ORIGINAL ARTICLE

Superoxide Radical Generation Mediated *Plasmodium berghei* Infection in Swiss Mice

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Abstract: *Aim:* This study was aimed to evaluate the duration dependent study to develop *Plasmodium berghei* infection in *in vivo* system. *Methods:* 200µl of infected blood containing 1×10^5 parasites was challenged in Swiss mice intraperitoneally for 5, 10 and 15 days. After sacrifice, parasitemia, O_2^{-} generation, NADPH oxidase activity, NO generation of lymphocyte; MDA level, TrxR activity and haemolytic activity of infected RBC; activities of LDH, CRP level, SGOT and SGPT, NO release of serum; the MPO activity of serum, liver, spleen and kidney were studied. *Results:* O_2^{-} generation, NADPH oxidase activity, NO generation of lymphocyte; LDH activity, CRP, SGOT, SGPT level and NO release of serum and TrxR, haemolytic activity were significantly increased than control; and also MDA level of infected RBC significantly increased as compared to control. These effects were the maximum after 10 days of challenge. *Conclusion:* The findings suggest that, *P. berghei* NK65 infection maximally develop after 10 days infection in Swiss mice.
Key words: Malaria**,** *Plasmodium berghei*, LDH, superoxide radical, NADPH oxidase, thioredoxin reductase

Introduction

Malaria caused by the agent, *Plasmodium*, a parasite that cycle through a mosquito and a mammalian host. *Plasmodium berghei*, a rodent parasite, a valuable model organism for the investigation of human malaria as the organisms are similar in most essential aspects of morphology, physiology and life cycle of these parasites is simple and safe [1].

Plasmodium berghei was first described by Vincke and Lips 1948 [2] and *P. berghei* NK65 strain was isolated from *Anopheles dureni millecampsi* in 1964 [3]. The multiplication of the parasite in the blood causes the pathology such as anemia and damage of essential organs of the host such as liver, spleen, alters the erythrocyte membrane and haemolysis occur by the parasite products [4].

Generally, the malaria-infected erythrocytes content higher lipid than the lipid content found in normal erythrocytes. Lawrence and Cenedella 1969 [5] calculated that "free" *P. berghei* might contain as much as 5 times more lipid than rat reticulocytes, but the value could also be as low as 1.5 times. It is generally accepted that superoxide, ROS, including NO and peroxynitrite kill intraerythrocytic malarial parasites [6-7] but parasite protect itself by antioxidant enzyme and it contains functional redox system comprising NADPH, thioredoxin, thioredoxin reductase (TrxR) [8].

TrxR have a great role in maintenance of redox homeostasis and anti-oxidant defense in Plasmodium infection [9]. From immunological perspective, clinical, biochemical and epidemiological studies suggest that innate immune mechanisms contribute to protection from malaria and modulate adaptive immune responses [10, 7] and it also proposed that the balance and timing of both innate and adaptive immune responses are the critical issues to malaria. Here we investigated the superoxide mediated *P. berghei* infection of the host, biochemical characters of serum, lymphocyte, RBC and tissues, elicited by different period using mouse- *Plasmodium berghei* model.

Material and Methods

Chemicals and reagents: Histopaque 1077, Hepes, DTNB, Phorbol mirested aceted, horse heart cytochrome-*c* were purchased from Sigma Chemical Co., USA. Tris buffer, Sodium chloride, Triton-X 100, potassium dihydrogen phosphate, di potassium hydrogen phosphate, EDTA, sodium hydroxide, chloroform, sodium acetate, ammonium acetate, potassium hydroxide, methanol, Gimsa were procured from Merck Ltd., Mumbai, India.

Parasite: The NK65 strain of *Plasmodium berghei* was kindly supplied from Dr. Pralhad Ghosh's Research laboratory, Department of Biotechnology, Delhi University, South campus, Delhi, India and maintained into sex and age-matched wild type mice by weekly passage and blood stage parasites were stored at -80ºC.

