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ASSESSMENT OF BIOCHEMICAL VARIATIONS AND SPLENOMEGALY DURING *FALCIPARUM* MALARIA IN MICE MODEL

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ABSTRACT
The present study was carried out to determine the serum biochemical and spleen external morphological changes in *Plasmodium falciparum* infected and control C57BL/6J male mice. Present work reports the levels of lipid profile, AST, ALT, ALP, total bilirubin, direct bilirubin, indirect bilirubin, total protein, albumin, globulin, GGT, serum creatinine and quantification of parasites in malaria infected group. Malaria parasitemia was monitored up to day 42 by observation of blood smears of mice. The peak level of parasitemia (35% on day 11) and condition of splenomegaly was observed in *falciparum* infected mice in comparison with control. Liver transaminases, lipid profile and total protein had shown statistically significant changes ($p < 0.05$) whereas total albumin, indirect bilirubin and serum creatinine were not significantly ($p > 0.05$) altered. Our observations strongly support the hypothesis that *falciparum* malaria infection affects serum biochemical profiles and enlargement of spleen as a valuable pathological sign in animal model.

INTRODUCTION

Plasmodium falciparum malaria alone is responsible for over 800,000 deaths a year, with many of these fatalities occurring in infants in Africa (WHO, 2009). Worldwide, majority of the deaths from malaria has been attributed to *P. falciparum*. Malaria pathogenesis is based mainly on extensive changes in biochemical and haematological parameters (Bidaki and Dalimi, 2003). The World Health Organization (WHO) criteria acknowledge that some biochemical and haematological features should raise the suspicion of severe malaria (WHO, 2000).

Infection by malaria parasites induce a dramatic, albeit variable splenic responses mostly characterized by splenomegaly. In fact, spleen size has been used as a tool to determine the intensity of malaria transmission in endemic regions (Chaves *et al.,* 2011). Malaria can frequently cause splenomegaly, but massive splenomegaly is rare. Hyperactive malarial splenomegaly is a complication of malaria that can cause chronic massive splenomegaly. It is thought to occur as a result of abnormal immune response to repeated malaria infections (Vinetz *et al*., 1998). Diagnosis of malaria is established by the finding of malarial parasites in the blood. The degree of parasitemia is useful as a prognostic factor; magnitude of parasitemia is related to the risk of death or complications (Krogstad, 2000).

The objective of the present work is to determine the effects of *falciparum* malaria on some biochemical parameters and study the morphological changes of the spleen that occurs due to *falciparum* infection in experimental animals would help in malaria diagnosis and management as well as understanding malaria pathogenesis. Hence the present investigation was done to study and assess the effect of *P. falciparum* parasite on AST (aspartate transaminase), ALT (alanine transaminase), and ALP (alkaline phosphatase), lipid profile, bilirubin, total protein, albumin, globulin, GGT (Gamma Glutamyl Transferase) and serum creatinine to determine the relationship between these biochemical parameters and parasitemia using experimental rodent.

MATERIALS AND METHODS

Animals

For the present experiment C57BL/6J male mice weighing between 25-30g, aged 10-12 weeks were randomly divided into two groups i.e., Group I (control) and Group II (infected) each with 6 animals.

Preparation and inoculation of *P. falciparum*

The *P. falciparum* antigen was obtained from an infected person. Initially the antigen was inoculated intraperitonially in to the three mice. The level of parasitemia was monitored from next day of inoculation on wards by observation of blood smears. When high level of parasitemia achieved in these mice, the blood samples of the mice were then collected and diluted in normal saline at the ratio of 75% parasitized blood and 25% PBS. This diluted parasitized blood was inoculated into Group II mice on day '0' via intraperitoneal (*i. p.*) route because malaria parasites penetrate the peritoneal wall enter into the blood stream within one minute of intraperitoneal inoculation (Thurston, 1953). Mice in Group I were inoculated with distilled water on day '0' and served as control.

Collection and analysis of blood samples

When the parasitemia reached peak level in Group II (infected), animals were sacrificed by cardiac puncture and blood samples were collected for analysis of the biochemical parameters.

