High dose of N-acetylcysteine increase $H_2O_2$ and MDA levels and decrease GSH level of HUVECs exposed with malaria serum

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Abstract. Dysfunction of endothelial cells in severe malaria may result from excessive activation of tumor necrosis factor (TNF)-α which leads to an increase in production of reactive oxygen species (ROS) and decrease of antioxidant level of endothelial cells. To investigate the effect of N-acetylcysteine (NAC) on hydrogen peroxide ($H_2O_2$), malondialdehyde (MDA) and glutathione (GSH) levels produced by endothelial cells exposed with serum of malaria falciparum patient, an in vitro model of human umbilical vein endothelial cells (HUVECs) culture was used. Sample groups were normal HUVECs (group A), HUVECs that was exposed with malaria serum without any treatment (group B), HUVECs that were exposed with malaria serum and treated with NAC 2µM (group C), HUVECs that were exposed with malaria serum and treated with NAC 4µM (group D), and HUVECs that were exposed with malaria serum and treated with NAC 8µM (group E). The level of MDA was measured by thio-barbituric acid reaction assay and $H_2O_2$ level was measured by NWLSS™ Hydrogen Peroxyde/Peroxydase Assay kit. The level of GSH was determined by using NWLSS™ Glutathione Assay kit. The level of $H_2O_2$ and MDA decreased after administration of low dose of NAC. Unfortunately, increased $H_2O_2$ and MDA levels were found on HUVECs treated with high dose of NAC (8µM). There was a positive correlation between NAC dose and $H_2O_2$ level ($r= 0.603$) and between NAC dose and MDA level ($r= 0.721$). A significant decreased level of GSH was found on HUVECs treated with high dose of NAC ($p = 0.023$). It can be concluded that the use of high dose of NAC as supportive therapy in severe malaria infection must be taken carefully.

INTRODUCTION

Severe malaria is associated with adhesion of parasitized red blood cells on endothelial cells. *Plasmodium falciparum*–infected erythrocytes adhere to CD36, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin (Turner, 1994; Silamut et al., 1999). Sequestration of *P. falciparum* favours the brain when circulating concentrations of TNF are high (Clark et al., 2006). Toxic mediator contained in malaria serum (TNF-α) is associated with adhesion of parasitized red blood cells on endothelial cells, this adhesion increases reactive oxygen species (ROS) production and induces oxidative stress conditions (Armuyanti et al., 2007).

Previous in vitro study has demonstrated that cytoadherence can promote ROS production in endothelial cells (HUVECs). The addition of TNF-α can also increase ROS production by
endothelial cells in vitro (Fitri, 2006). A previous study has revealed that parasitized red blood cells (PRBC)-induced apoptosis in endothelial cells is mediated through an oxidative stress pathway. The inhibition of nitric oxide (NO) synthesis and the administration of superoxide dismutase mimetic (MnTBAP) protected the human lung endothelial cells (HLEC) against PRBC-induced apoptosis (Pino et al., 2003). Endothelial cells apoptosis is not only caused by the adherence of *P. falciparum* infected erythrocytes but also caused by the ROS production and secretion of proteolytic enzyme from neutrophyl (Hemmer et al., 2005).

Superoxide radicals, a member of ROS dismutate spontaneously changes to hydrogen peroxide and water at neutral to slightly acidic pH, whilst they are relatively stable at alkaline pH. Hydrogen peroxide can also arise via the transfer of two electrons to molecular oxygen, this reaction is carried out by oxidases in the peroxisomes. A further one-electron transfer to hydrogen peroxide produces the hydroxyl radical, which is the most reactive ROS and triggers chain reactions with organic molecules (Halliwell & Gutteridge, 1999). The higher degree H$_2$O$_2$ is associated with higher cytoadherence process that occurs in capillary of vital organ (Fitri et al., 2006).

Malonic dialdehyde (MDA) is the end result of free radical attack on the integrity of cellular components. One of the components of the cell vulnerability to free radicals is the cell membrane containing poly-unsaturated fatty acids (PUFA). Free radicals cause peroxides fat component in the PUFA with the end results of MDA (Halliwell & Gutteridge, 1999). Lipid peroxidation products such as MDA is the widely used indicator of stress oxidative (Favier et al., 1995). MDA level in falciparum malaria with Acute Renal Failure (ARF) was be significantly higher than healthy controls, and when compared with falciparum malaria without ARF (Nanda et al., 2004).

