Original Research Article

ERYTHROCYTIC ANTIOXIDANT ENZYMES, PLASMA MALONDIALDEHYDE AND HAEMOGLOBIN LEVELS IN PLASMODIUM FALCIPARUM INFECTED MALARIA PATIENTS

ABSTRACT

This study investigated the effect of malaria parasitaemia on *Plasmodium falciparum* infected human erythrocytes oxidative stress biomarkers and haemoglobin levels. Seventy (70) human subjects of fifty (50) *P. falciparum* positive and twenty (20) negative control subjects between the ages of 10-60 years were selected for this study. Rapid Diagnostic Test (RDT) and microscopy were used to identify *P. falciparum.* The samples were matched based on age, sex and level of parasitaemia. Samples of blood were collected for the determination of *P. falciparum*, level of parasitaemia, anti-oxidant assay and haemoglobin levels; to assess the activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), malondialdehyde (MDA), total protein (PRO), reduced glutathione (GSH), haemoglobin and Parasite density. Haemoglobin level was determined using a Coulter A-T Pierce haematology analyzer (Beckman Coulter, Inc. Fullerton, CA, USA). This study showed that the mean level of PRO, CAT, MDA and SOD was significantly higher among the *P. falciparum* positive patients to those in the negative control while GPx level was lower, also, the mean level of HGB was significantly lower in the *P. falciparum* positive patients to those in the negative control. MDA, SOD, GSH and PRO level were higher among age group (10-20) in the *P. falciparum* infected patients and lower in the control subjects when compared to other age groups. MDA, SOD, GSH and PRO level were higher in the males than the females in both the malaria positive and negative controls. There was a negative correlation between parasitaemia and haemoglobin levels with ages of patients. This study indicates that high parasitaemic patients are at greater risk of anaemia and oxidative stress compared to low parasitaemic ones.

Keywords: P. falciparum; super oxide dismutase (SOD); malaria parasite density; parasitaemia; glutathione peroxidase (GPx*).*

1.0 INTRODUCTION

The devastating effect of malaria in the tropics, particularly in Sub-Saharan Africa is well known (1). About 200-400 million people get infected with the parasite worldwide annually (2). With all the concerted efforts by governments and donor partners on the prevention and treatment of malaria, the disease has remain persistent in some regions of the world due to the evolution of multi-drug resistant genes in the parasites, lack of vaccines, poor environmental hygiene and inadequate knowledge on prevention of the disease (1).

The causative organism of Malaria is a parasitic protozoan, *Plasmodium,* classified under the phylum Apicomplexa, with a specialized apical complex to invade host cells. Some species of plasmodium infect man, *P. malaria, P.vivax, P. ovale,* and P. *falciparum,* of which *P. falciparum* remains the most deadly (3). In humans, the parasites multiply in the hepatocyte and then infect the erythrocytes leading to anaemia and other clinical symptoms associated with the disease (4). Malaria infections sometimes develop into a series of cellular abnormalities and complications such as anaemia, thrombocytopenia, splenomegaly and hepatitis (5).

The host defense mechanism is usually triggered with the mobilization of phagocytes to the site of infection caused by the parasites (6). The parasite attacks the erythrocytes leading to the breakdown of hemoglobin in a series of complex biochemical reactions (7). This leads to the production of substantial amount of reactive oxygen species (ROS), which overwhelms the host antioxidant capacity, thus, leading to oxidative stress (8).

ROS are responsible for some oxidative stress related diseases including aging (9), cancer (10), atherosclerosis and diabetes (11). ROS are sometimes beneficial as they are utilized by the host defense mechanism to destroy pathogens (12).

In malaria infection, the role of oxidative stress is still unclear as some researchers have suggested an ameliorative role, whereas others believe it plays a significant role in the development of the disease (13). However, studies have postulated that the ROS and RNS generation associated with oxidative stress, plays an important part in the systemic complications development caused by malaria. The generation of hydroxyl radicals ($OH^{\bullet}$) in the liver is induced by malaria infection, which is probably the major reason for the induction of oxidative stress and apoptosis (14).

This study attempts to assess the effect of malaria parasitaemia on *Plasmodium falciparum* infected patients oxidative stress biomarkers levels such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), Total protein (PRO), reduced glutathione (GSH), malondialdehyde (MDA) and haemoglobin levels.

