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Antioxidant activity in HIV and malaria co-infected subjects in Anambra State, southeastern Nigeria

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ABSTRACT

Objective: To determine the antioxidant status of HIV and malaria co-infected participants. **Methods:** Blood samples collected from the 193 randomly recruited participants were used for HIV screening, *Plasmodium falciparum* antigen screening, malaria parasite density count, $CD4^+$ T cell count, glutathione reductase, glutathione peroxidase and total antioxidant status measurement. Standard laboratory methods were used for the analysis. **Results:** The results showed that glutathione reductase, glutathione peroxidase, total antioxidant status and $CD4^+$ T cell count were significantly lowered in symptomatic HIV participants with and without malaria co-infection ($P<0.01$) in each case compared with control participants. Also, glutathione reductase, glutathione peroxidise, total antioxidant status and $CD4^+$ T cell count were significantly lowered in asymptomatic HIV participants with and without malaria co-infection ($P<0.05$) in each case, compared with control participants without malaria. Similarly, these antioxidants were significantly lowered in control participants with malaria infection ($P<0.05$) compared with control participants without malaria. The malaria parasite density in symptomatic HIV infected participants was negatively associated with glutathione reductase ($r = -0.906$, $P<0.01$), glutathione peroxidase ($r = -0.719$, $P<0.01$) and total antioxidant status ($r = -0.824$, $P<0.01$). **Conclusions:** The antioxidant activity was affected in HIV infected participants with malaria co-infection. Malaria co-infection in HIV seems to exert additional burden on antioxidants. This calls for concern in malaria endemic areas with increasing prevalence of HIV infection.

1. Introduction

Malaria is endemic in Nigeria with stable transmission over the year. The spread of HIV is also high within this area. Various studies in Nigeria have shown that there is a high prevalence of malaria among HIV infected individuals[1,2]. In a population based cohort, HIV infected individuals had significantly higher risk of having malaria parasitemia and developing clinical malaria than HIV negative individuals[3].

Oxidative stress has emerged in recent years as a suspected component in the pathogenesis of HIV disease. Increasing number of researchers agree that even in the earliest stages of infection, a deleterious reductive-oxidative (redox) imbalance may occurr[4]. During infections, neutrophils liberate free radicals and highly reactive oxidants both intracellularly and extracellularly in their attempt to destroy pathogens in the process termed respiratory burst[5]. The oxidants released include superoxide anion (O_2), hydrogen peroxide (H_2O_2), singlet oxygen ($O_2^{\cdot}$) and hydroxyl radicals (OH^-). The respiratory burst consumes reduced nicotinamide adenine dinucleotide phosphate (NADPH) which must be continuously restored if respiratory burst is to be mopped-up. Restoration of NADPH is accomplished by way of

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hexose monophosphate (HMP) pathway, by various rate limiting antioxidant enzyme mechanisms. These antioxidant enzymes include: glutathione peroxidase, glutathione reductase, superoxide dismutase, catalase, G-6-PD[6]. Defficiency of any of these enzymes can cause oxidative stress because NADPH is not replenished. Oxidative stress, viral replication and destruction of $CD4^+$ T cells leads to progression of disease[7]. Furthermore it has been suggested that generation of reactive oxygen species (ROS) and associated oxidative stress play central role in development of systemic complications in malaria. This is because glutathione enzyme system, which includes glutathione peroxidase, glutathione S-transferase, and glutathione reductase are important antioxidants enzyme activity present in erythrocytes[8]. Therefore the present study was designed to evaluate the impact of HIV and malaria co-infection on antioxidant status of infected individuals in the South-eastern Nigeria.

2. Materials and methods

193 participants aged (39±18) years were recruited at the Voluntary Counselling and Testing unit of Nnamdi Azikiwe University Teaching Hospital Nnewi, Nigeria.

These participants were screened for HIV and *Plasmodium falciparum (P. falciparum)* infections, and grouped into: (i) asymptomatic HIV and malaria co-infected participants (n=39), (ii) asymptomatic HIV participants without malaria (n=35), (iii) symptomatic HIV and malaria co-infected participants (n=35), (iv) symptomatic HIV infected participants without malaria (n=25), (v) HIV seronegative control participants with malaria (n=29) and (vi) HIV seronegative control participants without malaria (n=30). Blood samples collected from the participants were used for HIV screening, *P. falciparum* antigen screening, malaria parasite density, $CD4^+$ T cell count, glutathione reductase, glutathione peroxidase and total antioxidant status. Standard laboratory methods were used for the analysis. Participants were staged using World Health Organisation guideline for HIV staging. Only those participants in HIV stage-1(asymptomatic HIV infected) and stage- 2(symptomatic HIV infected) participated in the study. The HIV infected participants were not on any anti- retroviral therapy.