The tripeptide reduced glutathione (GSH) is one of the most important endogenous antioxidants. It plays the role of a sulfhydryl (SH) group provider for direct scavenging reactions. In doing so, it acts both as a substrate in the scavenging reaction catalyzed by glutathione peroxidase and as a scavenger of vitamin E and vitamin C radicals (Ortolani et al., 2000). In *Plasmodium* infected erythrocytes, GSH activity decreased and oxidized glutathione (GSSG) activity increased compared with normal erythrocytes (Atamna & Ginsburg, 1997).

Previous researchers have indicated that malaria infection in mice induces oxidative stress and the use of antioxidants like vitamin C and N-acetylcysteine (NAC) as exogenous support therapy will help to accelerate the healing for malaria (Fitri, 2003; Iskandar, 2005). NAC is able to serve as antioxidants through two mechanisms, increase the degree of glutathione cells and as a direct scavenger of free radicals (Halliwell & Gutteridge, 1999). This research was conducted to know whether administration of NAC on HUVECs exposed with malaria serum will reduce H$_2$O$_2$ and MDA levels and increase GSH level.

**MATERIALS AND METHODS**

This laboratory experimental study was conducted at the Biomedics Central Laboratory, Faculty of Medicine Brawijaya University, from October to December 2008. Serum were collected from three cerebral *P. falciparum* patients (WHO criteria) with high degree of parasitemia (>24 o/oo) from Dr. Saiful Anwar Hospital Malang, after getting informed consent. Umbilical cord was derived from an elective sectio-caesarian delivery patient at several hospitals in Malang, after getting informed consent, and then processed as previously described (Kim, 2007). HUVECs from umbilical cord were divided into 5 groups, there were negative control group or HUVECs without treatment (Group A), positive control group or HUVECs that were exposed with serum of *P. falciparum* patient (Group B), HUVECs that were exposed with serum of *P. falciparum*
patient and treated with NAC 2 µM (Group C), HUVECs that were exposed with serum of P. falciparum patient and treated with NAC 4 µM (Group D) and HUVECs that were exposed with serum of P. falciparum patient and treated with NAC 8 µM (Group E). Each group was replicated three times.

To induce oxidative stress of the HUVECs culture, positive control group and treatment groups were incubated with serum of severe P. falciparum patients (Armiyanti et al., 2007). In the same time we added NAC 2 µM, 4 µM and 8 µM on the treatment groups (group C, D and E) respectively. After 90 minutes, the culture were rinsed with HEPES buffered saline three times and incubated again with complete medium for 4, 5 hours. Finally supernatant from culture were isolated and the cells were destroyed with triton X as described previously (Fitri et al., 2008). Supernatant from culture and lysed cells culture were measured for H$_2$O$_2$, GSH and MDA levels.

H$_2$O$_2$ analysis with NWLSS$^\text{TM}$ Hydrogen Peroxyde Assay (Northwest, NWK-HYPO1)

Briefly, 5 µL catalase was added to blank sample wells. 5 µL deionized H$_2$O was added to sample test wells and calibrator wells. 20 µL of diluted samples or calibrators were added to wells. The plate was agitated and incubated for 5 minutes at room temperature. 200 µL of XO:Fe reagent was added to each well and incubated for 45 minutes at room temperature. Plate was read at 595 nM. Standard curve was plotted using linear regression analysis. Sample H$_2$O$_2$ concentrations were calculated by comparing sample absorbance to standard curve obtained.

Lipid peroxidation Malondialdehyde (MDA) analysis (modification of Draper & Hadley’s method, 1990)

Briefly, homogenized cells culture were mixed with 15% (w/v) trichloroacetic acid (TCA) to precipitate the protein, 0.25% hydrochloric acid (HCl) to performed acid PH and 0.375% (w/v) thiobarbituric acid (TBA), and then incubated in waterbath 100°C for 20 minutes. Following that, the mixture was centrifugated at 3000 rpm for 10 minutes. After cooling, the absorbance of supernatant was read at 535 nm. Malondialdehyde a secondary product of lipid peroxidation, reacts with TBA in acidic medium to give a pink colored pigment. The pink color is extracted with butanol and the absorbance read at 535 nm. Values were expressed as nano-moles per deciliter.

