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Plasma glutathione and oxidized glutathione level, glutathione/oxidized glutathione ratio, and albumin concentration in complicated and uncomplicated falciparum malaria



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ABSTRACT

Objective: To compare the level of glutathione (GSH) and oxidized glutathione (GSSG), the ratio of GSH/GSSG and the concentration of albumin in plasma of patients with complicated and un-complicated falciparum malaria.

Methods: This research was a cross sectional study using comparison analysis with the plasma GSH and GSSG, the ratio of plasma GSH/GSSG and the concentration of plasma albumin as variables. The complicated malaria patients were obtained from Dr. Saiful Anwar Hospital Malang, whereas uncomplicated malaria patients were obtained from the Regency of Pleihari South Kalimantan. Plasma GSH and GSSG levels were determined by the spectrophotometer at the wave length of 412 nm, whereas the concentration of albumin was determined by bromocresol green method in the pH of 4.1.

Results: There were no significant differences between the level of plasma GSH and GSSG in complicated and uncomplicated malaria patients, as well as the ratio of plasma GSH/GSSG in the two groups ($P = 0.373$; $P = 0.538$; and $P = 0.615$, respectively, independent t -test). In contrast, the plasma albumin concentration in complicated malaria patients were significantly higher than uncomplicated malaria patients ($P = 0.000$, Mann Whitney U test).

Conclusions: It can be concluded that the average of plasma GSH and GSSG level, also plasma GSH/GSSG ratio in complicated malaria are not different from uncomplicated malaria. Although plasma concentration of albumin in both groups is below the normal range, there is an increase in complicated malaria that might be as compensation of oxidative stress.

1. Introduction

The main sources of oxidant during malaria infection are from hemoglobin digestion in the food vacuole of parasite inside

host erythrocyte, the synthesis and folding of proteins within the endoplasmic reticulum, as well as the production of the energy (adenosine triphosphate) in the mitochondria. However, parasites maintain the redox equilibrium using antioxidant systems [1]. Oxidative stress can be regarded as an imbalance between oxidant production and antioxidant defenses. Overproduction of oxidant or reactive oxygen species can be toxic to cells causing oxidation of macromolecules, including lipids, protein and nucleic acids followed by cellular and tissue damage [2].

Glutathione (GSH) is the most abundant antioxidant in all aerobic cells, presenting with high-concentrations in body fluids and tissue. GSH which is synthesized from L-glutamate, L-cysteine and L-glycine is critical for protecting the tissue from oxidative stress, acting as a free radical scavenger and inhibitor

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The study protocol was performed according to the Helsinki declaration and approved by the committees of research of the Faculty of Medicine, Universitas Brawijaya. Informed written consent was obtained from the patients.

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of lipid peroxidation [3]. Most of free radical forms are removed by GSH using enzymatic reduction, whereas the elimination of H_2O_2 requires enzymatic catalysis by GSH peroxidase and catalase. During this process GSH becomes oxidized glutathione (GSSG). This GSSG characterized by a disulfide bond between two molecules of GSH is efficiently reduced back to GSH by the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent catalysis of the flavoenzyme GSH reductase [4].

When a strong oxidative stress is exposed to mammalian cells, this condition may require not only enhanced GSH action to maintain redox status, but also augmented energy supply and precursors to enhance GSH content and transport it to the places where it is needed. Moreover, when oxidative stress becomes prolonged and cellular systems are no more able to counteract the abundant of oxidative stress, the ratio of GSH/GSSG will reduce as a consequence of decreased amount of free GSH [4]. The ratio of reduced GSH to GSSG can be used as an indicator of cellular health. Measuring the ratio of GSH/GSSG in pathological tissues and experimental models compared to those in normal tissues is an excellent procedure to know the efficacy of antioxidant therapeutics in maintaining cellular redox [3].

The invasion of malaria parasite in human erythrocytes causes a potential oxidant that will induce the antioxidant resistance of erythrocytes [5]. The GSH metabolism of the parasites is regulated by its biosynthesis, reduction and efflux. GSH biosynthesis in *Plasmodium falciparum* is related to the rapid efflux of GSH from the infected erythrocytes and the parasite's inability to scavenge sufficient amounts of the tripeptide from its environment to compensate that efflux [6]. GSH efflux from *Plasmodium falciparum* infected erythrocytes is actually greater than that from uninfected erythrocytes [7].

