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## Biochemical Indices of Severity in Human Malaria

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The present study highlights the potential of using lactate dehydrogenase (LDH, EC. 1.1.1.27) and aldehyde oxidase (AO, E.C.1.2.3.1.) activities and level of lipid peroxidation (LPO) in RBC as indices in monitoring the severity of *P. falciparum* malaria infections. Two hundred and forty one male patients from two major hospitals in Benin-City, Nigeria, with a history of malaria and confirmed to be infected with the *P. falciparum* malaria parasites by microscopic examination of Giemsa-stained thin blood slides were chosen for the study. These patients who fell within the 18-40 year age group were further grouped into low, moderate and high parasitemia based on the parasite density. A control group of healthy male adults in the same age range with the patients were also used for the study. Erythrocyte obtained from venous blood collected in heparinized tube after several centrifugations and appropriate washings with specified buffer solutions were assayed. The level of LPO in Infected Red Blood Cells (IRBC) was significantly higher ( $p < 0.05$ ) as compared to control with the level increasing with severity of malaria infection. Conversely, the LDH activity of IRBC was significantly ( $p < 0.05$ ) decreased relative to control and the decrease was also in the order of severity of the infection. However, no significant difference was observed in the AO activity in the different experimental groups. A significant ( $p < 0.0001$ ) negative correlation ( $r = -0.9516$ ) was observed between LDH activity and level of LPO. The results obtained suggest that LDH and LPO can be utilised as markers of malaria severity.

**Key words:** Malaria, lipid peroxidation, lactate dehydrogenase, aldehyde oxidase, red blood cells

## INTRODUCTION

Malaria is endemic in the tropical and subtropical regions and is therefore a disease of hot wet climates (Alumanah *et al.*, 2000) and is caused by *Plasmodium sp.* *P. falciparum* infection is the commonest cause of malaria (Samba, 1997), accounting for 300-500 million clinical cases annually (Amador and Patarroyo, 1996) with 90% of such cases occurring in Africa. The life cycle of this parasite in the human host includes the developmental cycle in Red Blood Cells (RBC) and the cycle taking place in the liver cell parenchyma which involves a series of transformations (Miller *et al.*, 2002). Some biochemical events that have been reported to occur in malaria include cellular changes in energy metabolism, heme metabolism, membrane lipid peroxidation (LPO) and stress enzymes.

Studies have reported an imbalance in the generation and removal of radical species in malaria and free radicals can cause membrane lipid peroxidation (Das and Nanda, 1999; Loria *et al.*, 1999). Activation of the body immune system causes release of reactive oxygen species as an antimicrobial action (Allison and Eugui, 1983). In addition to host's immune system, malaria parasite also stimulates RBC to produce reactive oxygen species, which enhance haemoglobin degradation the products needed for parasite survival. So ROS is necessary for the pathogenesis and defense of the malaria parasite (Rath *et al.*, 1991; Erel *et al.*, 1997; Das and Nanda, 1999).

The survival and the resistance of the red blood cell to infection depend largely on energy generation, which is via glycolysis. Lactate dehydrogenase (LDH, EC. 1.1.1.27) is important for constant energy generation by RBC and is also present in nearly all types of metabolizing cells but different cells have different forms of this enzyme, which exists in five forms (Plummer and Wilkinson, 1963). The enzyme is especially concentrated in the heart, liver, erythrocyte, kidneys, muscles, brain and lungs (Markert and Ursprung, 1962; Sevinc *et al.*, 2005). Consequently, diseases affecting these organs, such as renal infarction, myocardial infarction and haemolysis, have been reported to be associated with significant elevations in total serum LDH activity. Such elevations have been widely applied as diagnostic indices for kidney, liver, heart and red blood cell dysfunction (Plummer and Wilkinson, 1963; Castaldo *et al.*, 1994). In some disease conditions like Hodgkin's and non-hodgkin's lymphoma, and ovarian dysgerminoma (Pressley *et al.*, 1992), LDH has been found to be relevant in monitoring the progress and severity of the diseases. Recent reports also indicate that serum LDH activity is a potentially valuable enzymatic marker of acute, uncomplicated *P. falciparum*

malaria infection, especially in the absence of other complicating diseases known to be associated with LDH activities.