GSH analysis (Bioxytech$^\text{R}$ GSH/GSSG-412$^\text{TM}$, catalog number 21040)

Preparation of GSH samples: 50 µL supernatant of HUVECs culture was placed to the bottom of micro-centrifuge tube and 350 µL cold 5% MPA was added to the tube (1/8 dilution of original samples). Samples were vortexed for 15 second and centrifuged at 1000xg for 10 minutes. 50 µL samples MPA extract was added to 3 mL assay buffer (1/61 dilution of the acid extract).

Assay: 200 µL of standards, blank and samples were placed to the cuvettes. 200 µL of chromogen was added to each cuvette followed by 200 µL enzyme (glutathione reductase). Then they were mixed and incubated at room temperature for 5 minutes. Finally 200 µL NADPH was added to each cuvette. The change of absorbance at 412nm was recorded for 3 minutes.

RESULTS

The level of H$_2$O$_2$ in all groups

Results of H$_2$O$_2$ measurement on HUVECs exposed with serum P. falciparum patient was showed in Figure 1. H$_2$O$_2$ level of group E reached a similar level to control positive group.

Anova test results of H$_2$O$_2$ level in all treatment groups showed there were a significant differences among groups (p=0.000). LSD test showed there were a significant differences between group A and group B (p = 0.000), group A and group C (p = 0.000), group A and group D (p = 0.000), group A and group E (p = 0.000) as
well as group B and group D (p = 0.031). In contrast, there were no significant differences between group B and group C (p = 0.351), group B and group E (p = 0.652), group C and group D (p = 0.175), group C and group E (p = 0.623) and group D and group E (p = 0.073).

The level of MDA in all groups
The level of MDA in control and treatment groups were showed in Figure 2. The highest level of MDA occurred on group E, group that treated with the highest dose of NAC.

Anova test results of MDA level in all treatment groups showed there were a significant differences among groups (p= 0,000). LSD test showed, a significant differences were only found between group A and group E (p = 0.009) and between group C and E (0.007). However there were no significant differences between group A and group B (p = 0.091), group A and group C (p = 0.911), group A and group D (p = 0.070), group C and group D (p = 0.623) and group D and group E (p = 0.073). No significant differences also found between group B and group C (p = 0.074), group B

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and group D (p = 0.890), group B and group E (p = 0.254), group C and group D (p = 0.057) and group D and group E (p = 0.312).

The level of GSH in control and treatment groups are showed in Figure 3. The lowest level was found in group E, the group that received the highest dose of NAC.

Anova test results of GSH level in all treatment groups showed there were a significant differences among groups (p= 0.000). LSD test showed there were significant differences between group A and group C (p = 0.009), group A and group D (p = 0.004), as well as group B and group D (p = 0.030), group B and group E (0.023), group C and group E (p=0.0000) and group D and group E (p=0.0000). In contrast, there were no significant differences between group A and group B (p = 0.320), group A and group E (p=0.156), group B and group C (p = 0.067), group C and group D (p = 0.677).

Pearson correlation analysis indicated that the level of H₂O₂ of untreated exposed HUVECs increased as well as MDA level (r= 0.552). There was a positive correlation between NAC dose and H₂O₂ level (r= 0.603) and between NAC dose and MDA level (r= 0.721).

DISCUSSION

In group B, group that was only exposed with serum of *P. falciparum* patient without any treatment, the level of H₂O₂ increased significantly and the level of MDA increased although the later was not significant. The high endothelial cell production of H₂O₂ and MDA in the positive control group showed that there was an excessive oxidative stress on endothelial cells. This phenomenon is caused by several toxic mediators which can be found in malaria serum and this phenomenon is due to a suspected release TNF-α during malaria infection. As reported by Fitri (2006), there was increased production of ROS endothelial cells after being exposed to *P. falciparum* infected erythrocytes, and this increase was higher by several fold times when HUVECs were exposed to TNF-α.

High pro-inflammatory cytokine level that was found in serum of severe malaria patient can induce ICAM-1 expression on endothelial cells and associated with endothelial cell activation that contribute to the sequestration of erythrocytes and leukocyte in endothelial cells as a basic cause of cerebral vascular syndromes (Dondorp, 2005).
Increased H₂O₂ and MDA level is suspected to occur through various pathways including the production of ROS secreted by immune cells that will cause damage to normal cells and the other network through lipid peroxidation (Hunt et al., 1992).