Animals: Swiss male mice (6-8 weeks old, weight- 20-25 g) were used to full fill the experiments. Animals were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India, and approved by the ethical committee of Vidyasagar University. The animals were fed standard pellet diet with vitamins, antibiotic and water were given *ad libitum* and housed in polypropylene cage (Tarson) in the departmental animal house with 12 h light and dark cycle under standard temperature (25±2 ºC). The animals used in this study did not show any sign of malignancy or other pathological processes.

Experiment Schedule: Mice were randomized into control and experimental groups and divided into two groups of eighteen (18) animals each. Group 'A' served as control and group 'B' served as *P. berghei* challenged (200µl of infected blood containing 1 X 10^5 parasites) intraperitoneally (i.p.). The dose was selected as per reported by Chandel and Bagai, 2010 [11]. The experiment was terminated at the end of 5 days, 10 days and 15 days and all animals were sacrificed by an intraperitoneal injection of sodium pentobarbital (60-70 mg/kg body weight). After sacrifice blood (n=6/group) was used to prepare serum, to separate lymphocyte and RBC for biochemical estimation of different parameters.

Separation of Serum, lymphocyte and RBC: Serum was separated by centrifugation at 1500× *g* for 15 min of blood samples taken without anticoagulant. Serum was kept at -80ºC for the biochemical estimation of different parameters. Lymphocytes were isolated from heparinized blood samples using standard isolation techniques [12].

Blood samples were diluted with equal amount of PBS (pH 7.0) buffer and then layered very carefully on the density gradient (Histopaque 1077) in 1:2 ratios. Centrifuged at 500×g for 20 min and the white milky layer of mononuclear cells i.e. lymphocytes was carefully removed. The layer was washed twice with the same buffer and centrifuged at 3000×g for 10 min to get the required pellet of lymphocytes and rest pellet as RBC was transferred in another centrifuge tube and stored at -80 ℃ for further study. The pellets of lymphocytes were lysed in a hypotonic lyses buffer for 45 min at 37 ℃ then kept at -20 ℃ until biochemical estimation [13].

Separation and homogenization of tissue: After decapitation, liver, kidney and spleen were excised from experimental mice of different groups and washed with cold normal saline. The tissues were homogenized in the ice-cold buffer containing 0.25 M sucrose, 1 mM EDTA, and 1 mM Tris-Hcl, pH 7.4. The homogenate was centrifuge at 600×g for 10 min at 4°C, and the supernatant was stored at -80℃ for experiment. [14]

Parasitemia: Thin blood film was stained with Gimsa and the percentage of parasitaemia was determined by counting the number of parasitized RBC out of 1000 RBC in 10 random microscopic fields [15].

Biochemical estimation

Superoxide anion generation in lymphocytes: The superoxide production was measured according to the method of Boveris 1984 [16] by the SOD-inhibitable reduction of acetylated cytochrome c. Cytochrome c reduction by generated superoxide was monitored spectrophotometrically at 550nm wavelength with or without the addition of 3l M SOD. The superoxide generation assay was performed in room air conditions [17].

NADPH oxidase activity in lymphocytes: NADPH oxidase activity was determined by measuring cytochrome c reduction spectrophotometrically at 550 nm, using the method of Heyneman and Vercauteren 1984 [18].

Nitrite generation in lymphocytes and release in serum: No generation and release was measured according to Sanai et al. 1998 [19]. Reading was taken in a UV spectrophotometer at 546 nm. The levels of NO were expressed as μ mol/mg protein.

C-reactive protein level in serum: C-reactive protein level in serum was estimated using a sandwich ELISA Kit (Tulip, Mumbai, India). The assay was performed as per the detailed instructions of the manufacturer.

Lactate dehydrogenase activity in serum: LDH activity in serum was estimated using a sandwich ELISA Kit (Tulip, Mumbai, India). The assay was performed as per the detailed instructions of the manufacturer.

Serum glutamate Oxalate transaminase and Serum glutamate pyruvate transaminase activity in serum: To measure the liver-associated enzymes SGOT and SGPT in serum, a modified protocol of the standard colorimetric end-point method [20] was used. Reading was taken at 520 nm after 5 min.

Myeloperoxidase activity in serum and tissue homogenate: The myeloperoxidase activity in serum was determined by using the method of Bos et al. 1990 [21] and reading was taken at 492 nm.

