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Original Article

Uncomplicated malaria infection in pediatric patients in Ghana: relationship with superoxide dismutase

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ABSTRACT

Background: During malaria infection, the superoxide ion is produced as a by-product of hemoglobin digestion by *Plasmodium falciparum* as well as by the immune cells in defense against the malaria parasite. The ion is toxic to biological systems and adverse effect of the ion is limited when it is dismutated and made harmless by the enzyme superoxide dismutase (SOD). In this study, we investigated the activity of SOD in the context of protection against *P. falciparum* during uncomplicated malaria infection in pediatric patients. **Method:** Pediatric patients suffering from uncomplicated malaria and apparently healthy non-infected controls were recruited during malaria season into the study. Blood samples were taken from each participant; hematological indices were measured and parasitemia was quantified. In addition, red blood cells were separated from the blood, lysed, and SOD activity was measured. **Results:** Significant differences were recorded in levels of white blood cells, particularly lymphocytes, among the uncomplicated malaria patients and control participants. Statistically high levels of SOD activity were observed in uncomplicated malaria patients compared with apparently healthy controls. Partial eta analysis revealed significant and independent influence of polymorphonuclear neutrophils, platelets, mean cell hemoglobin concentration and hemoglobin levels on the SOD activity levels. **Conclusion:** Uncomplicated malaria was associated with high SOD activity when compared with apparently healthy controls in Ghanaian children.

KEY WORDS: Ghana, malaria, *Plasmodium falciparum*, superoxide dismutase

INTRODUCTION

Malaria associated deaths caused by severe forms of the disease kills more African children than infants from other continents [1]. The malaria infection also has high economic costs implications of approximately $12 billion each year in most sub-Saharan countries in Africa [2]. Understanding of the pathogenesis of the disease and control of infection are affected presently by the development of resistance by *Plasmodium falciparum* and mosquitoes to the commonly used anti-malarial drugs and insecticides respectively [3]. The parasite exhibits a complex life cycle involving sexual stage in the female anopheline mosquito vectors and pre-erythrocytic, erythrocytic and gametocytic stages in humans. During the intra-erythrocytic stage, the parasite ingests and digests the cytosol of the host cell which consists mostly of hemoglobin. The digested hemoglobin becomes oxidized, producing superoxide anion radical ($O_2^{\bullet -}$) upon exposure to the acidic environment of the food vacuole [4].

Being a major component of reactive oxygen species (ROS), superoxide is also effectively generated by polymorphonuclear neutrophils (PMN) during their oxidative burst activity in response to microbial infection. The released free superoxide is a potent antimicrobial agent which destroys biomolecules, and are highly toxic for intra-erythrocytic malaria parasites. *P.falciparum* thus encounters enhanced superoxide levels and increasing oxidative stress [5] with consequential damaging effect on it during its erythrocytic life cycle [6]. The parasite therefore becomes easily removed from circulation by phagocytes [7, 8]. Levels of ROS with its consequential oxidative stress correlate with quicker parasite clearance in pediatric *P.falciparum* malaria [9] and protection [10]. Regardless of its origin, superoxide is dismutated by the antioxidant enzyme superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) thereby limiting its adverse effects. Since, in addition to the superoxide produced from leucocytes, parasites get additional superoxide from its digestion of hemoglobin, higher amounts of superoxide may build-up in the infected red blood cells (RBC). The high level of superoxide may kill the parasite in absence of sufficient SOD. This may therefore confer protection to the host.

In this study we measured percentage SOD activity as a surrogate of superoxide levels in human host during infection with *P. falciparum*. Our results show that RBCs

from individuals suffering from uncomplicated malaria have high percentage of SOD activity compared with that of controls and this was influenced by PMNs, hemoglobin and mean cell hemoglobin concentration (MCHC).

MATERIAL AND METHODS

This project was carried out with ethics approval granted by the Research and Protocol Review Committee of the University of Ghana Medical School (UGMS).

Study design, case definition and samples

We conducted a case-control, single-center-based study with the participating children selected prospectively from May 2012 to February 2013 at the study site. This study was carried out at the Korle-Bu Polyclinic, a primary health care facility in Accra, Ghana. We used a primary care center to lessen the bias of referral hospitals contributing more severe cases with discrepant proportion of antioxidants and hematological alterations. One hundred children between the ages of 0.5 and 14 years reporting for medical care with symptomatic uncomplicated malaria were recruited into the study with 41 control participants.

