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Samuel A. Sakyi
Department of Molecular
Medicine,
School of Medical Sciences,
KNUST, Ghana

Tel: +233 244 530214

Lipid Peroxidation and Catalase Levels among Children Presenting with Severe Falciparum Malaria in the Sefwi Wiawso Municipality, Ghana

¹Samuel A. Sakyi, ²Richard K.D. Ephraim, ²Enoch O. Antoh,
¹Christian Obirikorang and ³Gifty O. Berchie

This study investigated the levels of oxidative stress in children presenting with severe falciparum malaria and also determined the relationship that exists between these measured biomarkers of oxidative stress and the World Health Organization (WHO) criteria for the classification of severe malaria in children. In a cross sectional study, serum Malondialdehyde (MDA) and catalase levels were measured in 75 parasitaemic and 30 non-parasitaemic children in the Sefwi Wiawso municipality. High MDA with low catalase, platelets, glycaemia and haemoglobin levels was observed in the severe malaria group compared to the controls and the uncomplicated malaria (UCM) group ($p < 0.05$). Severely parasitaemic children also had the highest mean MDA concentration and lowest mean Catalase ($p < 0.05$) among the three groups studied. We observed a positive correlation between MDA levels and malaria parasite density ($r = 0.936$, $p < 0.05$); serum catalase and Hb ($r = 0.850$, $p = 0.001$) and catalase levels and platelets count ($r = 0.464$, $p < 0.05$). However, a negative correlation was observed between MDA levels and Hb ($r = -0.941$; $p = 0.003$) catalase levels and malarial parasite density ($r = -0.920$, $p = 0.001$) and MDA and platelet count ($r = -0.694$, $p < 0.05$). Severe malaria creates oxidative stress proportionate to the degree of parasitaemia; these biomarkers of oxidative stress correlate with the WHO criteria on the classification of severe malaria.

Key words: Oxidative stress, malondialdehyde, catalase, severe malaria, parasitaemia

¹Department of Molecular Medicine, School of Medical Sciences, College of Health Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana

²Department of Laboratory Technology, University of Cape Coast

³Department of Nursing, School of Biological Sciences, University of Cape Coast

INTRODUCTION

Annually, about half a million (500 million) cases of clinical malaria are reported worldwide with *Plasmodium falciparum* infection as the most prominent cause accounting for over a million deaths yearly. Furthermore, it remains the major cause of morbidity and mortality in Sub-Saharan Africa with about 60% of clinical bouts and over 80% of deaths occurring in infants (Breman, 2001; WHO, 2005). The World Health Organization (WHO) in the year 2000, enumerated a number of criteria by which severe malaria could be identified in children: Prostration, fever, headache, nausea and vomiting, abdominal pain, convulsion and severe anaemia (WHO, 2000).

In the performance of their normal metabolic processes most cells are exposed to oxidative stress thus are provided with inherent anti-oxidative mechanisms to counter this effect (Saltman, 1989). Earlier studies by Eaton *et al.* (1976) and Becker *et al.* (2004) indicated that erythrocytes which are infected with plasmodium tend to undergo increased endogenous oxidative stress as a result of the malaria infection. Evidence from a number of studies have indicated that oxidative stress induced physicochemical changes in the membrane of the erythrocytes is the cause of the lipid peroxidation and haemolysis observed in malaria (Clark and Hunt, 1983; Clark *et al.*, 1984; Das and Nanda, 1999). Reactive oxygen intermediates and hydrogen peroxides produced by neutrophils and macrophages do play important role in the infected person's defense against malaria infection. (Kharazami, 1986; Egwunyenga *et al.*, 2004). Furthermore, Golenser and Chevion (1989) noted in mice infected with *Plasmodium berghei* there is an increase in the concentration of MDA. Similarly, free radical generators momentarily caused haemolysis in *Plasmodium vinckei*-infected mice (Clark *et al.*, 1984).