In the group that was treated with low level of NAC, the levels of H₂O₂ and MDA decreased, however in group that was treated with high dose of NAC, the MDA and H₂O₂ level increased. Thus, the use of NAC in a high dose might cause pro-oxidant accumulation. It means activities of NAC that can reduce oxidative stress depends on the dose.

N-acetylcysteine appears to be more effective in playing a role as antioxidants only in small doses. In low dose, NAC can reduce the level of H₂O₂ and MDA. This may be related to the ability of NAC as antioxidants that can suppress the occurrence of stress oxidative on various organs. N-acetylcysteine is an antioxidant from thiol group, that serves as a precursor of GSH in malaria treatment. N-acetylcysteine serves as SH donor in GSH synthesis. N-acetylcysteine is a cysteine donor that can increase the GSH level in erythrocyte. N-acetylcysteine works by stimulating production of antioxidant, to neutralize the effect of oxidant (Halliwell & Gutteridge, 1999). The direct scavenging of hydroxyl radicals by thiols has been suggested as their protection mechanisms. Nevertheless, the interaction of thiols with reactive radicals can generate thyl radicals, which, in turn, may impart a pro-oxidant function. Study of the influence of oxidants on membrane fluidity and the measurements of the changes in the fluorescence of bilayer probes, have shown the antioxidant and pro-oxidant effects of both NAC and GSH (Sagrista et al., 2002).

In this study a low dose of NAC (2 & 4 µM) cause an increase in GSH level. This increase is related to the decrease of H₂O₂ and MDA. Grimble (2006) showed that in the immune system, glutathione plays an important role in antioxidant defense to prevent cells from free radicals damage. Cysteine on the glutathione system works as scavenger to convert dangerous free radicals in serum of malaria patients to be non toxic substances. Reducing agent and antioxidant like NAC and GSH enhance phosphatase inhibitor and thus decrease the activation of NFκB and inhibit transcribed of pro-inflammatory cytokine (Staal et al., 1993). Unfortunately a high dose of NAC (8 uM) caused decrease of GSH level, it means in a high dose, NAC can act as pro-oxidant.

N-acetylcysteine is an antioxidant which is quite popular for its ability to minimize oxidative stress and the downstream negative effect thought to be associated with oxidative stress (Kerksick & Willoughby, 2005). NAC will inhibit TNF release and act potentially as scavenger for toxic free radicals that are produced as a response to TNF release (Zimmerman et al., 1989; Mohanty et al., 2006). Administration of NAC in malaria patients shows a rapid recovery (Watt et al., 2002). The used of combination of artemisin and NAC has been revealed to reduce the parasitemia and lactate serum in severe falciparum patient (Treeprasertsuk et al., 2003).

High dose of N-acetylcysteine was already used in medical emergency, especially on paracetamol (acetaminophen) intoxication. In this case, NAC is given as an initial dose:150mg/kg diluted in 200mL of 5% glucose and infused over 15 to 60 minutes and followed by second infusion 50 mg/kg NAC dilute in 500 mL 5% dextrose intravenously infused for the next 4 hours and finally followed by a continuous infusion of 100mg/kg of NAC in 1000 mL of 5% glucose over the next 16 hours (Daly et al., 2008). Anaphylactoid reactions including angioedema, bronchospasm, flushing, hypotension, nausea/vomiting, rash, tachycardia, and respiratory distress may occur 15–60 minutes into NAC infusion (20 hour intravenous regimen) in up to 10% of patients. Following accidental intravenous over-dosage, the adverse reactions of NAC are similar but more severe; fatalities have occurred (Flanagan & Meredith, 1991).
To repair endothelial dysfunction & ischemia in chronic renal diseases, the dose of intravenously NAC is 5 g/25 ml solution intravenously diluted in 500 ml of 5% glucose solution for 4 hours (Yogiantoro et al., 2005). However in severe malarial infection, a reduction in the loading dose of NAC may reduce the risk of adverse reactions while maintaining efficacy. It is recommended that administration of NAC is not over 300 mg/kg/3 days (Watt et al., 2002).

It can be concluded that administration of a low dose of NAC can reduce the level of H2O2 and MDA via glutathione (GSH) synthesis. The results suggested that a low dose of NAC gives positive effects by decreasing free radicals production. Unfortunately a high dose of N-Acetylcysteine increases H2O2 and MDA levels and decrease GSH level of HUVECs exposed with malaria serum. The influence of NAC on superoxide radical production, the ratio of GSH / GSSG, *OH, and NO need to be inspected further.

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REFERENCES


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