Aspartate and alanine transaminases (AST & ALT) were assayed by colorimetric method (Reitman and Frankel, 1957). ALP was determined by Phenolphthalein method (Babson *et al.,*1966). Total cholesterol concentration was determined by enzymatic end point method (Roeschlaolu *et al.,* 1974). HDL was determined by Schettler and Nusel (1975). Triglycerides by simple colorimetric method described by Sugiura *et al.* (1977). The concentration of the LDL was determined using Friedwald equation i.e., LDL = TC – (HDL + VLDL) and VLDL by LDL = TG ÷ 5. Total serum protein and albumin were determined by Biuret method (Kinsley,1972) and anionic bromocresol dye binding method respectively (Doumas and Biggs, 1972). Serum globulin was determined indirectly by difference between total serum protein and serum albumin (Watson, 1965). Serum bilirubin (total, direct and indirect) by diazo reaction method (Noslin, 1960). The activity of serum GGT was measured according to Szaz (1969). Serum creatinine was analyzed by alkaline picrate method (Tietz *et al.,*1986).

Course of infection

The presence and relative parasite count of *P. falciparum* in Group II was determined by preparing thin blood smear of freshly drawn blood from tail vein of mice upto one month. The smears were stained with JSB I & JSB II. The percent of infected RBCs (parasitemia) was determined by enumerating the number of infected RBCs in relation to the number of uninfected RBCs (Oyewole *et al.,* 2010) as follows:

$$\% \text{ Parasitemia} = \frac{\text{No. of infected RBCs}}{\text{No. of RBCs counted}} \times 100$$

Data analysis

Data was expressed as mean ± standard deviation. The comparison of control Vs. infected was performed with Student t-test with MINITAB 11.12, 32 Bit statistical package and graphs were drawn from MS Excel. The values are statistically significant at $p < 0.05$, $p < 0.001$ and $p < 0.0001$.

RESULTS

In the present investigation, parasitic stages were observed in mice RBCs. Merozoites entering the fresh RBCs were seen attached along the margin of the RBC. Two or three rings are attacking a single erythrocyte is a characteristic feature of *P. falciparum* (Fig. 1). Course of infection in infected mice was studied for 30 days. Parasitemia reached to peak level on day 11 with 35% and then gradually decreased up to day 18. But on day 19 parasitemia increased slightly with 20.7% then decreased up to day 21 and slightly increased on day 22. Then the parasitemia gradually decreased and completely disappeared on day 28 and no parasites were observed up to 30th day (Fig. 2).

In our study we also found palpable enlargement of spleen in *P. falciparum* infected experimental mice (Group II). Spleen has shown change in colouration and size. The spleen had increased enormously in size i.e., double the size of the normal spleen. Parasitized and unparasitized cells along with haemozoin are filled inside the spleen which leads to dark red colouration and enlargement of the spleen (Fig. 3).

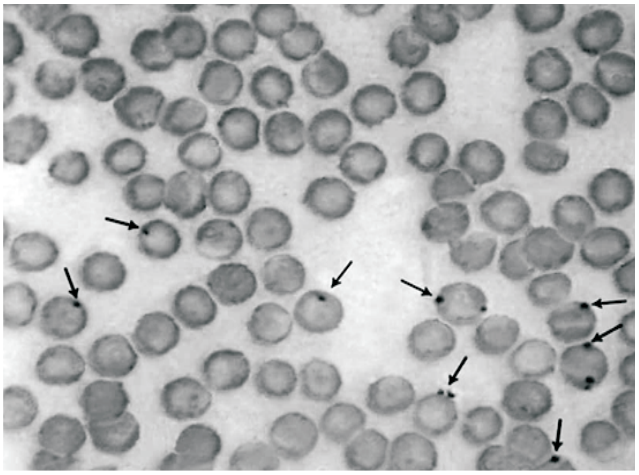

Figure 1: Early infection of *Plasmodium falciparum* in C57BL/6J experimental mice showing ring stages