A number of antioxidant enzymes have been studied in malaria infections (Mohan *et al.*, 1992b). Increase in lipid peroxidation is known to produce a corresponding effect in the levels of enzymatic anti-oxidants in severe malaria (Egwunyenga *et al.*, 2004). However, there is a paucity of data on the level of reactive oxygen species and antioxidants in Ghanaian infants with severe malaria. The present study was thus undertaken to investigate oxidative stress in children with severe falciparum malaria as well as establish the correlation between the biomarkers of oxidative stress and the WHO (2000) indicators of malaria.

MATERIALS AND METHODS

Study design/study site: This was a cross-sectional study that was carried out between December 2011 and March 2012 at the pediatric wards of Saint John of God hospital

(SJOGH) and Asawinso Seventh Day Adventist clinic (ASDAC) located in Sefwi Wiawso in the Western region of Ghana. This town is malaria endemic and has a total population of 148, 290 (<http://www.ghanaweb.com/GhanaHomePage/geography/population.php>) covers a total land mass of 118.73 square km (29338.73 acres) with an annual average rainfall of 2000 mm per annum.

Study population: Children 10 years of age and below, who self reported to these hospitals were screened for the study. The study population comprised 75 parasitaemic and 30 non-parasitaemic, sex and age matched children from the paediatric wards of the two hospitals. A questionnaire was used to obtain information on demographic data, antimalarial drugs used previously; the last episode of malaria and the use of insecticide treated nets. Informed consent was obtained from parents and guardians of the participants. Ethical approval was obtained from the Committee for Research Ethics of the University of Cape Coast (CRE/UCC) and the institutional ethics committees of the hospitals.

Inclusion and exclusion criteria: Participants with different level of *P. falciparum* parasitaemia (low, moderate and high) at an axillary temperature of at least 38.0°C and not having received antimalarial treatments before enrolment were included in the study as subjects. Healthy children, age and sex matched attending the outpatient departments of the selected hospitals for routine check-up were included as controls. The selected participants were stratified into Uncomplicated Malaria (UCM) group and severe malaria group.

The UCM group presented with usual malaria symptoms and a positive thick blood film. Severe malaria was defined by an asexual *P. falciparum* parasitaemia with at least one of the WHO (2000) criteria, and by the absence of detectable, non-malaria causes.

Children with sickle cell disease and non-malaria infections; Human Immunodeficiency Virus (HIV), hepatitis B and C infection, enteric fever and other pyrexia of unknown origin were excluded.

Blood collection and processing: Five milliliter of venous blood sample was collected from each participant out of which 2 mL was transferred immediately into dipotassium ethylenediaminetetraacetic acid (K₂ EDTA) tubes for haematological analysis and the remaining 3 mL into Vacutainer plain tubes for biochemical analysis. The clotted sample was centrifuged at 1500 g for 5-10 min and the serum stored at -80°C until assayed.

Haematological analysis: Hemoglobin (Hb), hematocrit (HCT), leukocyte count and platelet levels was assessed by using an automated haematological analyzer (Sysmex KX-21N, Japan).

Malaria diagnosis: Thick and thin Giemsa-stained blood smears were prepared and examined for the presence of *P. falciparum* parasites. The parasite count (parasites μL^{-1} of blood) was estimated by counting 200 White Blood Cells (WBCs) and the number expressed on the basis of 8000 WBCs μL^{-1} (Raza *et al.*, 2009).

Biochemical analysis

Malondialdehyde (MDA): Malondialdehyde (MDA) levels were determined by the MDA Thiobarbituric Acid (TBA) test which is the colorimetric reaction of MDA and TBA in acid solution. MDA, a secondary product of lipid peroxidation, reacts with TBA to generate a red-coloured product which was detected spectrophotometrically. The protocol used in this study was the Kamal *et al.* (1989) modification of the Schlafer and Shepard (1984) protocol. The absorbance of the mixture was measured at 535 nm with a spectrophotometer (Biomate 3S UV Visible spectrophotometer, Thermoelectron Inc. USA) and the results were expressed as μmol^{-1} , using the extinction coefficient of $1.56 \times 10^5 \text{ L mmol}^{-1} \text{ cm}^{-1}$ (Buege and Aust, 1978).