Albumin, a non-glycosylated, negatively charged plasma protein, with ascribed ligand-binding normally accounts for over 50% of total plasma protein content. Albumin is present as transport protein and serves multifunctional activities like antioxidant functions, and enzymatic activities. Albumin could potentially reduce oxidant effects through scavenging antioxidant actions, modifying redox balance, and regulating cell signaling moieties active in mediating pro-inflammatory response [8,9]. A previous study has shown that albumin resuscitation may reduce mortality rate in children with severe malaria. Mortality decreased in children receiving albumin than in those treated with gelofusine (Fisher's exact test, $P = 0.06$). The effect of albumin on mortality showed a pooled relative risk of death with albumin administration of 0.19 (95% CI: 0.06–0.59; $P = 0.004$) compared to other fluid boluses [10].

Based on the above facts and theories, this study will compare GSH and GSSG level, GSH/GSSG ratio and albumin concentration in plasma between complicated and uncomplicated falciparum malaria.

2. Materials and methods

2.1. Sample collection

This study was a cross sectional study with descriptive analysis data to determine the level of GSH, GSSG and the ratio of GSH/GSSG and the level of albumin in the plasma of malaria patients. This research was conducted at the Central Laboratory

of Biomedical, Faculty of Medicine Universitas Brawijaya and the Central Laboratory of Dr. Saiful Anwar Hospital Malang. The experiment was conducted from November 2009 to April 2010. Ethical clearance was provided on 16 November 2009 by the committees of research of the Faculty of Medicine, Universitas Brawijaya.

The research subject was blood plasma of complicated malaria falciparum patients ($n = 9$) from Dr. Saiful Anwar Hospital Malang (most of them were imported cases from Kalimantan island) and uncomplicated malaria falciparum patients ($n = 10$) from the district of South Kalimantan, Pleihari. Blood was taken with the informed consent. All blood chemical parameters [urea, creatinine, serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and bilirubin level] were determined in Central Laboratory of Dr. Saiful Anwar Hospital Malang.

Reduced GSH and GSSG level were detected using the Oxis kit/Research TM. Catalog number 21040. The level of the disulfide GSSG formed consisting of two molecules of GSH through a combination of the reaction between GSH and 5,5'-dithio-bis (2-nitrobenzoic acid) to form 5-thio-2-nitrobenzoic acid and GS-5-thio-2-nitrobenzoic acid (glutathione adduct of GSH), which immediately reduced to GSH. The ratio of GSH/GSSG was then counted by dividing the difference level between the GSHt and GSSG (reduced GSH) by the level of GSSG or (GSHt–2 GSSG)/GSSG. Albumin concentration in plasma was measured by bromocresol green method in Roche/Hitachi Cobas c 501-analyzer.

2.2. Preparing GSH samples

Fifty microliters of blood plasma was inserted into the base of microcentrifuge tube then frozen at $-70\text{ }^{\circ}\text{C}$. In the experiment day, samples were thawed and immediately mixed. A total of 350 μL of 5% metha phosphoric acid (MPA) was inserted into the tube (dilution 1/8 of the original sample). Samples were vortexed for 15–20 s and then centrifuged at 3500 r/min for 10 min. Then 50 μL of MPA extract was added to 3 mL of assay buffer (1/61 dilution acid supernatant). Supernatant was placed in a cooler for measuring in spectrophotometer.

2.3. Preparing GSSG samples

Ten microliters of 1-methyl-2-vinyl trivate pyridium were put into the tube then 100 μL blood plasma was carefully inserted to the bottom of centrifuge tube. The tube was mixed gently. The samples were frozen at $-70\text{ }^{\circ}\text{C}$. In the experiment day samples were thawed then incubated at room temperature for 2–10 min. A total of 290 μL of 5% MPA was inserted into a chilled tube containing the samples (1/4 dilution of the original sample). Samples were vortexed for 15–20 s and centrifuged at 3500 r/min speed for 10 min. Then 50 μL MPA extract was added to 700 μL of GSSG buffer (1/5 dilution acid supernatant). Supernatant was placed in a cooler for measuring in spectrophotometry (dilute 1/60).

2.4. Determination of levels of GSH, GSSG, and the ratio of GSH/GSSG

A total of 200 μL of standards and samples were added to the cuvettes. Then 200 μL of chromogen was added to each cuvette, and 200 μL of the enzyme was added to each cuvette, mixed and then incubated at room temperature for 5 min. A total of 200 μL

of NADPH was added to each cuvette. Changes in absorbance at 412 nm for 3 min were recorded and observed.