Aldehyde oxidase (AO, E.C.1.2.3.1.) is an enzyme which belongs to the family of molybdoflavoproteins along with xanthine oxidase, sulphite oxidase and nitrate reductase (Johns, 1967; Beedham, 1985). The enzyme oxidizes aldehydes to the corresponding acids using molecular oxygen (Johns, 1967; Al-Salmy, 2001; Yasuhara *et al.*, 2002). Experimental evidence shows that free radicals are generated from NADH and acetaldehyde oxidation by aldehyde oxidase during ethanol oxidation (Mira *et al.*, 1995). The involvement of AO in the generation of free radicals could affect the balance between free radical generation and their mop up. As free radicals are also implicated in both protection and pathogenesis of malaria, a study of the role of AO in the generation of free radicals and its link to the severity of malaria is desirable.

Some biochemical parameters in serum or plasma have been investigated as possible markers of malaria severity (Davis *et al.*, 1994); however there is scarcity of data in literature on the use of AO, LDH and levels of LPO in RBC as possible markers of malaria severity. The present study was therefore carried out in order to investigate the effect of *P. falciparum* infection in humans on these parameters with the objective of assessing the possible use of these biochemical parameters as markers of severity of malaria infection.

## MATERIALS AND METHODS

Two hundred and forty one male patients who visited the Outpatients Departments of the University of Benin Teaching Hospital and the Central Hospital, both in Benin-City, Nigeria, between May and June 2009, were chosen for the study. Patient selection and pre-qualification were done by simple random sampling of male individuals with a history of fever and malaise within a period of one to eight days and who were confirmed to be infected with the *P. falciparum* malaria parasites by microscopic examination of Giemsa-stained thin blood slides. Patients whose case history showed a concomitant presentation with the following conditions: Acquired Immune Deficiency Syndrome (AIDS), anaemia, liver cirrhosis, hepatitis, alcoholism and kidney disorders were excluded from the study. Similarly patients on self-medication with any anti-malarial drug prior to presentation were also excluded from the study. These patients who fell within the 18-40 year age group were further grouped into low, moderate and high parasitemia

based on the parasite density and with 67, 74 and 100 patients respectively in each group. Patients were classified as low parasitemia when the parasite density was below 1,000 cells dL<sup>-1</sup> blood. Moderate parasitemia had a density of between 1,000-10,000 cells dL<sup>-1</sup> of blood, while patients' parasitemia with over 10,000 cells dL<sup>-1</sup> blood were regarded as high parasitemia. A control group of 67 healthy male adults in the same age range with the patients were also used for the study.

Venous blood (5 mL) was obtained by venepuncture of the antecubital vein using a sterile needle and syringe. The blood samples were then transferred into heparinized tubes. The plasma was separated by centrifugation at 1000 g for 15 min. The erythrocytes remaining after the removal of the plasma were washed three times with 310 mM isotonic Tris HCl buffer (pH 7.4). Hemolysis was performed by pipetting out the washed erythrocyte suspension into polypropylene centrifuge tubes, which contained 20 mM hypotonic Tris HCl buffer (pH 7.2). The erythrocyte membranes were sedimented in a high-speed centrifuge at 20,000 g for 40 min. The supernatant was decanted and subsequently used for the biochemical assays.

LDH activity was assayed using kit from Quimica Clinica Applicada (Spain). The assay was carried out by incubating the sample with nicotinamide adenine dinucleotide (3 mg mL<sup>-1</sup>) and DL-lactic acid (0.45 M) with sodium pyrophosphate buffer, pH 8.8. This assay condition eliminates the contribution of parasite LDH, which has a different pH and substrate optima for activity. The activity of LDH is expressed in Units/L. AO activity was determined by monitoring the decrease in absorbance consequent upon the oxidation of benzaldehyde to benzoate (Johns, 1967). The activity of the enzyme is expressed in units per mL. One unit of the enzyme is defined as the amount required to form one micromole of benzoate under assay conditions. Level LPO in the supernatant was assayed by the procedure of Gutteridge and Willins (1982). The procedure involved the determination of concentration of malondialdehyde (MDA) which are indicators of LPO. The level of LPO is expressed in nanomoles MDA mL<sup>-1</sup>.

The ethical principles in the conduct of research with human participants of the American Psychological Association (APA, 1982) were taken into account during the course of the present study.

The results are expressed in Means±SEM. Analysis of variance was used to test for differences in the groups. Duncan's multiple range test was used to test for significant differences between the means. Statistical correlation analysis was carried out using Pearson's product moment correlation coefficient.

## RESULTS

Present results reveal that LDH activity decreased significantly, while level of LPO increased significantly in the various groups of malarial patients compared with the control (Table 1). The result in Table 1 also showed that increase in parasitemia resulted in a corresponding increase in LPO and decrease in LDH. Statistical analysis of the data failed to reveal any significant difference in the activity of AO in the test groups compared with the control. There was also no significant change in AO with severity of malaria. The result shows that red blood cell lipid peroxidation and lactate dehydrogenase activity are related to the severity of parasitemia.