The criteria for inclusion were as follows: fever (> 37.5°C) measured within 24 h of reporting to the hospital, microscopy confirmed presence of *P.falciparum* in blood film and at least one other sign of malaria (*e.g.*, vomiting, diarrhea, malaise). Uncomplicated malaria (UM) definition was based on the microscopic examination of parasitemia with hemoglobin (Hb) value of at least 8 g/dl and without clinical features of severe malaria in the cases.

Children with history of antimalarial treatment during the last 2 weeks, those on other treatments such as antipyretics for the recent febrile illness, or those who where positive were excluded from the study to limit bias for false negatives and resolution of antioxidants and other hematological alterations. Children on medications that may confer antioxidant properties, *e.g.* metformin, insulin *etc*, were also excluded. Similarly, patients identified with malnutrition, infectious diseases such as upper respiratory tract infections and typhoid or any cause of anemia other than malaria were also not included in the study.

Sampling methods

An active case-finding network was organized with visits to participating centers to identify and interview cases before treatment. Overall, children were examined by physicians, who documented the findings of clinical examinations, using the national guidelines for case management of malaria in Ghana, updated to reflect standard World Health Organization (WHO) recommendations [1]. Physician-identified children presenting with symptoms consistent with uncomplicated malaria and referred to the laboratory for a malaria tests were evaluated for inclusion in the study after informed consents were obtained from parents or guardians. Those with laboratory data confirming uncomplicated malaria were enrolled. Controls were drawn/obtained/selected/recruited from the same as are the cases and comprised apparently healthy children of similar age without detectable malaria parasites. Information relating to demography of study participants was documented using a standard questionnaire. Patients and/or guardians were requested to assist in the provision of information for the study. Laboratory records of children enrolled were also reviewed.

Hematological and parasitological measurements

Four milliliters of venous blood was aseptically taken into ethylenediaminetetraacetic acid (EDTA) tubes from each participant. Thick and thin blood film slides were prepared for all study participants stained with 10% Giemsa and examined microscopically for the detection and identification of *Plasmodium* species. Hematological parameters such as Hb level, total RBC count and mean corpuscular volume (MCV) were measured with an automatic hematological analyzer (Sysmex K21, Kobe, Japan). Samples were spun at 2000 rpm for 10 min at 4°C to separate the plasma from the cells. The cells were processed further to separate RBCs from the buffy coat. Plasma and buffy coat samples were stored at -20°C while RBC samples were stored at 4°C and processed later for the analyses of SOD activity.

Measurement of superoxide dismutase activity

Levels of superoxide dismutase activity were determined in the RBC lysate of the study participants using SOD Assay Kit-WST (Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instruction. Briefly, 50 μl of RBC pellets from each participant was lysed on ice for 5 min using double distilled ice cold sterile water followed by centrifugation at 13000g for 15 min at 4°C. The supernatant was diluted 100x using double distilled ice cold sterile water. Twenty microliters of the supernatant from each sample processed was added to the wells of a 96 microtiter plate. Two hundred microliters of water-soluble formazan (WST) working solution (from manufacturer of kit) was added to each well, and 20 μl of dilution buffer was also added to the sample blanks (double distilled water) included in the assay plate. Twenty microlitres of enzyme working solution (from manufacturer of kit) was added to the samples and blanks and mixed thoroughly by incubating briefly on a rocking platform. The microtiter plate with the samples and controls was incubated at 37°C for 20 min and absorbance was read at 450 nm using a spectrophotometer. The inhibitory concentration, IC_{50} (50% inhibition activity of SOD), was then determined [11, 12].

Statistical analysis

Results obtained from this study were analyzed using SPSS software (version 19.0). Basic descriptive statistics such as means, standard deviations (SD), frequencies and percentages (%) were determined for the study population

with regards to their hematological parameters. The test of Shapiro-Wilk normality test was used to confirm the assumptions of the test. The independent t-test was used to compare the mean values of hematological parameters and SOD inhibition activity levels among the study and control groups. The effect of hematological parameters on SOD was determined by generalized linear models (univariate analysis) using partial eta^2 estimates; partial eta squared statistic reports the 'practical' significance of each term, based upon the ratio of the variation (sum of squares) accounted for by the term, to the sum of the variation accounted for by the term and the variation left to error. A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Characteristics of study participants

Table 1 summarizes the independent assessment of hematological parameters in study controls and uncomplicated malaria cases, respectively. Comparing the levels of hematological parameters between the cases and controls showed significantly higher levels in the total white blood cell (WBC) (P = 0.001) and RBC (P = 0.002) count, Hb (P = 0.001), MCV (P = 0.006), platelets (P = 0.001) and lymphocytes (P = 0.001) in malaria cases. However, PMNs and eosinophil counts, and MCHC levels were not different from the uncomplicated malaria cases and the apparently healthy control group (P > 0.05) (Table 1).