2.0 MATERIALS AND METHODS

2.1 Study Laboratory and Period

This work was carried out in the Biochemistry Laboratory of the Nigerian Institute of Medical Research (NIMR), Yaba, Lagos, and the study site was in Ajegunle with Geo-coordinates, Latitude: 6°36'22" and Longitude: 3° 16' 57". The study site is an urban slum area with a significantly high episode of malaria infection.

2.2 Subject's Selection

Seventy (70) human subjects of fifty (50) P. *falciparum* malarial and twenty (20) non-infected (control) subjects between 10-60 years were chosen for the study. Simple random sampling of individuals who presented for malaria parasite test at Ajeromi General Hospital Laboratory, with headache, fever (temperature > 37ºC), and malaise within of 2-7 days and who were confirmed to be *Plasmodium falciparum* malaria positive by RDT Kits and later microscopic examination were chosen for this study. All the patients diagnosed with febrile condition suggesting the presence of malaria parasites, referred to the laboratory for investigation, were recruited. All patients who were not diagnosed with febrile conditions, suggestive of malaria parasites and were not referred to the laboratory were excluded. Similarly, patients on antimalarial drugs medication prior to presentation were also excluded from the study. Twenty (20) subjects who are malaria parasite negative by RDT and microscopy were used as controls. Before blood samples collection, the patients prior consent was sought at the Ajeromi General Hospital, Ajegunle, Lagos, and all the malaria positive patients were subsequently treated after blood samples were collected from them. The malaria positive and negative (control) patients were sex and age-matched.

2.3 Blood Samples Collection

Blood samples collection was done through the nurses at Ajeromi General Hospital. A 5ml blood sample was taken with 10ml syringe from subjects by venipunture into an EDTA vacutainer tubes to prevent blood clot. Only the samples intended for biochemical assay were centrifuged at

3000g for 10minutes at about 29-30ºC to obtain the plasma. The plasma was collected and placed in a separate labeled container and freeze stored until required for analysis.

2.4 Parasitological Examination

Randomization was done by selecting patients with malaria after microscopic examination of thin and thick blood smears by the method of Cheesbrough (15) as reported by Ozojiofor *et al*., (37). The number of parasites counted per 200 white blood cells was used to calculate parasite density on the basis of 8000 leukocytes per µl of blood for those slides that were positive. Level of parasitaemia was evaluated with the formula:

$$\frac{\text{Parasite count} \times 8000}{Count\ WBC\ 200}$$

Smears that were positive were grouped into:

Parasite density <1000 asexual forms per ml of blood- Low parasitaemia.

Parasite density of > 10,000 asexual forms per ml of blood- High parasitaemia.

2.4.1 Estimating and Grading of Parasitaemia

Malaria parasitaemia grading was done according to the WHO method (3). The amount of relative parasite count in positive smears was done using a simple code from one to four crosses (+ - ++++) (16).

2.5 Diagnosis of Malaria Parasite Using a Rapid Diagnostic Test Kit

A Rapid Diagnostic Kit (Acon) which works by chromatographic immunoassay in detecting *Plasmodium falciparum* in whole blood was used to screen for *P. falciparum* before microscopic examination was done. The procedure was as described in the manual by the manufacturer of the kit (Acon Laboratories, Inc.).

2.6 Antioxidant assay

Antioxidant assay was done for the estimation of glutathione *S*-transferase (GST), catalase (CAT), superoxide dismutase (SOD), malondialdehyde (MDA) and glutathione (GSH). Plasma protein was determined using Bovine Serum Albumin (BSA) as standard by the method of Gornall (17). Plasma antioxidant enzymes were assessed by estimating the levels of reduced glutathione (GSH) (18,19), glutathione *S*-transferase (GST) (20), catalase (21), malondiadehyde (MDA) (22) and superoxide dismutase (SOD) (23).

2.7 Measurement of Hemoglobin Levels.

Hemoglobin level was determined using an Coulter A-T Pierce hematology analyzer (Beckman Coulter, Inc. Fullerton, CA, USA).