Catalase: Catalase was assayed by the method of Takahara *et al.* (1960). To 1.2 mL of 50 mM phosphate buffer (pH 7.0), 0.2 mL of plasma was added and the enzyme reaction was started by the addition of 1.0 mL of 30 mM H_2O_2 solution. The decrease in absorbance was measured at 240 nm at 30 sec intervals for 3 min with an ultra violet-visible spectrophotometer. The enzyme blank was run simultaneously with 1.0 mL of distilled water instead of hydrogen peroxide. The enzyme activity was expressed as IU mL^{-1} .

Estimation of blood glucose: Blood glucose level was measured using glucometer (OneTouch® Ultraselect, manufactured for; Lifescan Inc. Milpitas, CA 950035, USA). Glucose concentration in mg dL^{-1} was then expressed as mmol^{-1} using a factor of 0.0556.

WHO criteria for classification of severe malaria: Manifestation of severe malaria in this study included severe anaemia, prostration, acute respiratory distress, cerebral malaria (unarousable coma), repeated generalized convulsions, impaired consciousness, hyperpyrexia, hypoglycaemia and thrombocytopenia (WHO, 2000).

Statistical analysis: Independent t-test was used to compare mean scores between two groups. One-way ANOVA was also employed to compare the mean scores of more than two groups. Correlation (Pearson correlation) and linear regression analyses were also performed. $p < 0.05$ were considered statistically significant. Data was analyzed with SPSS version 16 (SPSS Inc. Chicago).

RESULTS

Demographic, clinical, haematological and biochemical parameters of study participants are shown in Table 1. A high temperature of 39.96°C was observed among severe malaria patients compared to 37.04°C of control. Moreover, higher MDA concentration of $14.98 \mu\text{mol}^{-1}$ was observed among severe malaria patients compared to $8.38 \mu\text{mol}^{-1}$ of controls. A high leukocyte count of $10.40 \times 10^3 \mu\text{L}^{-1}$ was also observed among severe malaria patients compared to $5.54 \times 10^3 \mu\text{L}^{-1}$ of controls and the UCM group ($p < 0.05$). However, a

Table 1: Demographic, clinical, hematological and biochemical parameters of study participants

Parameters	CTRL (n = 30)	UCM (n = 30)	Severe malaria (n = 45)
Age (months)	97.34±0.18	98.47±0.30	95.42±0.11
Temperature (°C)	37.04±0.09	38.48±0.45	39.96±0.12"
Clinical manifestations			
Hyperpyrexia (Temp ≥ 40°C)	0 (0.0) ^f	0 (0.0)	9 (20.0)
Prostration	0 (0.0)	7 (23.3)	30 (66.7)
ARD	0 (0.0)	0 (0.0)	18 (40.0)
Coma	0 (0.0)	0 (0.0)	5 (11.1)
Convulsion seizure	0 (0.0)	0 (0.0)	22 (48.9)
Unconsciousness	0 (0.0)	0 (0.0)	7 (15.5)
S. anaemia (Hb < 5 g dL^{-1})	0 (0.0)	0 (0.0)	27 (60.0)
Hypoglycemia (< 2.2 mmol L^{-1})	0 (0.0)	0 (0.0)	12 (26.7)
Thrombocytopenia	0 (0.0)	0 (0.0)	25 (55.5)
Hematological data			
Parasitaemia (pa μL^{-1})	-	1400±1.37 ^a	40,000±4.51"
Haemoglobin (g dL^{-1})	14.75±0.14	11.98±0.19	5.98±0.11"
Platelet level ($\times 10^9 \text{ L}^{-1}$)	356±1.26	202±0.12	89.48±3.04"
Leukocyte count ($\times 10^3 \mu\text{L}^{-1}$)	5.54±0.05	9.45±0.43	10.40±0.30"
Biochemical parameters			
Glycaemia (mmol L^{-1})	8.30±0.09	5.94±0.21	2.98±0.13"
MDA ($\mu\text{mol L}^{-1}$)	8.38±0.27	10.66±0.99	14.98±0.23"
Catalase (IU mL^{-1})	46.86±0.25	43.82±0.28	43.30±0.52"