Relationship between SOD activity and hematological parameters

A generalized linear model using SOD inhibition activity values as dependent variable and hematological parameters as covariates showed that for uncomplicated malaria case group, Hb, PMN and MCHC levels were found to have significant effect on SOD activity (Table 2). Partial eta analyses showed that PMN exerted the greatest effect (eta = 0.144, P = 0.032) on SOD followed by Hb (eta = 0.123, P = 0.015), and then MCHC (eta = 0.115, P = 0.047). The other parameters did not show any significant effect (P > 0.05). Table 2 also shows summary of a similar generalized linear model for the control group, here PMN and platelet levels had significant effect on the SOD levels. Partial eta values showed that PMN exerted the greatest effect (eta = 0.197, P = 0.011) followed by platelets (eta = 0.129, P = 0.043). The remaining parameters did not show any significant effect on the SOD activity values.

Table 1. Comparisons of cases and controls by hematological parameters

Variables	Mean (SD)		P value
	Cases (n = 100)	Controls (n = 41)	
Age (years)	9.9 (3.1)	10.3 (2.1)	0.451
Hemoglobin (g/dl)	9.94 (0.84)	10.96 (0.54)	0.001*
Red blood cells (x 10^{12}/l)	4.7 (0.64)	5.17 (0.7)	0.002*
White blood cells (x10^9/l)	7.41 (3.41)	5.57 (0.85)	0.001*
Polymorphonuclear neutrophils (%)	72.01 (14.48)	71.96 (5.66)	0.989
Lymphocytes (%)	27.2 (14.26)	39.17 (7.48)	0.001*
Eosinophils (%)	0.28 (0.64)	0.2 (0.51)	0.477
Mean cell hemoglobin concentration (g/dl)	33.35 (1.59)	33.27 (1.57)	0.786
Mean corpuscular volume (fl)	80.29 (7.06)	84.71 (5.8)	0.006
Platelets (x10^9/l)	132.9 (66.7)	220.98 (53.85)	0.001*

SD, Standard deviation; **n,** sample size; *statisticilally significant

Table 2. Relationship between superoxide dismutase activity and hematological parameters

Source	Controls			Cases		
	Sum of squares	P value	Type III partial eta squared	Sum of squares	P value	Type III partial eta squared
Corrected model	15871.364	0.014	0.482	13305.272	0.119	0.366
Intercept	339.79	0.446	0.02	2055.19	0.112	0.082
Hb	587.94	0.318	0.033	5156.55	0.015*	0.123
RBCs	179.48	0.579	0.01	580.69	0.391	0.025
WBC	267.71	0.498	0.015	49.49	0.801	0.002
PMN	4186.46	0.011*	0.197	3870.02	0.032*	0.144
LYM	1573.51	0.107	0.084	2796.9	0.066	0.108
EOSI	17	0.864	0.001	11.91	0.902	0.001
MCHC	243.13	0.518	0.014	3278.64	0.047*	0.115
MCV	86.33	0.7	0.005	0.42	0.981	0.001
PLT	2532.8	0.043*	0.129	97.31	0.724	0.004

Hb, hemoglobinym**; RBC,** red blood cells; **WBC,** white blood cels; **LYM,** lymphocytes; **EOSI,** eosinophils; **PLT,** platetlets. **PMN,** polymorphonuclear neutrophils; **MCV,** mean corpuscular volume. *statistically significant

Percentage activity of SOD in study participants

Since the hematological parameters exert effect at varying degrees on the levels of SOD activity in both control samples and cases, we compared the levels between the control and uncomplicated malaria groups. The level of the SOD activity, as an index of the ROS in the RBC lysate of both cases and controls, was determined and it was found to be significantly higher ($P = 0.005$) in malaria cases compared to the controls (Figure 1).