2.8 Statistical Analysis

The results obtained were expressed as Mean ± Standard Deviation, Student t-test was used to compare means and Pearson Correlation was carried out. Statistical package for social sciences (version 16.0 SPSS) was used for analysis of results and a p-value of <0.05 was considered statistically significant at 95% confidence interval.

3.0 RESULTS

Table 1 shows a significant decrease ($p<0.05$) in reduced glutathione (GSH), glutathione peroxidase (GPx) and haemoglobin (HB) levels and a significant increase in catalase (CAT), superoxide dismutase (SOD), malondialdehyde (MDA) and protein in the *P. falciparum* positive subjects when compared to control subjects.

Table 2 shows the antioxidant markers levels in *P. falciparum* positive and negative control subjects based on sex. There was a significant increase in the mean level of PRO, MDA, CAT, and SOD among the *P.falciparum* infected females compared to control and a decrease in GPx level, also, there was a significant increase in the mean level of MDA and SOD among the *P. falciparum* infected males compared to controls and a decrease in GSH and GPx level. MDA, CAT, PRO and SOD were significantly increased among the males compared to the females in both the *P. falciparum* positive and negative subjects while CAT and GPx decreased.

Table 3 shows the age-based classification of the antioxidant enzymes levels in *P. falciparum* positive and negative control subjects. MDA and PRO level were significantly higher ($p<0.05$) among (10-20) age group in the *P. falciparum* infected patients and lower in the control subjects compared to other age groups.CAT level was significantly higher ($p<0.05$) among (31-40) age group in the *P. falciparum* infected patients and lower in the control subjects compared to other age groups.SOD level was significantly higher ($p<0.05$) among (10-20) age group in the *P. falciparum* patients and lower in the control subjects compared to other age groups.GSH level was marginally higher among (10-20) age group in the *P. falciparum* patients and lower among (21-30) age group in the control subjects compared to other age groups. GPx level was marginally higher among (41-50) age group in both *P. falciparum positive and negative control subjects* compared to other age groups.

Table 4 shows the mean antioxidant markers levels at different parasitaemic levels in *P. falciparum* positive and negative control subjects. Total protein level was lower in the moderate and high parasitaemic group when compared with low parasitaemic group but was not significantly lower and was marginally reduced in the high parasitaemic group compared to the moderate group. CAT level was found to be higher in the high parasitaemia group compared to both the moderate and low group, and they were not significantly lower in the moderate than in the low group. MDA and SOD levels were significantly lower ($p<0.05$) in the high parasitaemic group when compared to the moderate and lower group and were significantly lower in the moderate than in the low group.GSH level was higher in the high group when compared to the low and moderate group, and were higher in the moderate than in the low group but was not significant. GPx level was found to be higher in the moderate than in the low and high parasitaemic group and they were lower in the high compared to the low parasitaemia group.

Table 5 shows the correlation analysis of Haemoglobin and Parasitaemia level against ages among participants. Correlation analysis of Haemoglobin and Parasitaemia level was done against age. HGB and parasitaemia levels were both negatively correlated with age, and were not statistically significant.

Table 1: Level of antioxidant enzymes and haemoglobin in *Plasmodium falciparum* infected patients and control.

ANTI-OXIDANTS parameters	Sample	Control
PRO(g/L)	65.82±6.03	64.1±7.13
CAT(min/mg protein)	0.57±0.36	0.33±0.17
MDA(mg/ml)	33.06±21.65	22.69±6.79
SOD(min/mg protein)	22.43±10.56	10.44±3.44
GSH(µmol/ml)	0.28±0.11	0.39±0.06
GPX(µmol/ml)	1.52±0.22	2.74±0.48
HGB (g/dL)	10.15±3.29	12.83±2.25

Results were presented as Mean ± SD of three determinations.