Informed consent was obtained from those who participated in the study, while the ethical committee of Nnamdi Azikiwe University Teaching Hospital Nnewi approved the study design.

2.1. Sample collection

Blood sample (6 mL)was collected from the participants and aliquoted into lithium heparin, plain and EDTA tubes for the determination of glutathione peroxidase activity; total antioxidant status and glutathione reductase activity; HIV seroreactivity, $CD4^+$ T-cell count, malaria parasite count and malaria parasite antigen detection respectively.

2.2. HIV screenings by both immunoassay and immunochromatographic methods

2.2.1. Immunoassay method

HIV screening was performed using Abbot determine TM HIV-1 & 2 kit. (Abbot Japan Co ltd. Tokyo, Japan). The procedure was as described by the manufacturer. Briefly, 50 μL of plasma sample from participants were applied to appropriately labelled sample pads. After 15 minutes of sample application, the results were read. The inherent quality control of the kit validates the results. Two visible red lines occurs in the region labelled control and test represents HIV seropositive reaction while a single red colour in the region of control validates the test kit. Absence of red line in the test region represents HIV seronegative reaction.

2.2.2. Immunochromatographic method

HIV screening by immunochromatographic method using HIV 1 and 2 STAT-PAK Assay kit; for the qualitative detection of antibodies to HIV-1 and HIV-2 in human plasma (Chembio diagnostic system, INC. New York, USA). The procedure was as described by the manufacturer of the kit. 50 μL of plasma sample was dispensed into appropriately labelled sample wells, and then three drops of running buffer (supplied by the manufacturer) was added drop-wise into the appropriately labelled sample wells. The results of the test were read at 10 minutes after the addition of the running buffer.

2.3. P. falciparum antigen detection

The procedure was as described by the manufacturer (Para check, Orchid Biomedical systems Vena Goa, India). Briefly, 10 μL of the whole blood specimen from the participants were transferred into appropriately labelled specimen cassettes containing sample wells. Subsequently, 120 μL of clearing buffer was added into the sample wells. After 15 minutes, the results were read.

2.4. Determination of P. falciparum parasite density

Giemsa Stained Thick and thin blood smear for microscopic detection of *P. falciparium* parasites was made. Thin and thick blood films were prepared for each participant and allowed to dry. After drying, the films were examined using the ×100 oil immersion objective. The parasite density was determined by recording the number of malaria parasite detected within a high powered field count of 200 white blood cells (WBC). This was then multiplied by

the participants total white blood cell count (TWBC). The number of parasite per μL of blood was calculated. The procedure was repeated in two other areas of the field and an average of the three counts was taken, and malaria parasite density was calculated as follows:

Parasite count = TWBC count × number of parasites counted within 200 WBC/200.

2.5. Determination of $CD4^+$ T cell count

50 μL of whole blood in EDTA anti-coagulant was dispensed into a partec test tube and 10 μL of CD4 PE antibody was added. The reaction mixture was incubated in the dark for 10-15 minutes. After incubation, 800 μL of the already prepared diluted buffer (Xn 0.09% NaN_3) was added to each reaction tube and vortexed. The partec tubes containing these reactions were plugged in position in the Cyflow SL Green (Partec Germany), which has already been connected to flow max software, CD4 count template data file and CD4 count instrument. The test was run on the Cyflow for 90 seconds. The results were displayed as histogram and printed. The $CD4^+$ T-Cell count was read off the histogram correcting for the dilution factor.

