Blood from the tails were taken  $\pm 10\mu\text{L}$  and diluted  $10^4$  in RPMI. The number of erythrocytes were counted in *erythrocytes Naubauer*, using the formula  $N \times 5 \times 10^4 \times \text{dilution}$ , N is the number of erythrocytes. The number of parasites from mice donor was counted by multiplying the number of erythrocytes per mL of blood and the percentage of parasitemia. After *P. berghei* was transferred to mice, every two days parasitemia was checked. Approximately six days post infection, or when parasitemia reached  $\pm 10\%$  therapy was begun.

**Parasitemia.** Parasitemia was determined microscopically from blood smear by counting the percentage of infected erythrocytes from 1000 erythrocytes at 1000X magnification. The blood smear was stained with Giemsa Fluka Giemsa in buffer 1: 9 proportion. Giemsa staining was conducted for 20 minutes.

**Administration of Artemisinin and N-Acetyl Cysteine (NAC).** Artemisinin was given to mice orally 0.04 mg/g BW/daily for 7 days. Constant dose of NAC 1 mg/g BW/daily was given to mice orally for 7 days and tapering dose given to mice were 1 mg/g BW for 3 days continued with 0.5 mg/g BM per oral for 4 days.

**Measurement of IL-12p70.** Blood samples from mice collected from the heart were taken into BD Vacutainer Lithium Heparin (BD Franklin Lakes NJ USA, REF 367884). Plasma was separated from blood samples by centrifugation in  $4^\circ\text{C}$  at 3000 RPM for 5 minutes. Plasma IL-12p70 level was determined by quantitative sandwich enzyme-linked immunosorbent assay (ELISA; Bender Medsystems GmbH, Vienna, cat. BMS6004) following the manufacturer's procedure. The sensitivity of detection for cytokine was  $< 10$  pg/ml.

**Statistical Analysis.** Comparisons across multiple groups were performed by ANOVA one way, and when differences were significant, pair-wise comparisons between conditions were performed by Tukey. Pearson

correlation analysis was used to know the correlation between parasitemia and plasma IL-12p70 levels. Determination of significance was at  $\alpha = 0.05$ .

## RESULTS

### Parasitemia

All of the mice were treated at the time when parasitemia reached  $\pm 10\%$ . As shown in Figure 1.

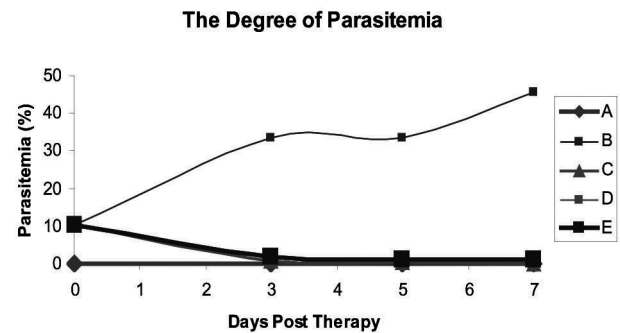


Figure 1. Parasitemia in mice infected with *P. berghei* and treated with combined artemisinin and NAC.

Normal mice group (A), non therapy of infected mice group (B) artemisinin therapy group (C) artemisinin plus NAC constant dose therapy group (D), and artemisinin plus NAC tapering dose therapy group (E). Parasitemia was observed on the 0, 3<sup>rd</sup>, 5<sup>th</sup>, and 7<sup>th</sup> day post therapy.

The degree of parasitemia in mice that were given artemisinin mono therapy and combined therapy were significantly decreased ( $P=0.000$ ). There was no significant difference ( $P>0.05$ ) of decreasing parasitemia among artemisinin mono therapy group (C), combination of artemisinin and NAC in constant dose therapy group (D), and combination of artemisinin and NAC tapering dose therapy group (E). The percentage of parasitemia among those groups on the 0, 3<sup>rd</sup>, 5<sup>th</sup>, and 7<sup>th</sup> days post therapy were not significantly different ( $P>0.05$ ). The highest parasitemia was observed in the non-therapy infected group (B) on the 7<sup>th</sup> day (45.47%) and the lowest parasitemia in the artemisinin therapy group (C), 0.17%.

### Plasma Interleukin-12p70 (IL-12p70) Level

Plasma Interleukin-12p70 (IL-12p70) level in the normal mice group (A) was 1021.810 pg/mL. Infection of *P. berghei* caused an alteration of the plasma IL-12p70 level. As shown in Figure 2, plasma IL-12p70 level in all mice infected with *P. berghei* decreased (277.048 pg/mL) day

0 and increased on the 3<sup>rd</sup> day post therapy. There were no significant difference among all groups on the 3<sup>th</sup> day post therapy. Plasma IL-12p70 level in the combination of artemisinin and NAC tapering dose therapy group (E) was higher than that in other groups on the 5<sup>th</sup> and 7<sup>th</sup> days post therapy. The highest plasma IL-12p70 level from all mice was found in the combination of artemisinin and NAC tapering dose therapy group (E) on the 7<sup>th</sup> day post therapy (1432.286 pg/mL). The plasma IL-12p70 level of artemisinin and NAC tapering dose therapy group (E) on the 7<sup>th</sup> day post therapy was significantly different ( $P < 0.05$ ) with that in normal mice group (A), non-therapy of mice infected (B) on the 3<sup>th</sup>, 5<sup>th</sup> and 7<sup>th</sup> days post therapy, artemisinin therapy group (C) on the 3<sup>th</sup>, 5<sup>th</sup> and 7<sup>th</sup> days post therapy and combination of artemisinin and NAC constant dose therapy group (D) on the 3<sup>th</sup> and 5<sup>th</sup> days post therapy. The plasma IL-12p70 level showed a weak inverse correlations with parasitemia ( $r = -140$ ).