Values are Mean±SE, Percentage is given in parenthesis; Pa: Parasites, ARD: Acute respiratory distress, s: Severe. "Then mean score in each row with the symbol are significantly different at $p < 0.05$, CTRL: Control, UCM: Uncomplicated malaria

reduced catalase level of 43.30 IU mL⁻¹ was observed among severe malaria patients compared to 46.86 IU mL⁻¹ of controls. Moreover decreased platelets level of 89.86×10⁹ L⁻¹ was obtained compared to 356×10⁹ L⁻¹ of controls (p<0.05). Reduced glycaemic levels of 2.98 mmol⁻¹ compared to 8.30 mmol⁻¹ was observed among severe malaria patients and controls, respectively. A further reduced haemoglobin of 5.98 g dL⁻¹ compared to 14.75 g dL⁻¹ of controls and UCM group were observed (p<0.05).

Table 2 shows the mean MDA and catalase levels in relation to gender and age. The mean MDA concentration was higher in the cases 13.32 µmol⁻¹ compared to the controls of 8.38 µmol⁻¹ (p<0.05). Furthermore, male and female cases had higher mean MDA levels of 13.29 µmol⁻¹ compared to mean male and female controls of 8.38 µmol⁻¹ (p<0.05). However, the MDA concentration was higher (p<0.05) in cases ≤60 months old compared to similar controls.

The mean catalase concentration was lower in cases 43.49 IU mL⁻¹ compared to controls of 46.86 IU mL⁻¹ (p<0.05). Furthermore, male and female cases had a mean lower catalase concentration 43.49 IU mL⁻¹ compared to mean male and female controls of 46.87 (p<0.05).

Table 3 shows the mean oxidative biomarker levels in relation to the degree of parasitaemia in the control and case groups. Children with severe parasitaemia has the highest mean MDA concentration of 14.98 µmol⁻¹ (p<0.05) compared with those with low parasitaemia 9.40 µmol⁻¹ with a statistically significant value (p<0.05) between the three groups of parasitaemic children and the non-parasitaemic group. However, the cases with severe malaria had the lowest mean catalase concentration 43.30 µmol⁻¹ (p<0.05) among the three groups of children with parasitaemia compared to the controls with 49.86 µmol⁻¹.

Table 4 shows a correlation between oxidative biomarkers and haemoglobin and malaria parasite density levels. A positive correlation was observed between MDA levels and malaria parasite density (r = -0.936, p<0.05), serum catalase and Hb (r = 0.850, p = 0.001) and catalase levels and platelets count (r = 0.464, p<0.05). However, a negative correlation was observed between catalase levels and malarial parasite density (r = -0.920, p = 0.001), MDA levels and Hb (r = -0.936, p<0.05) and between MDA and platelet levels (r = -0.694, p<0.05).

The relationship between oxidative biomarkers, blood glucose and clinical parameters is shown in Fig. 1. A positive correlation was observed between serum catalase levels and blood glucose concentration (r = 0.624, p = 0.003); and between MDA levels and clinical parameters per the WHO classification (r = 0.623, p<0.05).

Table 2: Mean MDA and catalase levels in relation to sex and age of studied participants

Characteristics	Control (n = 30)		Case (n = 75)	
	No.	MDA (µmol L ⁻¹)	No.	MDA (µmol L ⁻¹)
Mean MDA		8.38±0.27 ^a		13.32±2.45 ^b
Sex				
Male	21	8.39±0.28 ^a	44	13.35±2.56 ^b
Female	9	8.37±0.28 ^a	31	13.23±2.33 ^b
Age (months)				
≤ 60	26	8.41±0.27 ^c	59	14.06±2.02 ^d
≥ 60	4	8.24±0.31 ^e	16	10.61±1.96 ^f
Catalase (IU mL⁻¹)				
Mean catalase		46.86±0.25 ^e		43.49±0.37 ^d
Sex				
Male	21	46.85±0.26 ^a	44	43.51±0.33 ^b
Female	9	46.89±0.26 ^a	31	43.48±0.43 ^b
Age (months)				
≤ 60	26	46.86±0.27 ^c	59	43.39±0.23 ^d
≥ 60	4	46.85±0.16 ^e	16	43.89±0.52 ^e