2.6. Determination of glutathione peroxidase enzyme

The procedure was as described by the manufacturer of the kit (Randox Laboratories Ltd, UK). In brief, 0.05 mL of heparinized whole blood from each of the participant was dispenced in appropriately labelled test tubes containing 1 mL of diluting agent (0.18 mmol/L). This was incubated for 5 minutes. After the incubation, 1 mL of haemoglobin reagent (containing I volume of haemoglobin reagent with 4 volumes redistilled water) was added. This was mixed very well and assayed within 20 minutes for glutathione peroxidase as follows: 0.02 mL of the diluted sample was added in a labelled separate test tubes containing 1 mL of Reagent 1 [Glutathione 4 mmol/L, glutathione reductase 0.5U/L and NADPH 0.34 mmol/L in phosphate buffer PH 7.2 (0.05 mol/L, and EDTA, 4.3 mmol/L)], and 0.04 mL of Cumene solution (Cumene hydroxide, 0.18 mmol/L). The reagent blank was prepared similarly with 0.02 mL of distilled water in place of the sample. This was mixed gently and the absorbance of sample and reagent blank was read at 340 nm against air after 1, 2 and 3 minutes. The change in absorbance of both sample and reagent blank was calculated and the concentration of glutathione peroxidase calculated using a factor of 8 412 after subtracting the absorbance of blank from that of sample.

2.7. Determination of glutathione reductase enzyme

The procedure was as described by the manufacturer of the kit (Randox Laboratories Ltd, UK). 40 μL of serum from each of the participant was dispenced in appropriately labelled test tube containing 1 000 μL of substrate (2.2 mmol/L reduced glutathione in 5 mL of 250 mmol/L potassium phosphate buffer, PH 7.3 and 5 mmo/L EDTA) and 200 μL NADPH (0.17 mmol/L). This was mixed and the initial absorbance was read after 1 minute at 340 nm against air. This was read again after 2, 3, 4, and 5 minutes. The average change in absorbance was calculated and used to calculate the glutathione reductase activity in U/L by multiplying by a factor of 4 983.

2.8. Determination of total antioxidant status

This was as described by the manufacturer of the kit (Randox Laboratories Ltd, UK). 20 μL of serum was added into a test tube containing 1 000 μL of 2,2′-Azino-di-(3-ethylbenzthiazoline sulphonate), and 1 000 μL of chromogen (6.1 μmol/L of metmyoglobin in 10 mL of 80 mmol/L phosphate buffered saline (PH 7.4). Standard and reagent blank were prepared in the same way using a standard solution containing (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and double distilled water respectively. 25 μL hydrogen peroxide was added to all tubes and mixed. The absorbance was read after 3 minutes at 660 nm against air at 37 ℃. The change in absorbance of sample and blank was calculated and used to determine the total antioxidant status.

2.9. Statistical analysis

The variable were expressed in mean and standard deviation. ANOVA and Student-*t* test were used to check for significant differences in variables amongst and within the groups. Significant level were considered at $P<0.05$ and $P<0.01$.

3. Results

Table 1 shows significant differences in serum activities of glutathione reductase (U/L), glutathione peroxidase (U/gHb) and total antioxidant status (mmol/L) in asymptomatic HIV infected participants with and without malaria and in control participants with and without malaria co-infection ($P<0.01$). Group comparison shows that the antioxidants measured were significantly lower in asymptomatic HIV infected participants with and without malaria co-infection and in control participants with malaria compared with control participants without malaria ($P<0.05$) in each case (Table 1). The $CD4^+$ T cell count were also significantly lowered in both asymptomatic HIV infected participants with and without malaria compared with control participants ($P<0.05$) in each case.

Table 2 shows significant differences observed in mean

serum activity of glutathione reductase (U/L), glutathione peroxidase (U/gHb) and total antioxidant status (mmol/L) in symptomatic HIV infected participants with and without malaria co-infections and in control participants with and without malaria co-infection (P<0.01). Group comparison shows that the antioxidants measured were significantly lower in symptomatic HIV infected participants with and without malaria co-infection and in control participants with malaria compared with control participants without malaria (P<0.01) in each case.

Table 3 and Figure 1 shows that in asymptomatic HIV participants with malaria co-infection there was significant correlation between $CD4^+$ T cell count and glutathione reductase (r=0.953, P<0.01) and between $CD4^+$ T cell count and glutathione peroxidase (r=0.711, P<0.01). There was also a significant negative correlation between malaria parasite density and glutathione reductase (r = −0.492, P<0.05) and between malaria parasite density and glutathione peroxidase (r= −0.584, P<0.05) in asymptomatic HIV participants with malaria co-infection.