2.5. Data analysis

The statistical analysis was conducted using the independent *t*-test ($P < 0.05$). Checking distribution of data was performed by Shapiro–Wilk test due to normal distribution of data then unpaired *t*-test was held.

3. Results

3.1. Samples characteristic

Characteristics of malaria patients used in this study are presented in Table 1. There were some differences in the characteristics of laboratory values between the plasma of

Table 1

Characteristics of complicated and uncomplicated malaria patients.

Indicator	Complicated malaria	Uncomplicated malaria	<i>P</i> value
Age (year)	45 ± 13	41 ± 13	0.77***
Sex (males/females)	8/2	9/0	0.81***
Parasitemia (%)	32.17 ± 13.47	2.80 ± 1.55	0.00*
Urea (mg/dL)	65.93 ± 73.42	13.57 ± 5.55	0.03*
Creatinine (mg/dL)	1.24 ± 1.99	0.10 ± 0.09	0.08*
SGOT (IU/L)	28.11 ± 23.05	8.80 ± 2.27	0.01*
SGPT (IU/L)	9.22 ± 14.33	0.50 ± 1.20	0.01**
Total bilirubin (mg/dL)	2.25 ± 4.74	0.10 ± 0.11	0.17*
Bilirubin direct (mg/dL)	1.55 ± 3.35	0.06 ± 0.06	0.17*
Bilirubin direct (mg/dL)	0.69 ± 1.40	0.06 ± 0.06	0.17*

*: Independent *t*-test; **: Mann Whitney *U* test; ***: Fisher's exact test; The values were expressed as mean ± SD.

complicated malaria patients and that of uncomplicated malaria patients. Statistical analysis showed that the average concentration of urea, SGOT, SGPT and the degree of parasitemia of complicated malaria patients and uncomplicated malaria's plasma patients used in this study have significant differences ($P = 0.03$; $P = 0.01$; $P = 0.01$; $P = 0.00$).

3.2. GSH level in plasma patients of complicated and uncomplicated malaria

The average level of GSH in patients with complicated malaria was (81.67 ± 3.68) μmol/L, while in uncomplicated malaria patients, was (80.40 ± 1.80) μmol/L (Figure 1A). It was found that the average level of GSH in patients with complicated malaria was higher than those in patients with uncomplicated malaria, however, based on independent *t*-test it is known that the difference in average levels of plasma GSH of patients with complicated and uncomplicated malaria was not significant ($P = 0.373$).

3.3. GSSG level in plasma patients of complicated and uncomplicated malaria

GSSG level was obtained through the spectrophotometry method. The average level of GSSG in complicated malaria was higher than that of uncomplicated malaria *i.e.* (495.48 ± 98.80) μmol/L in complicated malaria compared to (474.54 ± 6.73) μmol/L in uncomplicated malaria patients (Figure 1B). Statistical analysis using independent *t*-test showed no significant difference ($P = 0.536$) on average of plasma GSH level between two groups (Figure 1A). Similar result was found on average plasma GSSG level (Figure 1B).

3.4. GSH/GSSG ratio in plasma patients of complicated and uncomplicated malaria

The average ratio of GSH/GSSG in the group of patients with complicated malaria was higher (4.29 ± 1.40) compared to those