We did not establish a significant (p>0.05) correlation in the level of LPO and the activity of LDH in control subjects with uninfected RBCs (Fig. 1).

Unlike in control subject, a perfect negative correlation (r = -0.9516) was observed between the level of LPO and activity of LDH in infected RBCs from malaria patients (Fig. 2). Thus it is proposed that LDH activity is responsive to the level of LPO in infected RBC (IRBC).

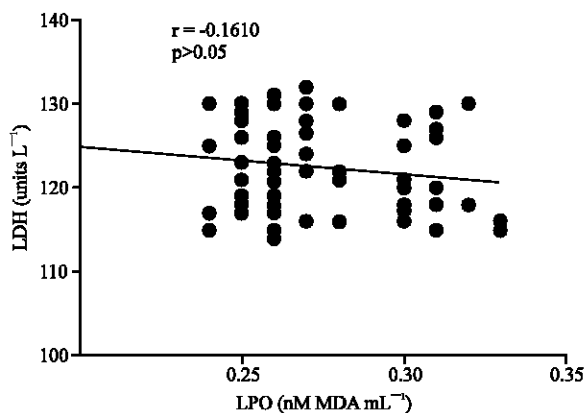


Fig. 1: Relationship between LDH activity and LPO in erythrocytes of normal male adults

Table 1: Effect of *P. falciparum* infection on AO and LDH activities and level of LPO in human erythrocytes

Results	Level of parasitemia			
	Control	Low	Moderate	High
LDH activity	122.290±0.65 <sup>a</sup>	73.880±0.92 <sup>b</sup>	53.700±0.38 <sup>c</sup>	31.320±0.87 <sup>d</sup>
AO activity	0.047±0.002 <sup>a</sup>	0.049±0.003 <sup>a</sup>	0.051±0.004 <sup>a</sup>	0.052±0.004 <sup>a</sup>
LPO	0.280±0.003 <sup>a</sup>	2.010±0.086 <sup>b</sup>	4.250±0.061 <sup>c</sup>	6.330±0.093 <sup>d</sup>

Means of the same row followed by different letters differ significantly (p<0.05). Values for LDH and AO activities are in units L<sup>-1</sup> and units mL<sup>-1</sup>, respectively. Values for level of LPO are in nmoles mL<sup>-1</sup>

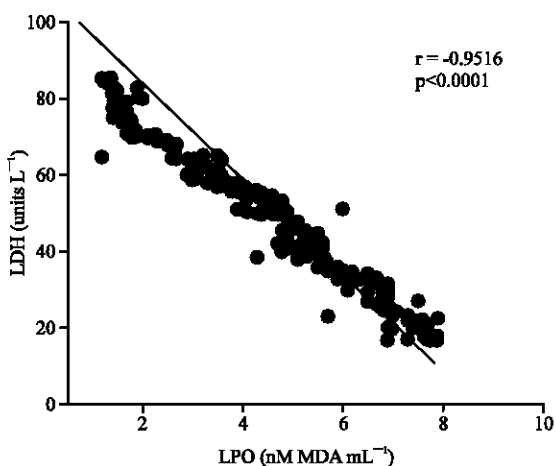


Fig. 2: Relationship between LDH activity and LPO in erythrocytes of malarial Patients

### DISCUSSION

The present study highlights the potential of using LDH and AO activities and level of LPO in RBC as indices in monitoring the severity of *P. falciparum* malaria infections. The observed increase in MDA concentration in the RBCs of malaria patients (Table 1) reflects the extent of LPO. Earlier studies have also shown increases in LPO in malaria (Hunt and Stocker, 1990; Rath *et al.*, 1991; Mishra *et al.*, 1994; Isamah and Asagba, 2003; Becker *et al.*, 2004). The production of ROS may be the combined effects of macrophage action in an attempt to fend off the parasite and the parasite's defense action. Earlier studies have shown that macrophage generated reactive oxygen species are known as nonspecific effector molecules in host's defense armoury, which not only damage the parasitized red blood cells but also non-parasitized red blood cells (Erel *et al.*, 1997; Das and Nanda, 1999; Loria *et al.*, 1999; Mohan *et al.*, 1994; Rath *et al.*, 1991). The accumulation of organic peroxides and oxidation of membrane lipids place a stress on cellular vitality ultimately leading to destructive effects on the cell. Moreover it has been reported that Plasmodium cells succeed in accumulating protective enzymes (catalase, glutathione peroxidase and superoxide dismutase) but depleting them in the red blood cells of the host (Stocker *et al.*, 1985; Erel *et al.*, 1997). Thus the increased level of lipid peroxidation observed in the RBCs of malaria patients in this study may be due to the production of reactive oxygen species and/or depletion of antioxidant enzymes. The significant ( $p < 0.05$ ) increase in level of LPO with the degree of severity of malaria (Table 1) implies that LPO could be used as a biomarker of malaria severity.

