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Original Research Article

Antimalaria therapy and changes in oxidative stress indices in falciparum malaria infection in Calabar metropolis, Nigeria

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Abstract

Purpose: *To assess the total antioxidant capacity (TAC), reduced glutathione (GSH), nitric oxide (NO), malondialdehyde (MDA), total plasma peroxides (TPP), oxidative stress index (OSI) and random plasma glucose (RPG) in falciparum malaria infection with and without antimalaria therapy.*
Methods: *Ninety subjects aged 18 to 60 years comprising 30 malaria patients without antimalaria therapy, 30 malaria patients on antimalarial therapy and 30 subjects without malaria (control) were studied. TAC, GSH, NO, MDA, TPP and RPG were determined using colorimetric methods, while parasite density (PD) and oxidative stress index (OSI) were computed. Anthropometric indices were obtained and the data analysed using analysis of variance and Pearson's correlation at $p < 0.05$.*
Results: *Higher levels of lipid peroxidation (MDA, TPP and OSI), lower antioxidant (GSH and TAC) and NO were observed in malaria patients with or without antimalaria therapy when compared to their respective controls ($p < 0.05$). Malaria patients without antimalaria therapy had higher PD and lipid peroxidation (TPP and OSI) and RPG and antioxidants (lower GSH and TAC) than those on antimalaria therapy ($p < 0.05$). Positive correlations were observed between PD and MDA ($r = 0.399$, $p = 0.029$) in malaria patients without antimalaria therapy, and between PD and TPP ($r = 0.660$, $p = 0,002$), and PD and OSI ($r = 0.717$, $p = 0.000$) in malaria patients on antimalaria therapy.*
Conclusion: *Falciparum malaria infection is associated with increased lipid peroxidation, depressed antioxidants and nitric oxide which may be ameliorated by antimalaria therapy.*

Keywords: *Malaria, Antimalaria therapy, Oxidative stress, Lipid peroxidation*

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INTRODUCTION

Falciparum malaria has been reported to account for 90 % of all cases of malaria in Sub-Saharan Africa, and is associated with *most severe forms of malaria leading to high morbidity and mortality* especially in young children and pregnant women [1]. Immune response to malaria infection results in increased generation of reactive oxygen species (ROS) from phagocytic cells recruited in the process of combating the infection [2]. Malaria parasite itself also

generates ROS via hemoglobin degradation resulting in the release of redox active by-products, free haeme and H_2O_2, conferring oxidative insult on the host cell. Reactive oxygen species are double aged sword, being necessary for vital physiologic functions at low concentrations, while leading to lipid peroxidation, oxidative DNA and tissue damage at higher levels.

Redox imbalance between ROS generation and their neutralization by antioxidants in the system leading to accumulation of ROS in the system results in oxidative stress (OS) [3]. Parasitemia induced OS has been implicated in the physio-pathology of malaria and the development of various complications associated with severe malaria [2].

Complex host-parasite interactions modulate the balance between antioxidants and ROS since both the host and parasite have the capacity of generating them. This is compounded by the observation that some antimalaria drugs such as chloroquine, primaquine and artemisinin and their derivatives induce ROS production and therefore constitute source of oxidation [4]. The first line of treatment for uncomplicated malaria according to WHO recommendations is artemisinin – based combination therapy (ACTs). Earlier studies have shown that artemisinins reduce the levels of reduced glutathione (GSH) and other antioxidants in the parasite thereby inducing OS in the parasite leading to their destruction [5].

Previous studies have reported elevations in ROS and depletion of antioxidants in malaria infection. These reports were mainly observations from animal studies and extrapolated to humans [3]. The level of ROS and RNS generated in malaria infection, and the extent of membrane peroxidation and ensuing tissue damage may be a function of the degree of parasitemia and severity of malaria. However, the systemic levels of biomarkers of oxidative stress, the relative or absolute effect of antimalarial therapy on their levels and their relationship with development of the various complications of malaria in individuals with malaria infection are still uncertain. Routine estimation of biomarkers of oxidative stress in malaria infection may therefore be useful prognostic markers of disease progression and response to antimalarial therapy.

This study assessed the random plasma glucose (RPG) and biomarkers of oxidative in relation to degree of parasitemia in individuals with malaria infection, with and without antimalaria therapy.