In Table 3 and Figure 2&3, a strong significant negative correlation was observed between malaria parasite density and the antioxidants glutathione reductase (r=−0.906, P<0.01), glutathione peroxidase (r=−0.719, P<0.01) and total antioxidant status (r=−0.824, P<0.01) in symptomatic HIV participants with malaria co-infection. Also a positive relationship was observed between $CD4^+$ T cell count and glutathione reductase (r=0.827, P<0.01), glutathione peroxidase (r=0.864, P<0.01) (Figure 2) and total antioxidant status (r=0.833, P<0.01) (Figure 3) in symptomatic HIV

Table 1

Serum/plasma activities of antioxidants, $CD4^+$ T cells and *P. falciparum* density in asymptomatic HIV infected and HIV seronegative control participants with or without malaria (Mean±SD).

Group	Glutathionereductase (U/L)	Glutathione peroxidase (U/gHb)	Total antioxidan (mmol/L)	CD4 T cell count (/μL)	*P. falciparum* density (/μL) of blood
Asymp HIVwith malaria [a] (n=39)	55.87±5.25	49.63±5.97	1.43±0.09	416.08±204.38	831.62±231.12
Asymp HIV without malaria [b] (n=35)	59.46±6.05	49.49±6.35	1.45±0.08	395.73±156.65	–
Control with malaria [c] (n=29)	54.79±3.47	52.56±1.95	1.54±0.07$^{*\$}$	950.00±142.58^{*R}	463.80±132.44*
Control Without malaria [d] (n=30)	68.77±5.96$^{*\$\triangle}$	66.58±2.71$^{*\$\triangle}$	1.60±0.07$^{\#R\triangle}$	1012.92±190.90$^{\#R}$	–
F (P) value	6.633[e]	6.814[e]	4.178[f]	12.366[f]	nc

F(P) = asymptomatic HIV with or without malaria and HIV seronegative control with or without malaria compared (using ANOVA), e= P<0.05, f=P<0.01, nc = not compared; a = Asymp HIV with malaria, b= Asymp HIV without malaria, c = Control with malaria; Group comparison was analyzed with student t test, Symp = symptomatic; *P<0.05 comparing with a; # P<0.01 comparing with a; $ P<0.05 comparing with b; R: P<0.01 comparing with b; △P<0.01 comparing with c.

Table 2

Serum/plasma activities of the antioxidants and *P. falciparum* density in symptomatic HIV infected and HIV seronegative control participants with or without malaria (Mean±SD).

Group	Glutathione reductase (U/L)	Glutathione peroxidase (U/gHb)	Total Antioxidant (mmol/L)	CD4 T Cell count (/μL)	*P. falciparum* density of blood (/μL)
Symp HIVwith malaria [a] (n=35)	43.93±6.27	42.95±4.57	1.39±0.06	171.56±49.04	1254.24±478.92
Symp HIV without malaria [b] (n=25)	49.03±6.05*	7.32±4.35*	1.41±0.04	155.27±44.00	–
Control with malaria [c] (n=29)	54.79±3.47$^{\#}$	2.56±1.95$^{\#}$	1.54±0.07$^{\#R}$	950.00±142.58$^{\#R}$	463.80±132.44$^{\#}$
Control Without malaria [d] (n=30)	68.77±5.96$^{\#R\triangle}$	66.58±2.71$^{\#R\triangle}$	1.60±0.07$^{\#R\triangle}$	1012.92±190.90$^{\#R\triangle}$	–
F (P) Value	8.229[f]	9.920[f]	8.426[f]	15.341[f]	nc

F(P) = asymptomatic HIV with or without malaria and HIV seronegative control with or without malaria compared (using ANOVA), f=P<0.01 nc = not compared; a = Asymp HIV with malaria, b= Asymp HIV without malaria, c = Control with malaria; Group comparison was analyzed with student t test, Symp = symptomatic; *P<0.05 comparing with a; # P<0.01 comparing with a; $ P<0.05 comparing with b; R: P<0.01 comparing with b; △P<0.01 comparing with c.

Table 3

Correlation between malaria parasite density, antioxidants and CD4 T cell counts in asymptomatic HIV infected participants with or without malaria.