SOD: superoxide dismutase, MDA: Malondialdehyde, CAT: Catalase, PRO: Total Protein, GSH: Reduced Glutathione, HGB: Haemoglobin, GPX: Glutathione Peroxidase,

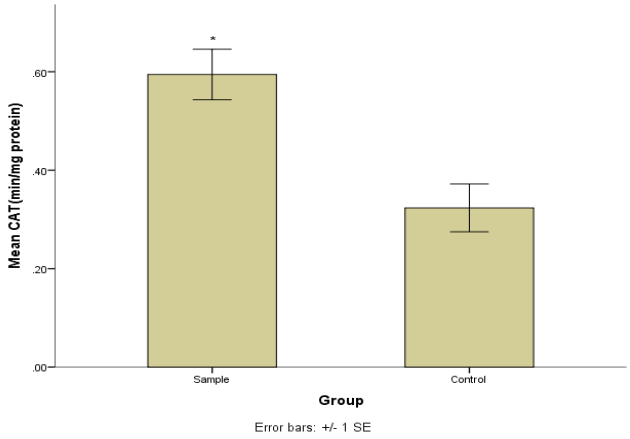

FIGURE 1: Mean level of Catalase (CAT) activity in *P. falciparum* positive and negative and control.

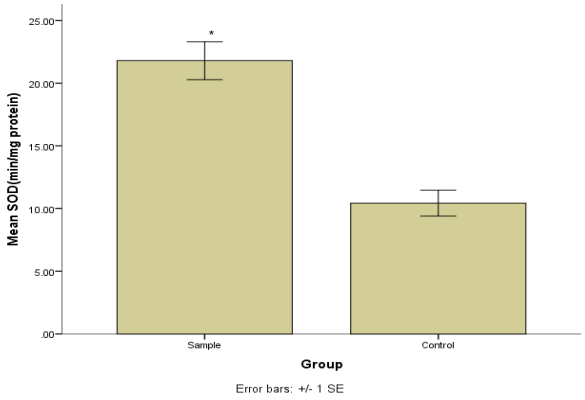

FIGURE 2: Mean level of Superoxide dismutase (SOD) activity in *P. falciparum* positive and negative and control.

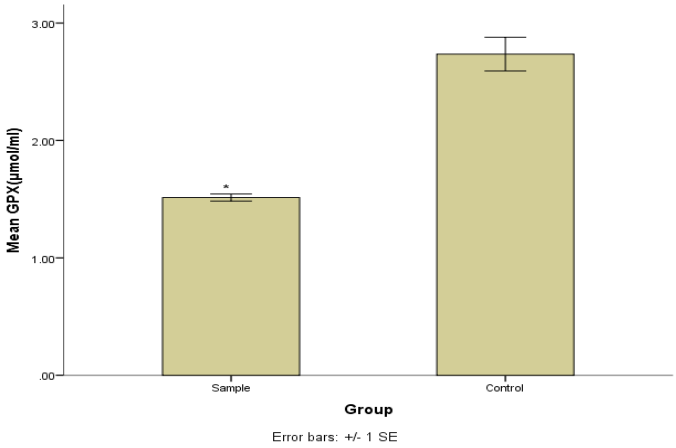

FIGURE 3: Mean level of Glutathione peroxidase (GPx) activity in *P. falciparum* positive and negative and control.

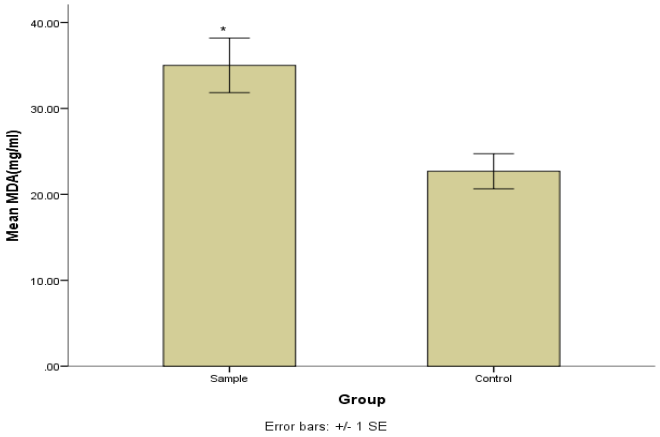

FIGURE 4: Mean level of Malondialdehyde (MDA) activity in *P. falciparum* positive and negative and control.