EXPERIMENTAL

Study design

This case control study was carried out in University of Calabar Teaching Hospital (UCTH) Calabar, Cross River State, Nigeria from February to July, 2016. The study population was made up of 90 subjects aged 18 – 60 years drawn from patients, hospital staff and students at UCTH. Informed consent was obtained from all subjects before recruitment into the study and the ministry of health, Calabar health research ethics committee approved the study protocol (REC no. CRSMOH/RP/REC/2017/489). The ethical principles for medical research involving human subjects according to the Helsenki Declaration in 1975 and subsequent revisions were strictly adhered to in this study [6].

Selection of subjects

The participants in this study were made up of 90 male and female subjects comprising 30 microscopy confirmed malaria infected subjects without antimalaria therapy, 30 microscopy confirmed malaria infected subjects on antimalaria therapy, and 30 subjects without malaria and who were not on prophylactic antimalaria treatment serving as controls, were recruited into the study. Subjects with history of hepatitis B virus, HCV, HIV infection and other chronic organ or systemic illness and prolonged medication were excluded from the study. Anthropometric data (height, weight, body mass index) of all participants were collected and used to calculate body mass index while socio-demographic and medical information such as age, diet, lifestyle, history of past disease, status of malaria infection, type of antimalaria drug taken, and presence of other illness other than malaria were collected via an interviewer-administered structured questionnaire.

Sample collection

Finger prick blood samples were aseptically taken, placed on clean grease free slides and used to prepare thick and thin films for identification of malaria parasites. Five milliliters of whole blood samples were also collected from same subjects via venipuncture, 2ml was dispensed into fluoride oxalate bottle for estimation of random plasma glucose, while 3 ml was dispensed into plain sample containers, kept away from direct sunlight, allowed to clot and retract and then spun at 500g for 5 minutes to obtain serum. The serum obtained was transferred into another clean dry plain sample

container and stored at -20 ˚C and analysed within one week.

Identification of malaria parasite

Thick and thin blood films were made on the same slide and air-dried for 2 to 3 min. Giemsa stain (5%) was used to stain the blood films for 30 min for detection of malaria parasite and parasite count [7].

Calculation of parasite density

The ratio of parasites to white blood cells (WBC) in thick film was recorded as the parasite density (PD). This was determined by counting the number of parasites per 200 white blood cells present in each thick blood film and multiplying by the total WBC count of each blood sample, as shown in Eq 1 [8].

PD = N/W …….. (1)

where PD is parasite density/µL blood, N is no. of parasites counted x 8000 WBC/µL, W is no. of WBC counted

Determination of plasma parameters

TAC

The TAC is estimated based on the reaction of a standard solution of Fe-EDTA complex with hydrogen peroxide, (H_2O_2) to form hydroxyl radicals [HO•]. These ROS degrade benzoate to release thiobarbituric acid reactive substances (TBARS). Antioxidants in the sample suppress the production of TBARS, and the inhibition of colour development measured at 532nm is described as the total antioxidant capacity of the sample [9].

TPP

Total plasma peroxide (TPP) concentration in the sample was determined using the ferrous-butylated hydroxytoluene-xylenol orange (FOX2) complex method. The FOX-2 test system is based on the oxidation of ferrous ions to ferric ions by various types of peroxides present in the serum samples to produce a coloured ferric-xylenol orange complex whose absorbance was measured at a wavelength of 560nm [10].

OSI

Oxidative stress index was calculated as the ratio of TPP to TAC expressed as a percentage, an indicator of the degree of oxidative stress [10].

NO

Griess test was used for determining the total levels of nitrite or nitrous acid in the samples. The NO-containing compounds in the serum combine with alpha- naphthylamine to produce pink azo dye whose absorbance was measured at 540nm. The total levels of nitrite and nitrate designated as total nitric oxide metabolites (NOx) is used as a direct marker of *in vivo* NO production [11].

GSH

Reduced glutathione estimation was done using the modified Ellman's method. The reagent, 5-5'-dithiobis [2-nitrobenzoic acid] (DTNB, Ellman's Reagent) reacts with GSH to form the chromophore, 5-thionitrobenzoic acid (TNB) and GS-TNB which is measured spectrophotometrically at 412 nm [12].

MDA

Estimation of MDA was done based on the reaction of MDA with thiobarbituric acid (TBA) to form MDA-TBA2 adduct which is measured at 532 nm [13].

RPG

The glucose in the sample in the presence of glucose oxidase is oxidized to hydrogen peroxide which reacts under catalysis of peroxidase, with phenol and 4-aminophenazone to form a red-violet quinoneimine dye indicator. The absorbance of dye is proportional to the concentration of glucose in the sample [14].