Participants		Asymptomatic HIV participants					Symptomatic HIV participants					Control HIV participants				
		GR cell	GP	TAS	MP ensity	CD4	GR cell	GP	TAS	MP density	CD4	GR cell	GP	TAS	MP density	CD4
Participants with malaria	MP Density	−0.492[b]	−0.584[b]	−0.157	1.0	–	−0.906[c]	−0.719[c]	−0.824[c]	1.0	–	−0.50[b]	−0.663[b]	0.124	1.0	–
	CD4 T cell count	0.953[c]	0.711[c]	0.572[b]	–	1.0	0.827[c]	0.864[c]	0.833[c]	–	1.0	0.312	0.164	0.040	–	1.0
Participants without malaria	CD4 T cell count	0.533[b]	0.326[b]	0.477[b]	–	1.0	0.855[c]	0.716[c]	0.812[c]	–	1.0	0.147	0.157	0.086	–	1.0

Correlation were analyzed by Pearson´s correlation, b = correlation significance at P<0.05, c = Correlation significance at P<0.01.

participants with malaria co-infection.

There was no significant correlation observed between $CD4^+$ T cell count and the antioxidants in HIV seronegative control participants with or without malaria but there was a significant correlation between malaria parasite density and glutathione reductase (r=−0.50, P<0.05) and glutathione peroxidase (r=−0.663, P<0.01) respectively in HIV seronegative control participants with malaria (Table 3 and Figure 4-6).

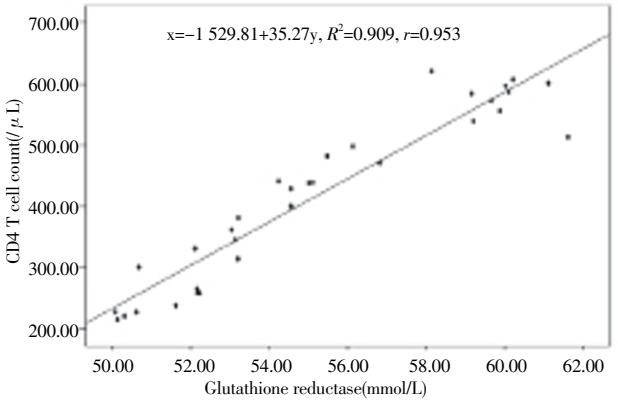

Figure 1. Scatter plot between CD4 T cell count and glutathione reductase in Asymptomatic HIV infected participants with malaria.

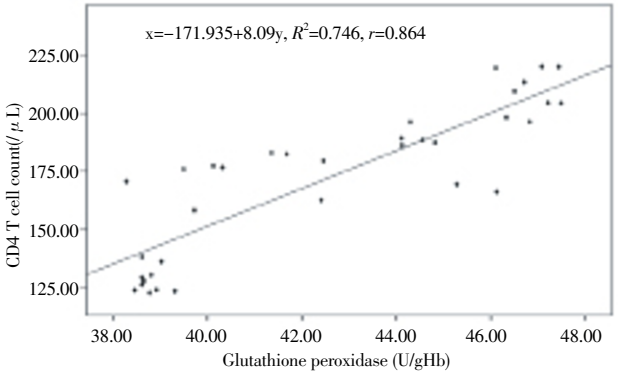

Figure 2. Scatter plot between CD4 T cell count and glutathione peroxidase in symptomatic HIV infected participants with malaria.

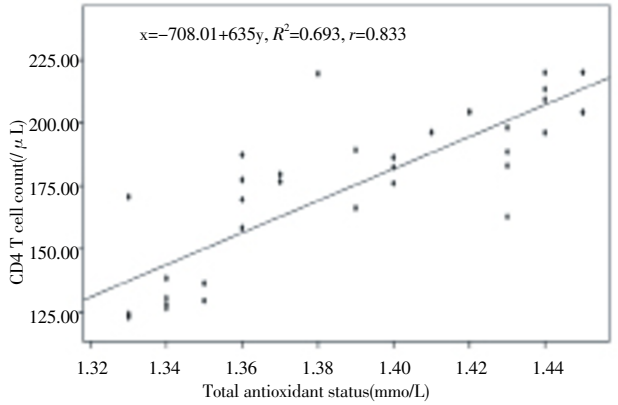

Figure 3. Scatter plot between CD4 T cell count and total antioxidant status in symptomatic HIV infected participants with malaria.