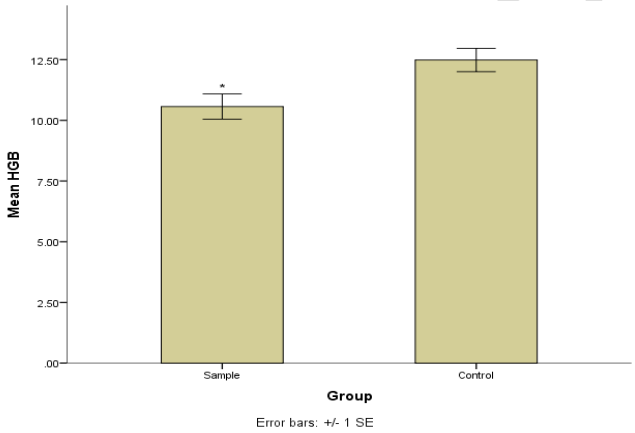

FIGURE 5: Mean levels of Haemoglobin (HGB) in *P. falciparum* positive and negative control.

Table 2: Sex-matched levels of antioxidant markers in *P. falciparum* positive and negative control subjects.

Antioxidant Parameters	Sex	Sample	Control
PRO(g/L)	MALE	65.89 ± 6.17	64.35 ± 5.77
	FEMALE	65.76 ± 6.11[c]	63.66 ± 10.12[d]
CAT(min/mg	MALE	0.53 ± 0.39	0.36 ± 0.2
protein)	FEMALE	0.61 ± 0.33[c]	0.28 ± 0.07[d]
MDA(mg/ml)	MALE	34.48 ± 22.75	25.08 ± 7.13
	FEMALE	31.81 ± 21.31[c]	18.51 ± 3.91[d]
SOD(min/mg	MALE	24.95 ± 11.82	11.62 ± 3.42

protein)	FEMALE	20.23 ± 9.15^{c}	8.38 ± 2.66^{d}
GSH(µmol/ml)	MALE	0.32 ± 0.14	0.39 ± 0.08
	FEMALE	0.25 ± 0.06^{c}	0.39 ± 0.02^{d}
GPX(µmol/ml)	MALE	1.51 ± 0.22	2.6 ± 0.55
	FEMALE	1.52 ± 0.22^{c}	2.98 ± 0.22^{d}

Results were presented as Mean ± SD of three determinations.

(a, b) Values with superscript in the same row for a particular gender are significantly different,

(c, d) Values with superscript in the same column for a particular group is significantly different

Table 3: Age-matched levels of antioxidant enzymes in *P. falciparum* positive and negative control subjects.

Parameters	Age groups	Samples	Controls
PRO(g/L)	10-20	75.73 ± 2.98	53.11 ± 0
	21-30	63.23 ± 4.87	62.28 ± 2.98
	31-40	65.73 ± 6.33	68.46 ± 11.15
	41-50	68.13 ± 3.98	66.15 ± 0
	51-above	62.92 ± 2.77	64.87 ± 7.12
	Total	65.27 ± 5.57	64.1 ± 7.13
CAT(min/mg protein)	10-20	0.42 ± 0.11	0.32 ± 0
	21-30	0.55 ± 0.35	0.45 ± 0.32
	31-40	0.70 ± 0.41	0.45 ± 0.17
	41-50	0.51 ± 0.29	0.31 ± 0
	51-above	0.65 ± 0.34	0.23 ± 0.09
	Total	0.60 ± 0.35	0.33 ± 0.17
MDA(mg/ml)	10-20	61.46 ± 23.71	13.74 ± 0
	21-30	30.8 ± 22.65	24.3 ± 6.76
	31-40	36.83 ± 18.78	29.62 ± 13.14
	41-50	37.54 ± 22.59	25.58 ± 0
	51-above	30.4 ± 21.53	20.49 ± 2.64
	Total	35.02 ± 21.34	22.69 ± 6.79
SOD(min/mg	10-20	25.51 ± 12.51	6.81 ± 0

protein)	21-30	20.47 ± 9.80	12.6 ± 2.39
	31-40	24.88 ± 12.12	9.96 ± 5.86
	41-50	20.56 ± 9.42	5.96 ± 0
	51-above	18.91 ± 7.77	11.39 ± 2.76
	Total	21.8 ± 10.15	10.44 ± 3.44
GSH(µmol/ml)	10-20	0.31 ± 0.08	0.39 ± 0
	21-30	0.27 ± 0.1	0.32 ± 0.15
	31-40	0.27 ± 0.11	0.40 ± 0.01
	41-50	0.27 ± 0.06	0.42 ± 0
	51-above	0.30± 0.15	0.41 ± 0.03
	Total	0.28 ± 0.11	0.39 ± 0.06
GPX(µmol/ml)	10-20	1.33 ± 0.05	2.76 ± 0
	21-30	1.46 ± 0.18	2.12 ± 0.97
	31-40	1.45 ± 0.17	2.83 ± 0.09
	41-50	1.71 ± 0.18	3.05 ± 0
	51-above	1.62 ± 0.24	2.89 ± 0.29
	Total	1.52 ± 0.21	2.74 ± 0.48

Results were presented as Mean ± SD of three determinations.