Lipid peroxidation in RBC: The extent of lipid peroxidation was estimated as the concentration of thiobarbituric acid reactive product malondialdehyde (MDA) by using the method of Ohkawa et al. 1979 [22]. The reading was taken at 530 nm with the use of the Hitachi U-2000 spectrophotometer.

Thioredoxin reductase activity in RBC: By using the method of Holmgren and Bjorsnstedt, 1995 [23], thioredoxin reductase (TrxR) activity was measured. The reading was monitored at 412 nm at room temperature and specific activity was calculated as units of enzyme per mg protein.

Haemolytic activity in RBC: Haemolytic activity was measured by using the method of Gupta and Saxena, 1980 [24] and the reading was measured at 540 nm.

Protein estimation: Protein was determined according to Lowry et al. 1951 [25], using bovine serum albumin as standard.

Statistical analysis: The data were expressed as mean ± SEM, n=6. Comparisons between the means of control and *P. berghei* infected groups (using a statistical package, Origin 6.1, Northampton, MA 01060 USA) with student's t-tests, $p < 0.05$ as a limit of significance.

Results

Parasitemia: In our experiment, the infected group showed a significantly ($p < 0.05$) increasing of parasitemia on 9.175% (5 days), 23.725% (10 days) and on 15 days infection parasitemia found 20.25%. All the mice survived during the experiment (Fig. 1).