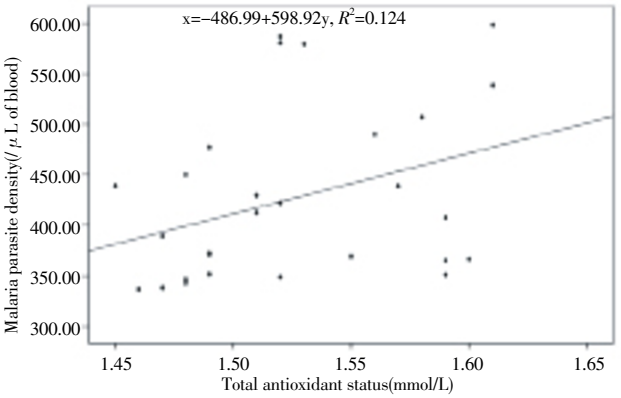

Figure 4. Scatter plot between malaria parasite density and total antioxidant status in HIV seronegative control participants with malaria.

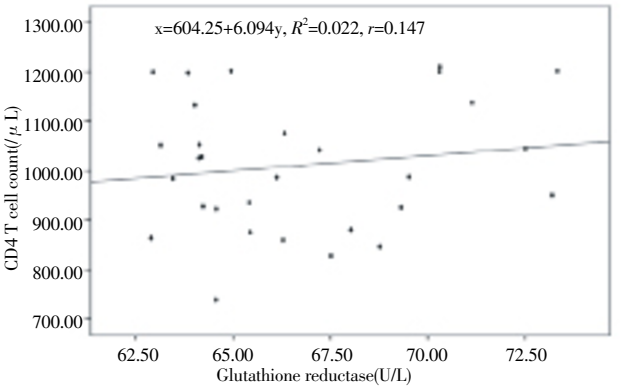

Figure 5. Scatter plot between CD4 T cell count and glutathione reductase in HIV seronegative control participants without malaria.

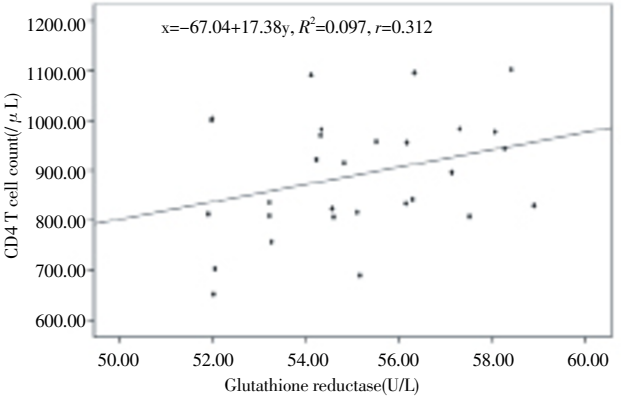

Figure 6. Scatter plot between CD4 T cell count and glutathione reductase in HIV seronegative control participants with malaria.

4. Discussion

This study examined the antioxidant activity of asymptomatic and symptomatic HIV infected participants with or without malaria co-infection. The present study showed that the concentrations of glutathione peroxidase, glutathione reductase, and serum levels of total antioxidant status were significantly lowered in asymptomatic and symptomatic HIV seropositive participants with or without

malaria co-infection compared with control participants. This reduction increases as the HIV disease progresses as observed from the study; symptomatic HIV infected participants having lower level of these antioxidants compared with asymptomatic HIV infected participants. The lowered antioxidant level may compromise the ability of HIV infected individuals to scavenge free radicals and oxygen reactive species, which might predispose them to disease progression. Progression of disease have been associated with conditions that predisposes to high production of free radical and oxygen reactive species and those that predisposes to depletion of antioxidants. In people living with HIV and AIDS, decreased levels of antioxidants and increased oxidative stress have been documented [9-11]. The compromised status of glutathione reductase, glutathione persoxidase and TAS could be attributed to greater utilization of antioxidant micronutrients subsequent to increased oxidative stress, and/or inadequate availability of carrier molecules that may influence circulation of antioxidant concentrations[12].