The significant ( $p < 0.05$ ) decrease in LDH activity that was observed in the RBCs of malaria patients relative to controls (Table 1) might be due to leakage of the enzyme and is not unconnected with the significantly increased level of LPO in the RBC. The life cycle of *P. falciparum* includes the developmental cycle in the RBCs. Plasmodium merozoites invade the RBCs in the erythrocytic phase producing more merozoites; thereafter the erythrocyte undergoes rapid and marked deformation by free radicals. A decrease in LDH of RBCs in *P. falciparum* has also been reported by Basco *et al.* (1995). A low LDH activity in RBCs also infers a drop in energy generation since RBCs derive their energy entirely via glycolysis releasing lactate into the plasma for liver to generate glucose for its constant fuel supply. Lactate dehydrogenase is thus elaborated in mature RBC and its activity would be directly related to the level of energy production. The low energy availability to RBCs will increase the propensity for their destruction not only by free radical, but such cells become easily turgid and lyse. It was not surprising therefore that the significant ( $p < 0.05$ ) increase in LPO was associated with a significant ( $p < 0.05$ ) decrease in LDH and it correlated positively with severity of malaria (Table 1). This study also shows significant ( $p < 0.0001$ ) negative correlation ( $r = -0.9516$ ) between the level of LPO and activity of LDH (Fig. 2) in IRBCs of malarial patients. Therefore as the level of LPO increases with the degree of severity of malaria, the activity of LDH decreases unlike in normal patients where there is no significant ( $p > 0.05$ ) correlation ( $r = -0.1610$ ) between the level of LPO and the activity of LDH (Fig. 1). Thus the results imply that like LPO, LDH can also be used as an index of malaria severity.

Some earlier studies have also attempted to relate the degree of the severity of malaria to certain enzymes. Kulkarni *et al.* (2003) related increases in erythrocyte adenosine deaminase and oxidants with *P. falciparum* malaria severity. Piper *et al.* (1999) showed that the level of Plasmodium LDH can be used to test for the severity of malaria. They reported an increase in parasite LDH activity with increased parasitemia. Our study attempted to relate the severity of the disease with erythrocyte AO and LDH activities. Our finding does not agree with the findings of Piper *et al.* (1999) although we measured erythrocyte LDH while Piper *et al.* (1999) measured the enzyme in the parasite. Other studies have also reported increases in LDH activity in the serum (Garba and Ubom, 2005) and the plasma (Nanda and Das, 2000) with the severity of *P. falciparum* infection. It is possible that a rise in LDH in plasma or serum of malaria patients may be due to leakage from the erythrocyte as a result of free radical induced damage on the membrane.

Available reports indicate that AO oxidizes aldehydes to the corresponding acids using molecular oxygen during which superoxide anions (free radicals) are generated (Mira *et al.*, 1995; Kundu *et al.*, 2007). This enzyme is elaborated in the liver and kidney but not as elaborated in RBC. However, the lack of significant alteration in the activity of AO in the test groups relative to the control (Table 1) is a likely indication that this enzyme may not be involved in malarial induced free radical production in RBC. Besides, as the enzyme is not elaborated in RBC, the possible damage to membranes of RBCs occasioned by rise in lipid peroxidation may not have caused significant leakage of the enzyme out of the RBC.

The availability of rapid, simple and specific diagnostic tools will assist in the control of malaria by allowing therapy to be accurately and aggressively administered. This has led to the recent development of rapid immunochromatographic dip stick assays as an adjuvant to or a replacement for classic microscopy for the diagnosis of malaria (Moody and Chiodini, 2002). However a major draw back of these assays is that they do not provide any information about the level of parasitemia. Therefore these test are unhelpful in assessing malaria severity. A major benefit of our findings is that RBC's LDH activity and level of LPO can form a basis for assessing malaria severity in humans once a diagnosis of *P. falciparum* malaria has been established.

In conclusion the findings of the present study indicate that LPO and activity of LDH are influenced by the severity of malaria. Thus these two parameters may not only be used in the diagnosis of *P. falciparum* infection, but may also be useful as determinants of the level of parasitemia in IRBCs.

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