Fig-1: *(a)* Parasitemia in infected blood of 5, 10, 15 days infection. Values are expressed as mean±SEM, n=6* Significant difference (P<0.05) compared to control. Gimsa-stained blood smear of non-infected and infected groups. *(b)* non-infected blood smear *(c)* 5 days infected blood smear *(d)* 10days infected blood smear *(e)* 15 days infected blood smear. Images are photographed at ×100 magnification.
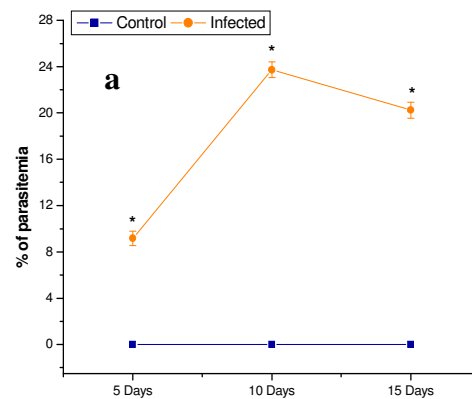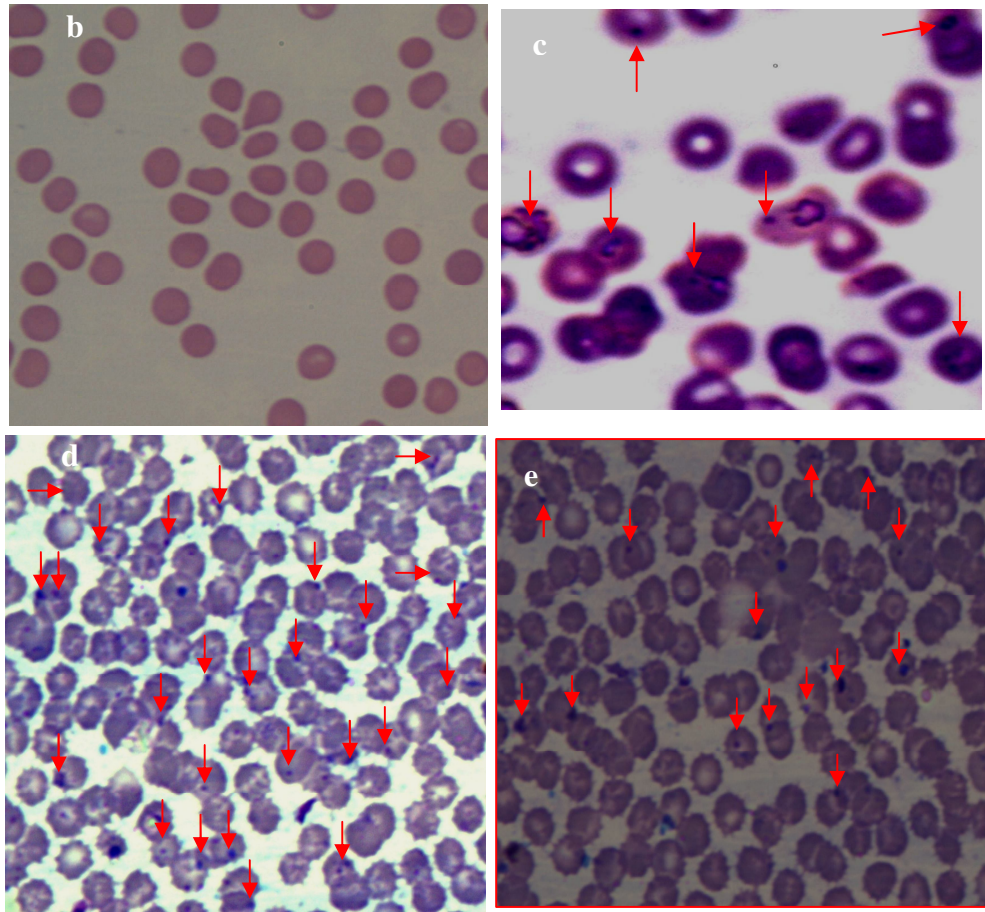
Super oxide radical generation and NADPH oxidase activity in lymphocyte: Superoxide anion (O_2^-) generation was significantly ($p < 0.05$) increased in lymphocyte of *P. berghei* infected mice blood on 5 days, 10 days and 15 days by 51.38%, 100.55% and 91.71% respectively, as compared to control (Fig. 2). NADPH oxidase in lymphocyte was found to be increase significantly ($p < 0.05$) by 48.30%, 115.58% and 107.6% respectively, as compared to control (Fig. 3).
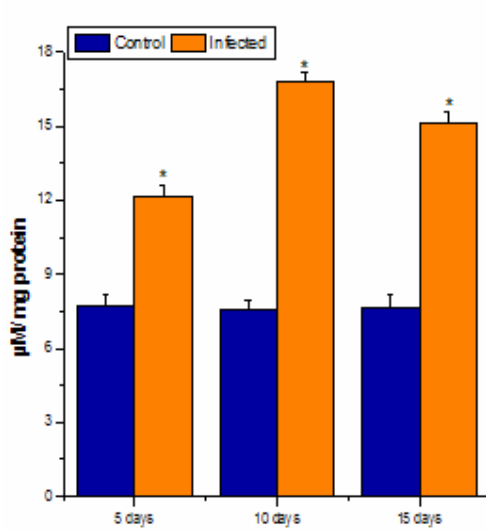