Statistical analysis

The data are presented as mean ± SD. Data were analysed using Statistical Package for Social Sciences, SPSS version 20.0. Student's t-test was used to determine group mean differences, analysis of variance for determination of variation within and among groups and Pearson correlation for association among variables at 95% probability level.

RESULTS

The comparison of age, BMI, PD, TPP, TAC, OSI, NO, RPG, GSH and MDA in malaria patients without antimalaria therapy, malaria patients with antimalaria therapy and controls is shown in Table 1. Significant variations were observed in BMI, PD, TPP, TAC, OSI, NO, RPG, GSH and MDA levels of the 3 groups studied ($p < 0.05$). No significant variation was observed in

the mean age of the 3 groups (p > 0.05). Significantly higher PD, TPP, OSI and MDA and lower RPG, TAC, NO and GSH were observed in malaria patients without antimalaria therapy when compared to the controls (p < 0.05). Higher levels of MDA, OSI and PD and lower BMI, TAC and NO were also observed in malaria patients with antimalaria therapy when compared to the controls (p < 0.05). Malaria patients on antimalaria therapy had higher RPG, GSH and TAC and lower PD, TPP and OSI when compared to those without antimalaria therapy (p < 0.05).

Figure 1 shows correlation plot of PD against MDA in malaria patients without antimalaria therapy. Significant positive correlation was observed between PD and MDA (r = 0.399, p = 0.029) in malaria patients without antimalaria therapy.

Table 1: Comparison of Age, BMI, PD, TPP, TAC, OSI, NO, RPG, GSH and MDA in malaria patients on antimalaria therapy, malaria without antimalaria therapy and control subjects

Index	Control (n = 30)	Ma (n	lue
Age (y)	24.43±3.079	25.6	)9
BMI (kg/m2)	22.78±4.39 ‡	21.6	7*
PD (μL^{-1})	0.00±0.00† ‡	187.7	0*
TPP ($\mu molH_2O_2/L$)	216.98±56.17†	369.4	0*
TAC (µmol/L)	1666.89±171.92† ‡	1298.4	0*
OSI (%)	13.16±3.73† ‡	29.4	0*
NO (µmol/L)	35.02±24.48† ‡	21.7	6*
RPG (mmol/L)	5.99±1.19†	4.97	0*
GSH (µmol/L)	26.94±6.014†	23.5	5*
MDA (nmol/mL)	24.70±2.76† ‡	68.9	0*

Data presented as mean ± SD, * = indicate significant variations among groups at p < 0.05, † = indicate significant difference between controls and malaria patiemts without antimalaria therapy at p < 0.05, ‡ = indicate significant difference between controls and malaria patients with antimalaria therapy at p < 0.05, ϕ = indicate significant difference between malaria patients without antimalaria therapy and those with antimalaria therapy at p < 0.05, BMI = Body mass index, PD = Parasite density, TPP = Total plasma peroxide, TAC = Total antioxidant capacity, OSI = Oxidative stress index and NO = Nitric oxide, RPG = Random plasma glucose, GSH = reduced glutathione, MDA = Malonedialdehyde, Antimal = malaria patients on antimalaria therapy

Figure 2 shows correlation plot of PD against TPP in malaria patients on antimalaria therapy. Positive correlation was observed between PD and TPP (r = 0.660, p = 0.002) in malaria patients on antimalaria therapy. Correlation plot of PD against OSI in malaria patients on antimalaria therapy is depicted in Figure 3. A significant positive correlation (r = 0.717, p = 0.000) was observed between PD and OSI in malaria patients on antimalaria therapy.

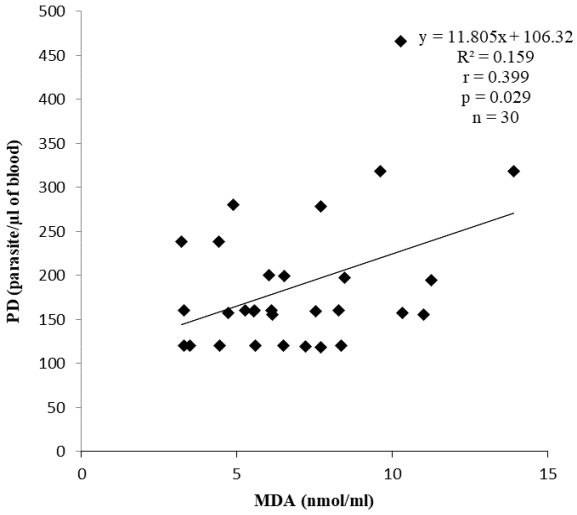

Figure 1: Correlation plot of PD against MDA in malaria patients not on antimalaria therapy