DISCUSSION

The clinical outcome of malaria infection in African children depends on multiple factors and is particularly influenced by age, immune status, genetic variables of the host, and to a lesser extent, the geographical origin of the parasite [13]. In the first 6 months of life, children born in malaria-endemic areas are protected from severity of the disease due to the passive transfer of maternal immunoglobulin and by expression of fetal hemoglobin [13, 14]. In areas where transmission of *P. falciparum* is intense, almost all children become infected with the parasite and subsequently acquire a form of partial immunity termed premunition. This allows them to harbor the parasites without experiencing clinical malaria. Attainment of premunition with subsequent protection against clinical malaria is slowly achieved in age- and exposure-dependent manner and it plateaus around adolescence [15-18]. During this period of acquisition of adaptive anti-malarial immunity, efficiency and effectiveness of innate immune response mediated amongst others by polymornuclear neutrophils and other myeloid cells may influence both the frequency and severity of malaria.

The critical respiratory burst activity carried out by PMN to mediate anti-malarial immune response [4,19] involves the catalytic conversion of di-molecular oxygen into superoxide anion [20]. Together with other reactive oxygen intermediates, superoxide serves to combat pathogens [21]. SOD activity levels correlates with the concentration of superoxide in sample. In this study, the percentage SOD activity was higher in uncomplicated malaria cases compared to healthy controls. This is suggestive of higher concentration of superoxide in malaria cases than in controls. SOD catalyzes the reaction of the unstable and toxic superoxide to hydrogen peroxide which can then be removed by catalase and glutathione enzyme systems [25]. Extracellular (EC)-SOD mediates this conversion in the extracellular matrix of tissue and intracellular SOD may therefore protect against collateral damage to intracellular tissues. Since *Plasmodium* concentrates the host SOD in the infected RBC as reported by several authors [22-24], this could probably be an effort to circumvent the adverse effects of the possibly increasing levels of superoxide on itself. [25].

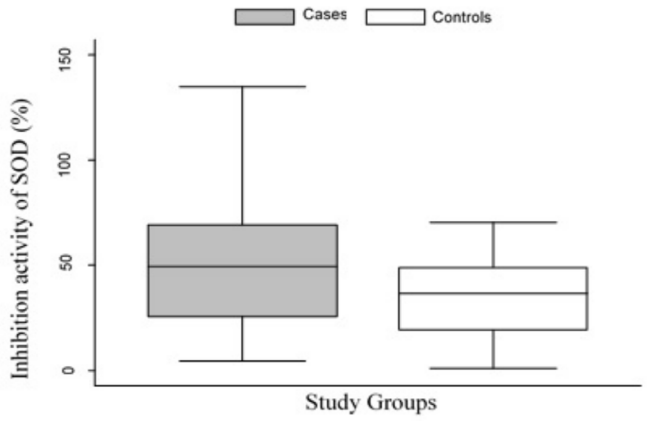

Figure 1. Percentage activity of superoxide dismutase (SOD) in the study participants (cases, n = 100; controls, n = 41). Difference between the groups is statistically significant.

In this study, activity of SOD was influenced by platelets and PMN in the study controls while PMN, Hb and MCHC levels influenced the levels in the cases (Table 2). The association of PMN and Hb levels, especially in the cases, is not unexpected as PMN are good sources of ROS. Also, if decreasing Hb levels is mediated by hemolysis, a detrimental but cardinal feature of malaria infection, it could lead to release of free iron that could catalyze hemoglobin oxidation to generate more superoxide. Increasing superoxide production would therefore subject the parasites to increasing oxidative stress to cause the parasite to take up and concentrate endogenous SOD in the infected RBC in order to protect itself [26]. Platelets levels in study cases were high and this has been ascribed both protective and pathogenic roles in malarial infection. The study has a number of limitations including the in ability to measure and relate the extracellular levels of SOD in the plasma to the levels in RBC and also to compare the levels across different disease phenotype expressions. We also did not correlate SOD activity with parasitemia levels.

In conclusion, in this study it was shown that the reasonably high SOD activity levels in uncomplicated *falciparum* malaria occur compared with the apparently healthy controls in a pediatric population in Ghana. Given our findings, and the fact that much of the pathology of malaria can be immune-mediated, we suggest additional investigations of the roles of cytokines in oxidative stress and their relationships with other hematological indices to provide further insights into the immunological status of patients with malaria.

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Conflict of Interest: None declared