Fig-2: Superoxide radical (O_2^-) generation in lymphocytes of control and *P. berghei* infected groups. Values are expressed as mean±SEM, n=6* Significant difference (P<0.05) compared to control group.
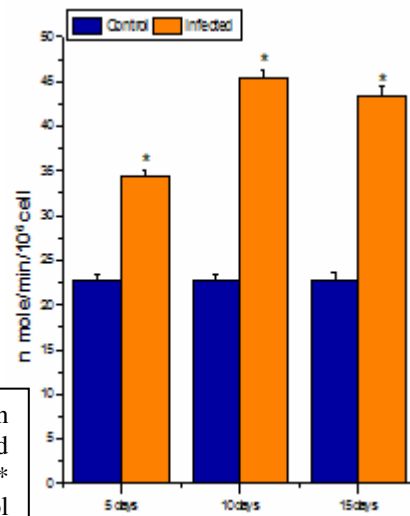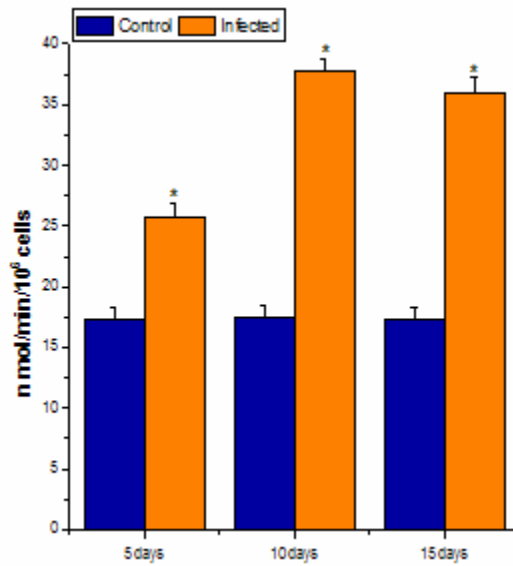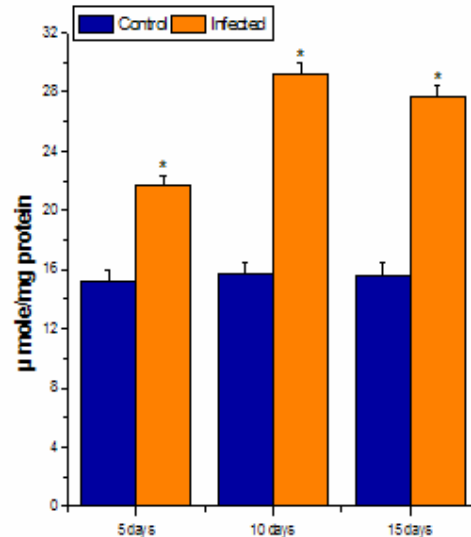
Fig-3: NADPH oxidase activity in lymphocyte of control and *P. berghei* infected groups. Values are expressed as mean±SEM, n=6* Significant difference (P<0.05) compared to control group.

Nitrite generation in lymphocyte and release in serum: In this experiment lymphocyte showed significantly (p < 0.05) increasing of NO generation and release by 56.44%, 122.10%, 97.58% and 42.26%, 85.54% 76.66% from 5, 10 and 15 days infected group respectively, as compared to control (Fig. 4 and Fig. 5).
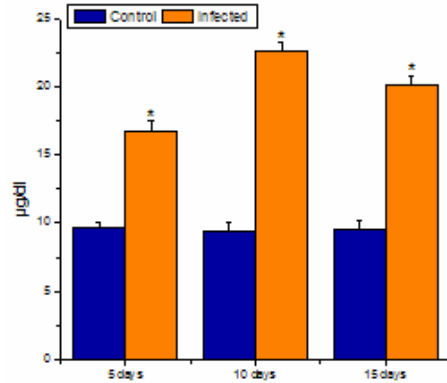

Fig-4: NO generation in lymphocyte of control and *P. berghei* infected groups. Values are expressed as mean±SEM, n=6* Significant difference (P<0.05) compared to control.

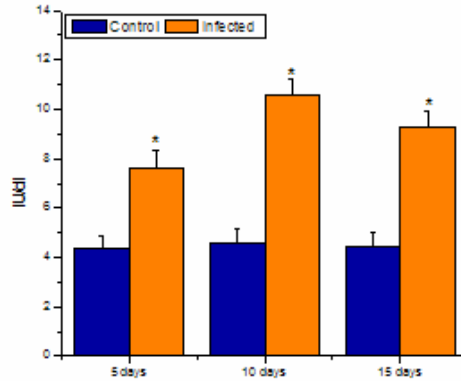
Fig-5: NO release in serum of control and *P. berghei* infected groups. Values are expressed as mean±SEM, n=6* Significant difference (P<0.05) compared to control.

Serum C-reactive protein level and lactate dehydrogenase activity: C-reactive protein level was significantly ($p < 0.05$) increased in infected serum on 5 days, 10 days and 15 days by 74.69%, 140.15% and 112.87% respectively, as compared to control (Fig. 6). LDH activity in serum was also significantly ($p < 0.05$) increased in infected mice after 5, 10 and 15 days infection by 73.94%, 132.35% and 109.87% respectively, as compared to control (Fig. 7).
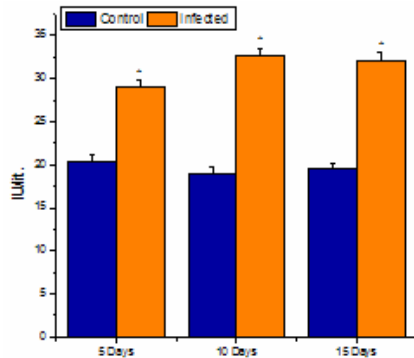