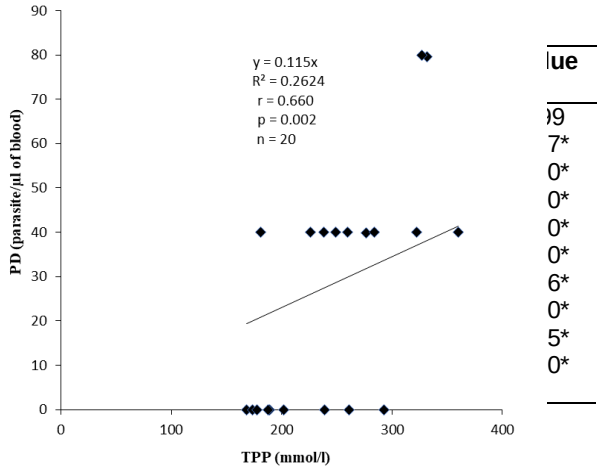

Figure 2: Correlation plot of PD against TPP in malaria patients on antimalaria therapy

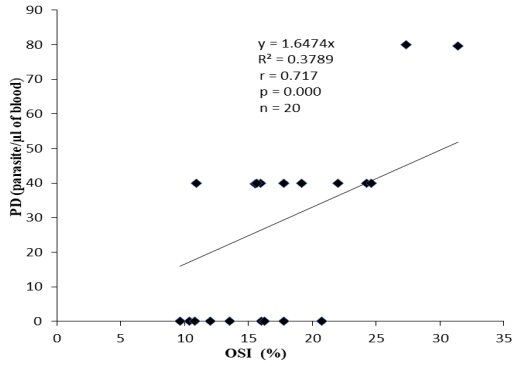

Figure 3: Correlation plot of PD against OSI in malaria patients on antimalaria therapy

DISCUSSION

Falciparum malaria has been associated with increased generation of ROS and RNS which has been implicated in the disease progression and development of complications.

In this study, lower TAC and higher TPP, MDA and OSI were observed in malaria patients without antimalaria therapy when compared to controls. Malaria results in production of reactive species, release of redox active substances and degradation of hemoglobin by the parasites resulting from cell – parasite adherence and anemia triggered by infection *P. falciparum* infected red cell has been shown to produce twice as much H_2O_2 and OH^- when compared to normal uninfected erythrocytes. The H_2O_2 and OH^- radical produced as a result of the infection leads to increased peroxidation of lipids and membrane proteins [15]. Higher levels of lipid peroxides therefore seen in malaria subjects without antimalaria therapy may be the result of the toxic effect of increased generation of ROS and RNS resulting from inflammatory process initiated in the host in response to infection [16].

The elevated MDA levels observed is also an indication of increased production of ROS, OS and lipid peroxidation, MDA being a biomarker of lipid peroxidation [15]. Malaria induced lipid peroxidation and OS in infected red cells reduce cell membrane deformity [4]. The ensuing cell rigidity results in microcirculatory obstruction, tissue hypoperfusion and the ultimate removal of the rigid cell by the spleen exacerbating anemia in malaria infection [17]. Accumulation of lipid peroxidation products affects cellular vitality often leading to molecular and cellular destruction and contributing to pathogenesis of malaria [2]. Positive association was observed between parasite density and MDA in malaria patients without antimalaria therapy. This shows that the extent of lipid peroxidation (MDA) increased with increase in the parasitaemic load. It has been 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 have been implicated in the development of various complications associated with malaria [18]. The lower TAC levels seen in malaria subjects compared to controls may result from their increased utilization in buffering the deleterious effects of excess ROS and RNS generated in the course of malaria infection.

Lower TAC and higher TPP, MDA and OSI were also observed in malaria patients on antimalaria therapy when compared to controls. Antimalarial therapies used by subjects in this study were predominantly ACT and its derivatives. Rapid clinical responses have been observed with ACTs with a parasite reduction ratio of approximately 10,000 per erythrocytic cycle [5]. The parasiticidal actions of ACTs has been attributed to the presence of the endoperoxide bridge in the trioxane pharmacophore of artemisinins since replacement of one peroxidic oxygen with a carbon results in a derivative devoid of antimalaria effect [5]. Proposed mechanisms of action of ACTs involve heme mediated cleavage of the endoperoxide bridge with subsequent formation of ROS and carbon – mediated radicals which destroys the malaria parasites [19]. Heme's involvement in ACTs activation may explain the selective toxicity of ACTs to parasite infected red cells when compared to normal red cells. The generation of ROS through parasite-antimalaria drug interaction adds to the overall oxidative burden of the parasitised cell and is responsible for higher MDA and OSI with lower TAC seen in malaria patients on antimalaria therapy compared to controls [19]. This also accounts for the positive correlation observed between parasite density and TPP and OSI seen in these subjects.