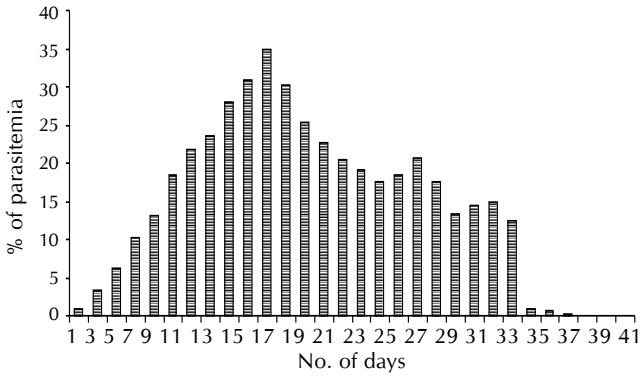

Figure 2: Multiplication of *P. falciparum* in experimental C57BL/6J mice

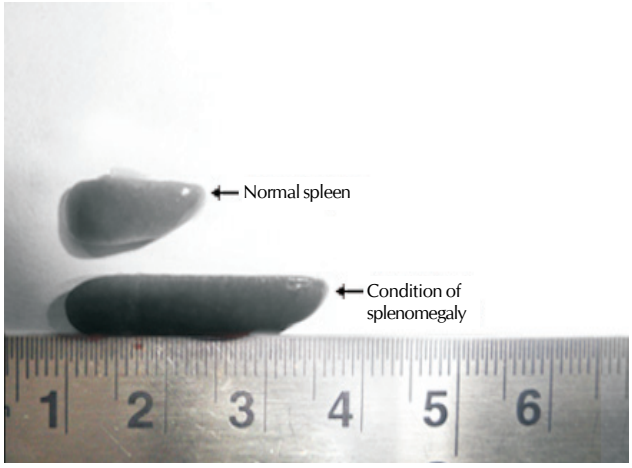

Figure 3: Photograph showing splenomegaly in *P. f.* infected mice in comparison with the spleen of control mice

Table 1: The Mean ± SD values of the serum hepato-specific markers in both control and experimental mice

Parameter	Control(Group I)(M ± SD)	Infected(Group II)(M ± SD)	t-value	p-value
AST (IU/L)	115 ± 2.58	183 ± 2.64	45.22	< 0.0001***
ALT (IU/L)	52 ± 2.32	85 ± 2.94	21.90	< 0.0001***
ALP (IU/L)	65 ± 2.58	98 ± 2.58	21.91	< 0.0001***

The values are expressed as mean ± standard deviation (SD), *** $p < 0.0001$, $t > 2.306$ represent significant change.

Table 2: Changes in various biochemical parameters of different experimental mice

Parameter	Control mice (Group I)(M ± SD)	Infected mice(Group II)(M ± SD)	t – value	p – value
Total Cholesterol(mg/dL)	76 ± 2.42	85 ± 2.66	6.24	< 0.0001***
HDL (mg/dL)	25 ± 2.79	36 ± 2.64	7.45	< 0.0001***
LDL (mg/dL)	24 ± 2.04	16 ± 2.83	6.20	< 0.0001***
VLDL (mg/dL)	27 ± 3.06	33 ± 3.92	2.96	> 0.05*
Triglycerides (mg/dL)	135 ± 3.03	165 ± 2.56	18.61	< 0.0001***
Total Protein (g/dL)	6.4 ± 0.303	5.8 ± 0.264	3.15	> 0.05*
Albumin(g/dL)	3.9 ± 0.232	3.0 ± 0.319	5.18	< 0.001**
Globulin (g/dL)	2.5 ± 0.259	2.8 ± 0.216	2.06	> 0.05
Total Bilirubin (mg/dL)	0.3 ± 0.234	0.8 ± 0.392	2.77	< 0.05*
Direct Bilirubin (mg/dL)	0.1 ± 0.014	0.6 ± 0.228	5.36	< 0.05*
Indirect Bilirubin(mg/dL)	0.2 ± 0.212	0.2 ± 0.075	0.42	> 0.05
GGT (IU/L)	8.0 ± 0.288	10.0 ± 24.6	9.97	< 0.0001***
Serum Creatinine(mg/dL)	0.4 ± 0.228	0.7 ± 0.217	1.17	> 0.05

The values are expressed as mean ± standard deviation (SD), * $p < 0.05$, **$p < 0.001$, ***$p < 0.0001$ and $t > 2.306$ represents significant change, HDL-High Density Lipids, LDL-Low Density Lipids, VLDL-Very Low Density Lipids, GGT- Gamma Glutamyl Transferase.

Impairment of hepatic function as a result of malaria infection was assessed by measurement of liver transaminases. The mean AST, ALT and ALP levels in infected (Group II) mice were increased significantly when compared with the control mice (Table 1).

Table 2, shows changes in biochemical parameters from control group to infected group. Serum total cholesterol, HDL, LDL and triglycerides have increased significantly ($p < 0.05$) in infected mice but VLDL level has decreased significantly ($p < 0.05$) when compared with the control value. The total protein and albumin levels in *P. f.* infected mice have shown significant decline ($p < 0.05$) but globulin level has not significantly increased ($p > 0.05$) in infected group than in control group. Serum total bilirubin and direct bilirubin levels were elevated significantly ($p < 0.05$) in mice with *falciparum* malaria when compared with non - parasitemic controls. But indirect bilirubin (unconjugated bilirubin) levels were same as in control mice. The serum gamma glutamyl transferase (GGT) has increased in infected mice significantly ($p < 0.05$) whereas serum creatinine has not increased significantly in *P. f.* infected mice when compared to control values.