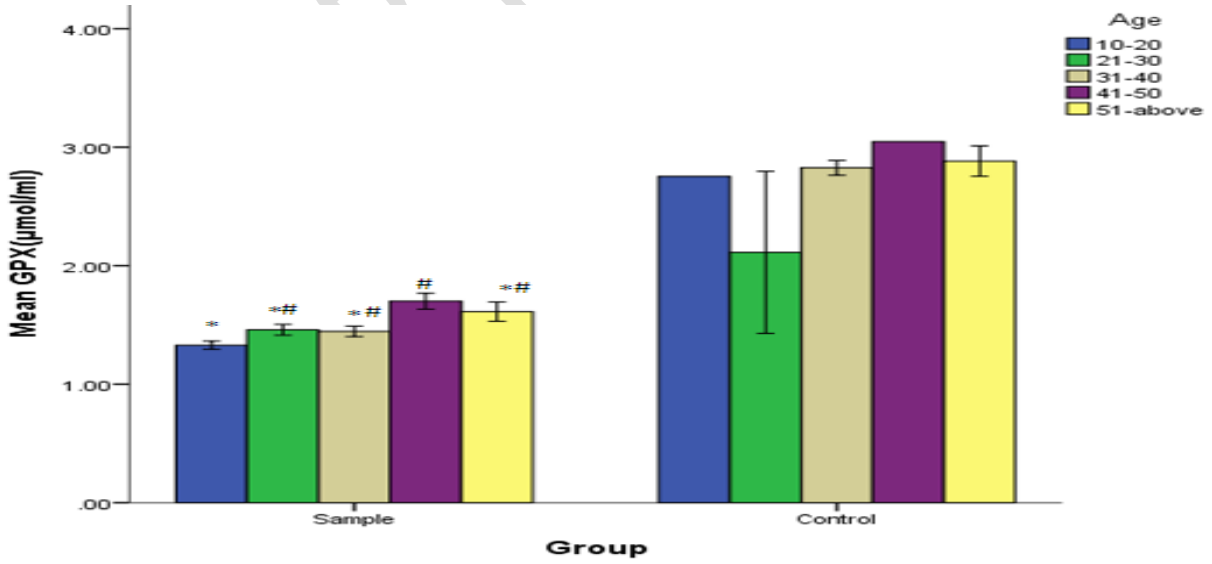

FIGURE 6: Mean level of Glutathione peroxidase (GPX) activity *P. falciparum* positive and negative control subjects based on age.