Higher TAC and lower PD, TPP and OSI demonstrated in malaria patients on antimalaria therapy when compared to those without antimalaria therapy, may be due to reduction in ROS generation by malaria parasites as a result of parasite clearance by antimalaria drugs [15]. *Plasmodium faciparum* infection has been shown to deplete TAC levels and antimalaria therapy with enhancement of TAC. Artemisinin - based combination therapies have been shown to enhanced serum TAC by 25.6 % when compared with the value obtained from malaria infected patients yet to receive treatment. Thus, treatment of malaria with ACT significantly improved the TAC levels of infected patients, hence their defense against ROS [20]. A decline in TPP levels after three days of chloroquine treatment when compared to pre-treatment levels in malaria patients has been reported. Reduced parasite density has also been reported in malaria patients treated with antimalaria drugs when compared to untreated patients [21].

Lower NO levels observed in malaria patients with or without antimalaria therapy when compared to the controls may be implicated in higher parasite density and malaria seen in these subjects. Nitric oxide has been shown to confer protection against malaria and also contribute to resolution of malaria infection [4]. The parasiticidal activity of NO has been attributed to oxidative destruction of malaria parasites by

peroxynitrite, formed from the reaction of NO with O_2 [22]. Reduction in production of NO by inhibition of inducible nitric oxide synthase (iNOS) has been shown to impair intrahepatic parasite destruction [22], leading to the development of severe forms of the disease (cerebral malaria) suggesting that NO inhibits development of severe complications in malaria [4]. However, excess NO production has been shown to be cytotoxic not only to the invading parasites but also to the host's cells. It has been reported that high concentrations of plasma NO have been positively correlated with depth of coma in cerebral malaria [23]. In contrast to the findings of this study, increased NO levels in malaria was not associated with a corresponding decrease in parasite density in infected subjects [4].

Lower levels of reduced glutathione (GSH) observed in malaria patients without antimalaria therapy may be due to increased utilization of GSH in the neutralization of increased ROS associated with *P. falciparum* infection. Moreover, GSH efflux from *P. falciparum* infected erythrocytes has been shown to be greater than that from uninfected erythrocytes [24]. Reduced glutathione is critical for protecting the tissue from oxidative stress, acting as a free radical scavenger and inhibitor of lipid peroxidation [18].

Malaria patients without antimalaria therapy had lower RPG levels when compared to those with antimalaria therapy and controls. Malaria infection has been reported to be associated with pleiotropic changes in glucose metabolism with the level of hypoglycemia correlated with severity of infection [25]. The metabolism of the parasite in infected erythrocytes has been shown to utilize up to 75 times more glucose than uninfected erythrocytes [25]. Thus, the risk of hypoglycemia increases as infection progresses because host glucose production becomes insufficient for host/parasite demand. Heavy parasitemia have been associated with high glucose requirements by malaria parasites and therefore lower glucose levels [25].

Lower BMI was observed in malaria patients on antimalaria therapy when compared to control. Transient weight loss and lower BMI seen in subjects with malaria may be attributed to poor appetite, decreased food intake, vomiting, diarrhea and abdominal pain associated with malaria [26]. Parasitemia also induce production of proinflammatory cytokines such as TNF-α which is a known mediator of anorexia and cachexia seen in many human disease states [26]. This relationship suggests that TNF-α production in *P. falciparum* malaria infection may contribute to decreased nutritional status which will reflect in the BMI of malaria patients.

Limitations of study

The major limitation of this study is the small sample size which will not allow for a definite conclusion to be drawn from the study. The authors were unable to ascertain the effect of dose, patient's adherence to drug prescription and the use of herbal remedies in combination with the ACT's.

CONCLUSION

The findings of this work suggest that malaria infection is associated with increased lipid peroxidation, depressed antioxidants and nitric oxide which may lead to oxidative stress and increased risk of development of severe complications in malaria. The use of artemisinin - based combination therapies ameliorates malaria induced oxidative stress by reducing lipid peroxidation and increasing antioxidant capacity. Assessment of biomarkers of oxidative stress may therefore serve as useful indices for monitoring the severity of *P. falciparum* infection and response to treatment.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors contributed to the conceptualization and design of the research, data analysis and final approval of manuscript.

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