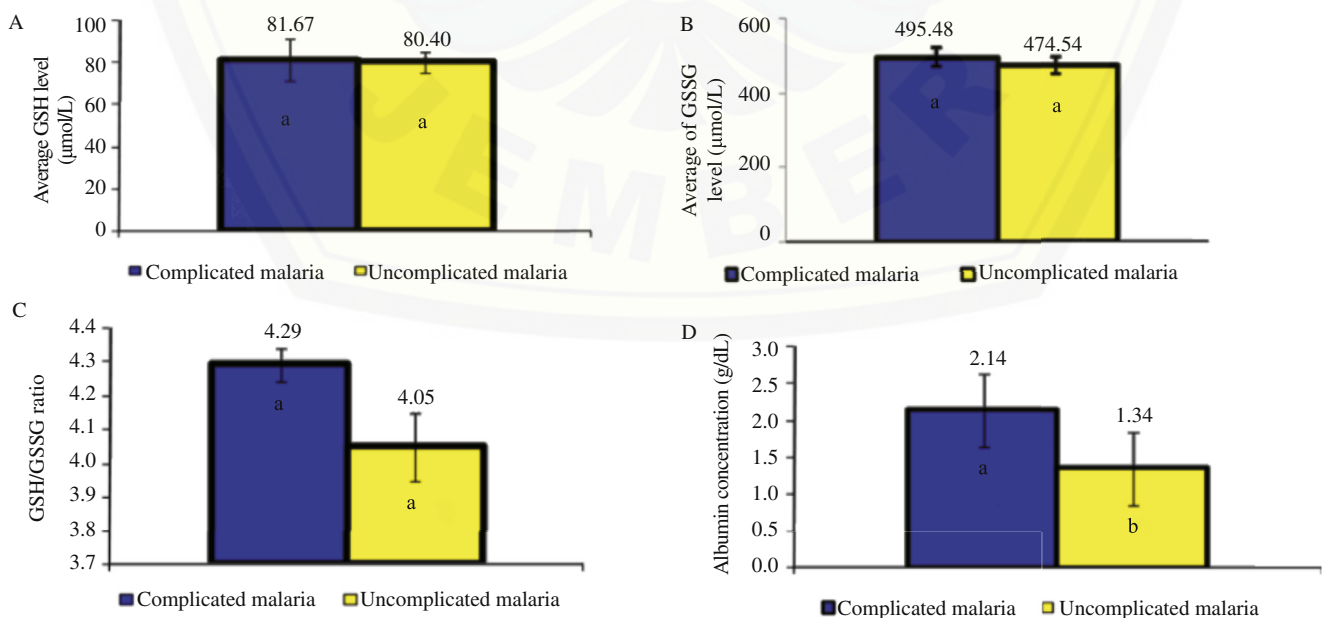


Figure 1. Average GSH level (A), average GSSG level (B), GSH/GSSG ratio (C), and albumin concentration (D) on plasma of complicated malaria patients ($n = 9$) and uncomplicated malaria patients ($n = 10$).

Similar notation showed there was no significant difference.

of uncomplicated malaria. (4.04 ± 0.52). The independent *t*-test, yield no significant difference ($P = 0.615$) on average of GSH/GSSG ratio between two groups (Figure 1C).

3.5. Albumin concentration in plasma patients of complicated and uncomplicated malaria

Plasma albumin levels showed to be below the normal range in both groups. However, the average albumin concentration in complicated malaria patients was higher (2.14 ± 1.17) g/dL compared to those in uncomplicated malaria patients (1.34 ± 0.45) g/dL as shown in Figure 1D. Statistical analysis using Mann-Whitney *U* test showed a significant difference ($P = 0.000$) on average concentration of plasma albumin between groups.

4. Discussion

A potential source of free radical production in malarial disease is from hemoglobin degradation which done by parasites, resulting in the liberation of large amounts of circulating heme. Another source is from activated macrophages and neutrophils which generate large amounts of reactive oxygen species and reactive nitrogen species, causing an imbalance between the formation of oxidizing species and the activity of antioxidants. This imbalance triggers alterations in redox status, which is an important mechanism of human hosts in response to malaria infection [11].

Oxidative stress induced by inflammatory process during falciparum malaria infection leads to increase lipid peroxidation due to an imbalance between plasma oxidant and antioxidant system [12]. This inflammatory process that mediated by effector immune response mechanisms is required to eliminate malaria parasite. However, these mechanisms are not sufficient and to some extent, may further harm the host cell. These mechanisms lead to the decrease of the antioxidant capacity, therefore, reflecting the severity of the disease [11]. Sibmooch *et al.* reported that during malaria infection, the lipoproteins are oxidatively modified, and the degree of oxidation is related to the severity of the disease. These oxidized lipids may have a role in pathomechanism of complicated malaria by increasing the endothelial expression of adhesion molecules [13]. Previous study reported that oxidative stress and antioxidant defense system were also altered in cerebral malaria. Some parameters such as reduced GSH, catalase, malondialdehyde, triglycerides and very low-density lipoprotein cholesterol were increased significantly in cerebral malaria patients compared to that of control [14].