DISCUSSION

In our study we found relation among parasitemia, splenomegaly and currently selected parameters in control and *P. falciparum* infected mice. As the parasites multiply and reached the peak level; liver transaminases, lipids, total protein, albumin, globulin, bilirubin (total, direct and indirect), GGT, serum creatinine levels were altered by the *falciparum* parasite.

During the study of course of infection, the peak level of was 35% on day 11 which correlates with the studies of LaCrue (2011) where 40% of parasitemia was observed at peak level of *P. falciparum* infection and Sunita *et al.* (2006) peak level of infection appeared on day 10 in *P. pinottii* infected experimental chicks.

The present investigation reveals palpable enlargement of spleen which is one of the main clinical symptoms. The main change was that of congestion, but later the spleen becomes dark by the accumulation of pigment in parasitized cells in the capillaries and sinusoids (Bruce-Chwatt, 1978). This is confirmed by Lawson *et al.* (1969), Rifkind (1965) and La Celle (1970) that abnormal red cells, such as red cells with rigid inclusions (Heinz bodies) or red cells with rigid membranes or contents (sickle red cells, spherocytes) encounter great difficulty traversing through the narrow cords and especially the small basement membrane fenestrations between cord and sinus and lead to congestion of the spleen later the spleen becomes dark by the accumulation of pigment in parasitized cells in the capillaries and sinusoids.

AST, ALT and ALP activities are used as markers in hepatic diagnosis. In the present study, the increase in the levels of AST, ALT and ALP in *P. falciparum* infected mice reflects impairment of the liver. When the liver is impaired the liver cells release the enzymes into the blood raising the enzyme levels. Our results are in consistent with other studies which reported that majority of the patients shown elevation in serum activities (AST, ALT and ALP) indicating liver damage (Oyewole *et al.,* 2010; Onyesom and Onyemakonor, 2011; Faheem *et al.,* 2011). The liver damage may have been caused by the free radicals generated by the *P. falciparum* parasite. The levels of hydroxyl and peroxide radicals induced by *P. falciparum* parasites may be responsible for the changes in the enzyme levels (Nnodium *et al.,* 2010).

In the present study, the serum total cholesterol, HDL-C, VLDL and triglycerides in malaria infected mice was higher than those in control mice. This finding is consistent with the studies which shown elevated levels of lipoproteins like HDL, total cholesterol and triglyceride in patients suffering from malaria infection (Vial *et al.,* 2003; Onogbu and Onyeneke, 1983). However, in infected mice, the LDL-C level was low but other serum lipid levels were rather found to be higher compared

with the control group.

Serum total proteins were reduced in malaria infected mice which corroborates with the previously reported (Etim *et al.,* 2009; Adebisi *et al.,* 2002) and these may have resulted from increased protein utilization by the parasite for the building of new protoplasm during multiplication and the host cells for the synthesis of immunoglobulins and acute phase proteins in response to the invading malaria parasites. Significant higher globulin protein fraction was also observed in our study which confirms earlier reports (Butcher, 1998; Etim *et al.,* 2009).

The relationship between serum protein levels and malaria infection has also been reported earlier (Adebisi *et al.,* 2002) indicating declining protein level and hypoalbuminemia to be responsible for the oedema during severe malaria infection, which supports the present finding.

Various authors have reported close relationship between incidence of severe malaria and liver damage characterized by jaundice (Mishra *et al., 1*992, Dondorp and Day, 2007). Observed elevation of bilirubin in the present study correlates with the studies of Oyewole *et al*. (2010) showing that increased red blood cell haemolysis and is associated with hepatocellular damage, biliary tract obstruction, haemolysis and jaundice (Renner, 1995; Yokoto and Calisei, 2006).

In our findings GGT levels are raised in infected mice than control mice. Our results confirm that severe malaria in adults is associated with hepatic dysfunction with increased AST, ALT, GGT and total bilirubin (WHO, 2000). The increase in these enzymes is indicative of hepatic cholestasis.

Elevation of the serum creatinine in the present study is an indication of abnormal renal function (Mouton and Holder, 2006). This is in agreement with those of Weber *et al*. (1999) who made similar observation in a study of renal involvement in Gambian children in malaria and also in another study in children reported by Raphael *et al*. (2010). Hence, the present investigation reveals the significant change in liver i.e., splenomegaly during peak level of infection and associated biochemical changes during malaria in experimental mice.

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