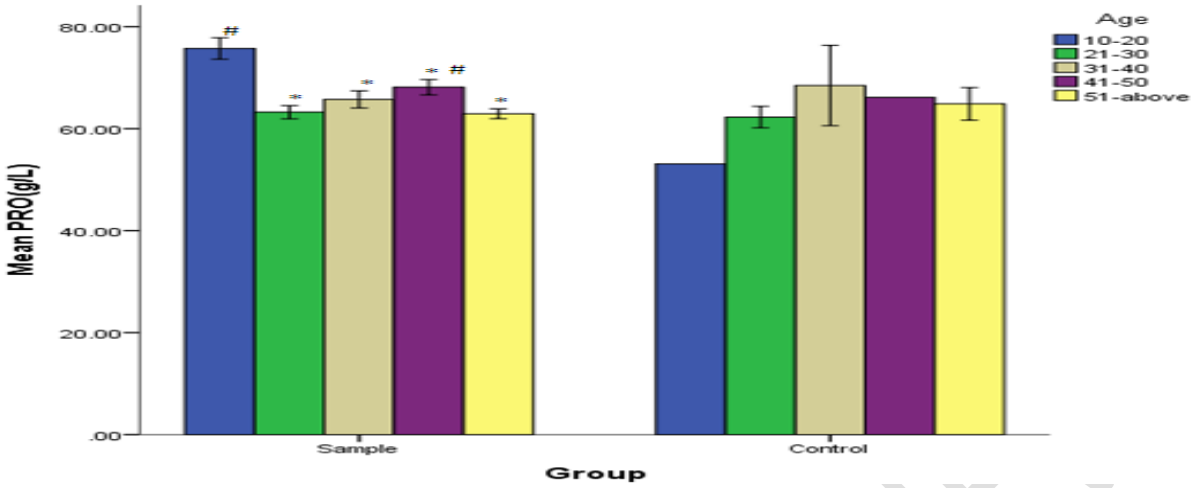

FIGURE 7: Mean level of protein in *P. falciparum* positive and negative control subjects based on age.

TABLE 4: Mean level of antioxidant enzymes activities at different levels of parasitaemia in *P. falciparum* positive and negative control subjects.

Parameters	Parasitaemia level	Samples
PRO(g/L)	Low	64.35 ± 8.4
	Moderate	62.74 ± 10.1
	High	62.53 ± 6.3
	Total	63.22 ± 2.4
CAT(min/mg protein)	Low	0.59 ± 0.8
	Moderate	0.51 ± 0.10
	High	0.61 ± 0.6
	Total	0.56 ± 0.24
MDA(mg/ml)	Low	43.37 ± 8.1
	Moderate	33.32 ± 3.10
	High	22.61 ± 2.6
	Total	33.99 ± 2.4
SOD(min/mg protein)	Low	24.16 ± 8.3
	Moderate	18.74 ± 1.0
	High	15.82 ± 6.1
	Total	19.82 ± 2.4

GSH(µmol/ml)	Low	0.24 ± 0.8
	Moderate	0.28 ± 0.10
	High	0.31 ± 0.6
	Total	0.28 ± 0.24
GPX(µmol/ml)	Low	1.53 ± 0.8
	Moderate	1.69 ± 0.10
	High	1.43 ± 0.6
	Total	1.57 ± 0.24

Results were presented as Mean ± SD of three determinations.

TABLE 5: Correlation analysis of HGB and Parasitaemia against ages of participants.

Parasitaemia		**Haemoglobin**	
Pearson coefficient (r)	p value	Pearson coefficient(r)	p value
-0.07035	0.650	-0.07679	0.433

4.0 DISCUSSION

In the tropic, malaria, a major parasitic disease responsible for high rate of mortality especially among infants is endemic in Africa (24). *P. falciparum* accounts for almost all the cases of complications and deaths that occur in malaria infection. Of all the complications associated with *P. falciparum* infections; oxidative stress, hepatic dysfunctions and anaemia occur in both children and adults in malaria ravaged regions of the globe (25-26).

Malaria triggers the body defence system, leading to the release of reactive oxygen species (ROS) and the breakdown of the erythrocytes (27). Oxidative stress induction by the parasites during its erythrocytic stage as a result of metabolism of hemoglobin, leads to the body building up defense against the oxidative insults by producing enzymatic antioxidant to cope with the build-up of free oxygen radicals e.g. Catalase (CAT), Super Oxide Dismutase (SOD),

Glutathione peroxidase (GPx), and reduced Glutathione (GSH). Under cellular conditions, ROS are removed from parasitized cells by detoxifying enzymes like SOD, GSH and CAT (28). These highly reactive oxygen radicals can cause some biochemical changes in cells including membrane lipid peroxidation, enzymes inactivation, alteration of intracellular redox state and damage to red blood cells and DNA (28).

This study revealed an increased level of MDA in the *Plasmodium falciparum* patients to the negative control subjects. The significantly higher MDA level in *P. falciparum* patients than control subjects as reported by our work may be suggestive of the damage elicited by free oxygen radicals against hepatocytes and erythrocytes cell membrane (29). Increased levels of MDA may suggest an increase in peroxidation of membrane lipids in *P. falciparum* patients; this agrees with a previous study by Araujo et al. (30). Some studies have reported an increase in the lipid peroxidation biomarkers like MDA as being responsible for some diseases like malaria (31). Plasmodium sp. is deficient in a triglyceride biosynthesis pathway, and depends on its host to obtain all its lipids (32). The parasite disorganizes the red blood cells membrane, to obtain lipids (29) which accounts for the increased MDA levels.