MDA: Malondialdehyde, Values are Mean±SD, Values in a row and in each column having different superscripts are significantly different at p<0.05

Table 3: Mean oxidative biomarkers in relation to the degree of parasitaemia

	Degree of parasitaemia			
	Control (N = 30)	L (N = 13)	M (N = 17)	H (N = 45)
MDA (µmol L ⁻¹)	8.38±0.27 ^a	9.40±0.20 ^b	11.92±1.78 ^c	14.98±0.91 ^e
Catalase (IU mL ⁻¹)	49.86±0.25 ^m	44.12±0.45 ⁿ	43.51±0.11 ^o	43.30±0.12 ^p

Values in a row and in each column having different superscripts are significantly different at p<0.05. L: Low parasitaemia, M: Moderate parasitaemia, H: Severe parasitaemia

Table 4: Correlation between oxidative biomarkers and malaria parasite density, haemoglobin level and platelet count.

Parameters	Malaria parasite density	
MDA (µmol L ⁻¹)	Correlation coefficient	0.941
	p-value	0.005
Catalase (IU mL ⁻¹)	Correlation coefficient	-0.920
	p-value	0.001
Hb (g dL⁻¹)		
MDA (µmol L ⁻¹)	Correlation coefficient	-0.936
	p-value	0.001
Catalase (IU mL ⁻¹)	Correlation coefficient	0.850
	p-value	0.001
Platelet level		
MDA (µmol L ⁻¹)	Correlation coefficient	-0.694
	p-value	0.001
Catalase (IU mL ⁻¹)	Correlation coefficient	0.464
	p-value	0.001

Conversely, a negative correlation was recorded between serum MDA levels and blood glucose concentration (r = -0.770, p<0.05), as well as between catalase and clinical parameters (r = -0.485, p<0.05).

DISCUSSION

The main objective of this study was to evaluate the severity of childhood falciparum malaria using oxidative biomarkers and also to establish whether a correlation exists between these biomarkers and the WHO (2000) criteria for severe malaria classification. Oxidative stress

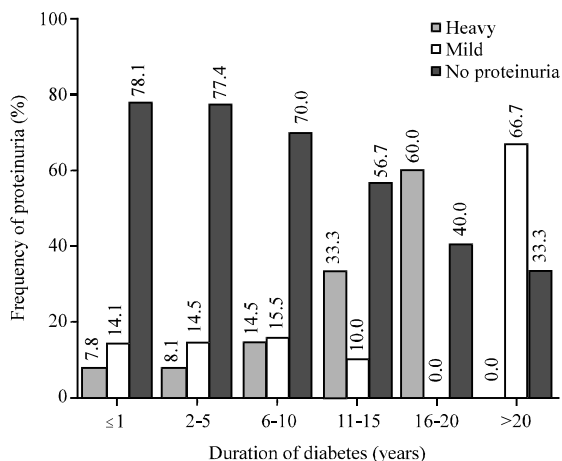


Fig. 1: The relationship between study parameters

was assessed by measuring MDA, a marker of lipid peroxidation and serum catalase, an antioxidant. Oxidative stress was observed amongst the participants with malaria depicted by the high lipid peroxidation and reduced catalase levels, furthermore the biomarkers of oxidative stress correlated significantly with malaria parasite density, platelet levels, haemoglobin concentration.

Severe malaria infection results in inflammation with its associated increase in ROS production and an imbalance in the generation and removal of free radicals (Loria *et al.*, 1999; Egwunyenga *et al.*, 2004; Akanbi *et al.*, 2010). The high levels of lipid peroxidation observed in this study among the participants with severe malaria (Table 1) validates this finding. MDA levels were significantly elevated in children with moderate and high parasitaemia than in non-parasitaemic controls (Table 1) in accordance with observations made by Egwunyenga *et al.* (2004). However, there was a significant difference in MDA levels between children with severe malaria who had low parasitaemia and malaria-free controls (Table 1). This observation however contradicts the findings of Egwunyenga *et al.* (2004). Whereas we used the Kamal *et al.* (1989) modification of the Schlafer and Shepherd method to estimate MDA, Egwunyenga and colleagues used the method of Satoh (1978). This could account for the inconsistencies in the concentration of MDA between the low parasitaemic group and the controls. As both markers are apparently correlated with parasitaemia, it is worth noting that the marker's strength lies in the severity of disease in young children and maybe so also in (non) immune adult travellers returning with malaria to non-endemic countries. Regarding adult semi-immune individuals, one may assume that the correlation with severity may not be so