Our results clearly show that severe antioxidant depletion occurs in HIV seropositive individuals in comparison with controls and with the progression of disease. This is confirmed with the finding of a strong correlation between $CD4^+$ T cell count and the antioxidants. The lower the $CD4^+$ T cells count the lower the antioxidants. This assertion is considered valid considering the role of antioxidants in normal immune function. Exposure to oxidants challenges cellular systems and their responses may create conditions that are favourable for the replication of viruses in HIV and decrease in $CD4^+$ T cells. As oxidative stress increases so does viral replication which increases the destruction of $CD4^+$ T cells and progression of disease [7]. Since the immune system is constantly stimulated in HIV infection and antioxidant level is lower in HIV seropositive individuals than in control participants as observed from the study, adequate intake of antioxidants and phytochemcals is critical in minimizing oxidative stress. The significantly lower concentration of antioxidants among symptomatic HIV infected participants than asymptomatic HIV infected participants suggests that these antioxidants may be utilized in the face of increased viral load.

The low activity of glutathione reductase and glutathione peroxidase may be influenced by the intake of nutrients required for enzyme activity; one example is selenium which is strongly required for glutathione peroxidase activity. Selenium a crucial component of glutathione peroxidase is important in the progression of HIV disease because it is involved in gene regulation and viral expression. The HIV virus replicates more slowly with adequate selenium and selenium rich glutathione peroxidase than when selenium and glutathione peroxidase is deficient. Viral load affect the progression of HIV disease. HIV infected individuals experience chronic immune stimulation from fighting the virus with a corresponding increase in free radical production and a decrease in antioxidant nutrients and antioxidant enzymes. This results in oxidative stress which causes an increase in the production of HIV virus and promotes cell death particularly of $CD4^+$ T lymphocytes[13, 14].

Further analysis of our finding show that glutathione reductase and glutathione peroxidase were significantly lower in asymptomatic and symptomatic HIV infected participants with malaria compared with control participants with or without malaria. This means that malaria exerted a great burden on the antioxidant activity. Even between the control participants, those with malaria presented with lowered antioxidant activity. Hence malaria infection seem to present with a higher demand on antixidant. Glutathione reductase and glutathione peroxidase deficient erythrocytes serve as host cells for malaria parasites[15] and they are among the important antioxidant enzyme present in the erythrocytes. Evidence has been shown that HIV seropositive individuals and those infected with malaria suffer from oxidant/antioxidant imbalance[3,12]. There was a significant negative correlation between malaria parasite density compared with glutathione reductase and glutathione peroxidase. This pattern of antioxidant status is a reflection of the malaria pathogenesis, which involves the invasion of human erythrocytes by the malaria parasite. This brings about metabolic changes in the host cell. The host cells may then become vulnerable to damage due to toxic metabolites derived from both host and parasites. Reactive oxygen species generated in the host-parasite interaction cause lysis of erythrocytes and alteration of the antioxidant enzymes. This has been documented by[16,17].

In HIV and malaria co-infection there may likely be greater generation of reactive oxygen species and resultant depletion of antioxidants as they scavenge the surge in the latter. Since the lowered activity of the antioxidant enzymes and total antioxidant status are much pronounced in symptomatic HIV than asymptomatic HIV it is a possible indication that reduction of antioxidants in HIV seropositive individuals increases as the disease progresses. This shows that as HIV infection progresses there may be greater demand for antioxidants. This finding is further skewed in cases of malaria co-infection in HIV participants. This calls for greater concern in malaria endemic area where incidences of HIV- malaria co-infection has been reported[1,2].

Malaria infection may accelerate the degree of reduction of glutathione reductase, and glutathione peroxidase in HIV positive individuals as the present study have shown. Thus

malaria infection depletes the erythrocytes of its defence against oxidative stress due to the reduced activities of glutathione reductase and glutathione peroxidase.

The study showed significant increase in malaria parasite density and a decrease in $CD4^+$ T cell count in symptomatic HIV infected participants with malaria compared to asymptomatic HIV infected participants with malaria. This shows that the incidence of clinical malaria may increase with decreasing $CD4^+$ T cell count. HIV weakens the body's immune response to malaria and malaria has been shown to induce HIV-1 replication and increase HIV viral load[18-22]. Various studies in Nigeria have shown that there is a high prevalence of malaria among HIV infected individuals[1,2,19]. This means that the impact of the interaction of malaria and HIV/AIDS will be most apparent in areas with generalized HIV/AIDS epidemic and malaria endemic areas such as Nigeria. Therefore strategies to reduce the burden of malaria morbidity and mortality especially in those infected with HIV must be re-enforced. Antioxidant treatment in combination with antimalaria may be a successful strategy for HIV infected individuals residing in malaria endemic areas.

Conflict of interest statement

We declare that we have no conflict of interest.

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