The increased plasma MDA level points to lipid peroxidation resulting from the formation of super oxide radicals by the action of malaria parasites (33). MDA, SOD, PRO and GSH were higher in the males than in the females and also among age group (10-20) of *P. falciparum* patients. This could mean that, such groups are more exposed to the parasite and hence, to lipid peroxidation due to free radical build-up in malaria.

This research also showed an elevated level of CAT, SOD and PRO in the *P. falciparum* positive patients when compared to the negative control subjects. Specifically, SOD is a key intracellular antioxidant enzyme in aerobic cells with neutralizing effect against superoxide radicals while catalase (CAT) protects the cells from the build-up of hydrogen peroxide by breaking it down to water and oxygen. The increase in SOD and CAT observed in this research could be due to an early stage oxidative insult by ROS in *P. falciparum* patients. Pujar *et al* (34) reported decreased CAT and SOD levels in *P. falciparum* positive patients. PRO, MDA and SOD were lower in age group (51-above) while GPx and CAT was lower in age group (10-20).The decrease PRO and SOD activity with age is in line with studies that shows that reactive oxygen species brings about ageing. The decreased CAT and GPx levels in age group (10-20) is still not well understood and there is a need for further works to ascertain this.

Plasma level of CAT, GPx, PRO and SOD were lowered significantly in the moderate and high parasitaemia compared to the low parasitaemia in this work. The significant lower level of plasma antioxidant enzymes in moderate and high parasitaemia patients might be the contributing factor to higher oxidative stress damage to erythrocyte membranes (35). This leads to cellular deformability, signaling the removal and degradation of the red blood cells by macrophages. This leads to anemia and hypoxia observed in malaria (35).

Catalase was lower in high parasitaemic patients than in low parasitaemic patients. This could be a survival strategy by the high parasitaemic erythrocytes resulting in reduction of plasma catalase. This is in tandem with the findings that catalase is required by *Plasmodium* parasites for growth and protection against free radical assaults (34-35). In this study, a significant decrease in the haemoglobin (HGB) level was observed. Akogwu et al (36) reported decreased levels of PCV/HGB (anaemia) and Ozojiofor et al (37) also reported a decrease in the mean level of haematocrit (HCT/PCV), haemoglobin (HGB), red blood cells (RBC), and platelets (PLT) in *P. falciparum* infected patients compared to the healthy subjects. The decrease in HGB could be due to oxidative stress (38) and the breakdown of haemoglobin by the parasite in malaria infected patients and the subsequent removal of parasitized erythrocytes from circulation by the reticuloendothelial system. Malaria infection leads to a reduction in the oxygen supplied to tissues and carbondioxide removed due to decreased red blood cells (39). This study further reiterates the findings that high parasitaemia predisposes humans to oxidative stress as observed in severe malaria. This demands for early diagnosis and treatment of malaria to prevent endogenous damage of erythrocyte until there is a breakthrough in malaria vaccine research.

CONCLUSION

The result of our study suggested that high parasitaemia predisposes malaria parasite patients to higher oxygen free radical production and anaemia. Age groups from 10-20 could be at a greater risk to developing oxidative stress and anaemia, as shown by a significant increase in MDA levels and decreased PRO, CAT, SOD and GPx in high and moderate parasitaemia, which buttresses the higher oxidative stress hypothesis in malaria. The increased antioxidant enzymes activities in some cases may be a compensatory regulation by the cell to respond to increased oxidative stress. Oxidative stress could play a role in the pathogenesis of malaria as shown by the increased level of MDA and reduced antioxidants. Therefore, there is need for prompt diagnosis and treatment to prevent damage to the red blood cells.

CONSENT

The patients prior consent was sought before blood samples collection from them at Ajeromi General Hospital, Ajegunle, Lagos between August 2018 and January, 2019.

ETHICAL APPROVAL

Ethical approval for this study was obtained from the Ethical Review Board of the Nigerian Institute of Medical Research, Lagos. Permission was obtained from the hospital used and all the participants gave written consent. All authors declare that all experiments were performed in accordance with the ethical standards.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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