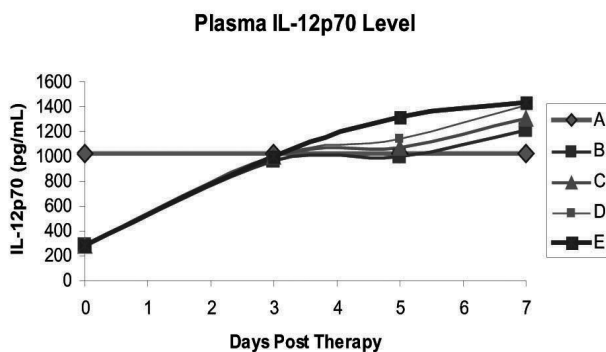


Figure 2. IL-12p70 Plasma level in mice infected with *P. berghei* and treated with combined artemisinin and NAC.

Normal mice group (A), non therapy of infected mice group (B) artemisinin therapy group (C) artemisinin plus NAC constant dose therapy group (D), and artemisinin plus NAC tapering dose therapy group (E). Level of plasma IL-12p70 was measured on the 0, 3<sup>th</sup>, 5<sup>th</sup>, and 7<sup>th</sup> day post therapy.

## DISCUSSIONS

*N-acetyl-cysteine* (NAC) is an acetylated cysteine residue. NAC is deacetylated to yield L-cysteine in cells. NAC can promote intracellular glutathione (GSH) synthesis,<sup>13</sup> while GSH promoted IL-12 production by antigen presenting cells. This could trigger a differentiation of the immune response to the pathway of type 1 T helper.<sup>9</sup> Using NAC without an antimalaria was investigated in mice infected with *P. berghei*, but single therapy of NAC had no effect in decreasing parasitemia, it was proposed that NAC acts as cytostatic.<sup>8</sup> Therefore, this

research used NAC with an antimalaria, artemisinin. Artemisinin was recommended in combination with other antimalaria or supportive drugs to avoid resistance.<sup>2</sup> Artemisinin derivatives act as blood schizontocidal agents that effectively inhibit parasites growth.<sup>14</sup>

All mice groups that were given artemisinin showed decreasing parasitemia, but inactive parasites were still found in the erythrocytes, in the crisis form and the degenerate form. Decreasing parasitemia in therapy groups was observed on the third day until the seventh day post therapy. This evidence revealed that artemisinin is an effective antimalaria. This research found that there was no significant difference in decreasing parasitemia between artemisinin mono therapy group and combination of artemisinin and NAC therapy group, because NAC does not kill parasites directly but acts as cytostatic to inhibit the growth of parasites.<sup>8</sup>

In malaria infection, type 1 immune responses control the parasitemia, while type 2 immune responses prevent enhanced pathology through attenuation of pro-inflammatory cytokine production for successful resolution of malaria.<sup>16</sup> IL-12 is associated with severity of disease in children with hyperparasitemia.<sup>17</sup> IL-12 polarizes the inflammation towards the pathway of type 1 immune response.<sup>18</sup> Ingestion of hemozoin by monocytes promotes the overproduction of IL-10 and TNF- $\alpha$  related to low production of IL-12, which correlates with an enhanced severity of mild malaria, showing the inverse correlation of IL-12 with parasitemia.<sup>5</sup>

Interleukin-12 (IL-12) is a disulfide-linked heterodimeric cytokine, composed of two subunits, 40-kDa (p40) and 35-kDa (p35) subunits. The two subunits form the 70- to 75-kDa (p70) protein, which is the biologically active form of IL-12. The results of this research showed that 10% parasitemia decreased the IL-12 level. It agrees with another study that showed that the infection of *P. falciparum* causes low IL-12 activity.<sup>5</sup> Hemozoin suppresses IL-12 by augmenting IL-10 and TNF- $\alpha$ .<sup>7</sup> NAC is a precursor of glutathione. The production of IL-12 by antigen presenting cells was influenced by glutathione.<sup>9</sup> So, NAC increases IL-12 level in malaria infection. Plasma IL-12 level in the combination of artemisinin and NAC therapy groups (constant and tapering dose) were significantly higher than that in artemisinin mono-therapy group. However, this research found that plasma IL-12 level has a weak inverse correlations with parasitemia. Plasma IL-12 level in mice that were given combined artemisinin

and NAC therapy was higher than that in mice that were given artemisinin mono-therapy. Mice treated with artemisinin and NAC tapering dose had the highest plasma IL-12 level. However, it was not significantly different with the plasma IL-12 level of artemisinin and NAC constant dose mice. This revealed that artemisinin and NAC therapy influenced the level of IL-12 in the animal model of malaria.

## CONCLUSION

The conclusion of this research is that artemisinin mono-therapy effectively decreased parasitemia as well as combination therapy consisting of artemisinin and NAC. Artemisinin and NAC combination therapy, constant and tapering dose, increase plasma IL-12p70 level more than artemisinin mono therapy does. The highest plasma IL-12p70 level was found in the combination of artemisinin and NAC tapering dose therapy group with a duration of seven days. For further research we suggest the evaluation of IL-12 and other cytokines as means for monitoring the success of therapy and the elimination of the pathology of malaria infection.

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