well as they exhibit a parasitic threshold before progressing towards severe disease.

A number of protective antioxidants including catalase are known to accumulate in the Plasmodium parasite leading to their depletion in the red blood cells of the host. Furthermore, increased production of hydrogen peroxide and free oxygen radicals (Elkin and Eaton, 1975) and a decrease in antioxidant enzymes (Nair *et al.*, 1984; Stocker *et al.*, 1985; Mohan *et al.*, 1992a) has been observed in the plasma of persons with malaria parasitaemia. This is confirmed by findings made in this study (Table 2). The observed decrease in catalase levels is given further impetus by the lipid peroxidation observed in the participants with malaria parasitaemia.

Serum MDA concentration increases in severe malaria infection due to activation of phagocyte cell population which generates ROS and hence lipid peroxidation (Ganguly *et al.*, 1997). In this study we observed a positive correlation between MDA and malaria parasitaemia (Table 4) which is in agreement with earlier reports (Hunt and Stocker, 1990; Egwunyenga *et al.*, 2004; Akanbi *et al.*, 2010). This implies that the MDA level is directly proportional to the degree of parasitaemia. On the contrary however, serum catalase was reduced in malaria parasitaemic patients (Table 4). This indicates that catalase activity was reduced in the participants with malaria due to red cell destruction by the parasite.

Serum MDA levels correlated positively with WHO (2000) clinical parameters for malaria severity whereas a negative correlation was noted between catalase and the WHO clinical parameters (Fig. 1). The implication of this observation is that as patients' present with more of these clinical criteria per the WHO, there is increased oxidative stress indicated by low catalase and high MDA levels.

Anaemia indicated by low haemoglobin levels is one of the major complications of severe malaria. In this study we established a relationship between oxidative stress indicated by high MDA and low catalase levels and haemoglobin concentration (Fig. 1). The significant positive correlation observed between MDA and haemoglobin in parasitaemic participants is consistent with the work of Kulkarni *et al.* (2003). Conversely, we observed a positive correlation between serum catalase and haemoglobin levels in participants with severe malaria which could be attributed the metabolic activities of the *P. falciparum*.

In this study we observed that higher malaria parasitaemia with hypoglycemia was associated with increased oxidative stress indicated by increased lipid peroxidation and reduced antioxidant enzyme

(catalase) activity (Fig. 1) consistent with earlier reports (Becker *et al.*, 2005). This shows that the malaria parasite may be using up the antioxidant proteins in the red cell for its metabolic processes.

Erel *et al.* (2001) concluded that there was a negative and significant correlation between whole blood platelet count and lipid peroxidation in *P. vivax* malaria infection. Observations made in this study in a way confirm this finding only that we worked on *P. falciparum* (Fig. 1). However, a positive correlation ($r = 0.464$ $p < 0.05$) was observed with serum catalase levels. The decreased platelet count (thrombocytopenia) in severe malaria is due to first, adherence of infected red cell to platelet and the resulting clearance by the spleen. Secondly, shortened life spans in the peripheral blood and, thirdly, some interactions between platelets and malaria plasmodia or parasitized red cells.

CONCLUSION

It can be concluded from this study that severe malaria creates an imbalance in the levels of oxidants (increased MDA) and antioxidants proportionate to the degree of parasitaemia (reduced catalase) culminating in oxidative stress. Furthermore, these biomarkers of oxidative stress correlate with the WHO criteria on the classification of severe malaria. Thus in managing malaria supplementation of diet or drug with antioxidants should form part of the treatment plan.

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