The antioxidant level may be a useful marker of oxidative stress during malaria infection [15]. In this study, the average of GSH level on plasma of complicated and uncomplicated malaria patients showed no significant difference. The explanation is that high oxidative stress will stimulate the body to achieve a new balance. GSH is moderately stable in the intracellular environment because intracellular peptidases can cleave peptide bonds formed by the α -carboxyl groups of amino acids. The steady-state level of intra-cellular and the extracellular of GSH are provided by the balance between production and consumption, as well as by extrusion from the cell as reduced, oxidized, or bound forms [16]. GSH is oxidized through antioxidant reactions by an active glutathione reductase redox

cycle. Efflux, detoxification and antioxidant reactions led to a constant loss of GSH, is compensated by active GSH biosynthesis [17].

Even though the statistical test of independent *t*-test showed no significant difference in the average level of GSSG, these results indicated that the higher the severity of malaria disease, the higher the levels of GSSG in plasma. In a study by Barrand *et al.* there was no export of GSSG detected under physiological conditions from uninfected/unchallenged cells, in contrasts with the situation in infected cells in which there were GSSG export under adenosine triphosphate-depleting conditions. The increasing of GSSG levels inside isolated parasites using 200 μ mol/L diamide produced no change in the total GSH content, but it reduced the total efflux [7]. Both GSH and GSSG are circulated and are used to supply other organs. The circulation is related to GSH functions, and at least two principles may be implicated. The main GSH function is directed to detoxification of injurious external agents to prevent damage to the vital organ. The second function is related to high intensity oxygen-based metabolism and detoxification of certain compounds by internal organs. The problems with extracellular and systemic GSH are related with the concentrations of extracellular GSH which are in a large extend higher than intracellular levels, thus correct redox ratios are often difficult to determine [16].

GSH/GSSG ratio also showed no significant difference between complicated and uncomplicated malaria patients. To maintain redox homeostasis, aerobes are completed with enzymatic and nonenzymatic antioxidants. Two central thiol/disulfide couples are involved in controlling the redox state of the cells, there are GSH/GSSG and dithiol/disulfide of thio-redoxins (Trxred/Trxox). GSH/GSSG is the major redox couple that determines the antioxidative capacity of cells [18,19]. GSH/GSSG homeostasis involves intra- and extracellular mechanisms. GSH is synthesized from amino acids by the action of γ -glutamylcysteine synthetase and GSH synthase. The GSH conjugates and GSSG are transported out of the cell via GS-X/GSSG pumps. The NADPH-dependent GSH reductase is responsible for the intracellular recycling of GSH, while extracellular GSH gets sequentially hydrolyzed by γ -glutamyl transpeptidase and dipeptidase, with glutamate, cysteine and glycine being recycled for GSH synthesis (γ -glutamyl cycle) [19].

This study showed that the higher level of plasma albumin in complicated malaria patients might be a compensation effect of oxidative stress. This contrasts with the previous study by Sibmooch *et al.* where the concentration of albumin in non-complicated malaria, was higher than those in complicated malaria [13]. However, Abdagalil and ElBagir reported that the albumin fraction levels similar in both lightly infected patients and heavily-infected patients [20]. Another study remains unexplained why 40% children which were confirmed to be suffering from *Plasmodium* infection had the plasma albumin levels above normal [21].

Albumin is a complex molecule which acts as the main transporter of plasma fatty acids. Vascular endothelial cells express specific binding sites for albumin which may assist in its ability to prevent endothelial cell apoptosis, an important mechanism of malaria complication. Albumin regulates pyruvate dehydrogenase enzyme in astrocytes and thus helps in efflux of glucose and lactate that can influence the microenvironment. Albumin infusion in severe malaria increase the formation of

lipoxins and 10,17S-dihydroxydocosatriene or neuroprotectin D1 a bioactive mediators derived from docosahexaenoic acid (DHA). This substance causes the mobilization of DHA produced neuroprotection [22,23]. Undurti showed that intravenous albumin administration is useful in critical care on those because of medical conditions, surgery, and sepsis. Cytoprotective action of albumin is due to increased formation protectin of DHA and polyunsaturated fatty acids [23].

It can be concluded that the average concentration of GSH and GSSG, GSH/GSSG ratio, in plasma patients with complicated malaria are not different from that of uncomplicated malaria. Plasma albumin levels showed to be low in both groups. The higher plasma concentration of albumin in complicated malaria might be a compensation response. Further study with larger sample size is needed to draw a definite conclusion. The needs of albumin as antioxidants could be a consideration in the management of nutritional therapy to accelerate the healing process during malaria infection.

Conflict of interest statement

We declare that we have no conflict of interest.

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