Fig-6: CRP level in serum of control and *P. berghei* infected groups. Values are expressed as mean±SEM, n=6* Significant difference (P<0.05) compared to control.
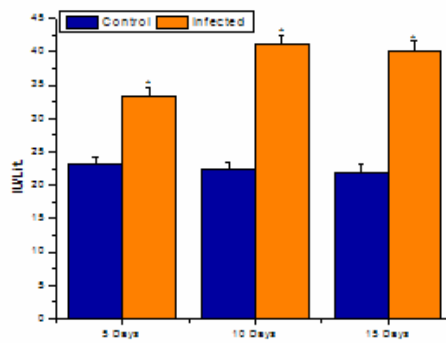

Fig-7: LDH level in serum of control and *P. berghei* infected groups. Values are expressed as mean±SEM, n=6* Significant difference (P<0.05) compared to control.

Serum glutamate oxalate transaminase activity and serum glutamate pyruvate transaminase activity: SGOT activity was significantly ($p < 0.05$) increased in serum of *P. berghei* infected mice on 5 days, 10 days and 15 days by 42.53%, 72.03% and 63.92% respectively, as compared to its control (Fig. 8). SGPT activity was significantly ($p < 0.05$) increased after 5, 10 and 15 days by 43.41%, 84.35% and 82.23% respectively, as compared to its control group (Fig. 9).
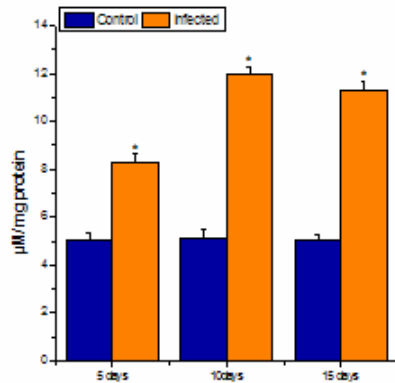

Fig-8: SGOT level in serum of control and *P. berghei* infected groups. Values are expressed as mean±SEM, n=6* Significant difference (P<0.05) compared to control.

Fig-9: SGPT level in serum of control and *P. berghei* infected groups. Values are expressed as mean±SEM, n=6* Significant difference (P<0.05) compared to control.

*Myeloperoxidase activity in serum and tissue homogenate:* Myeloperoxidase activity of serum increased significantly ($p < 0.05$) on 5 days, 10 days and 15 days infection by 64.28%, 135.99% and 124.71% respectively by comparing to control (Fig. 10). MPO of liver of infected mice was increased significantly ($p < 0.05$) on 5 days, 10 days and 15 days by 79.43%, 102.08% and 75.42% respectively by comparing to control (Fig. 11). MPO activity of spleen increased significantly ($p < 0.05$) on 5 days, 10 days and 15 days infection by 79.48%, 186.34% and 158.32% respectively by comparing to control groups (Fig. 12). MPO activity of kidney was increased significantly ($p < 0.05$) on 5 days, 10 days and 15 days infection by 63.64%, 85.7% and 57.8% respectively, as compared to control (Fig. 13).
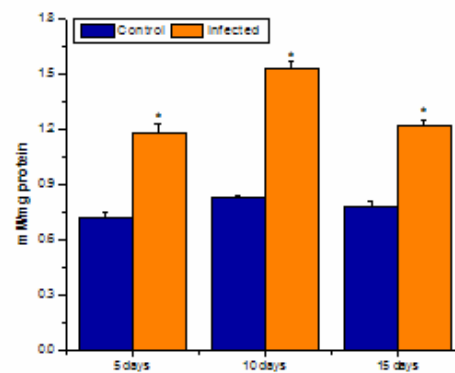

Fig-10: MPO activity in serum of control and *P. berghei* infected groups. Values are expressed as mean±SEM, n=6* Significant difference (P<0.05) compared to control.
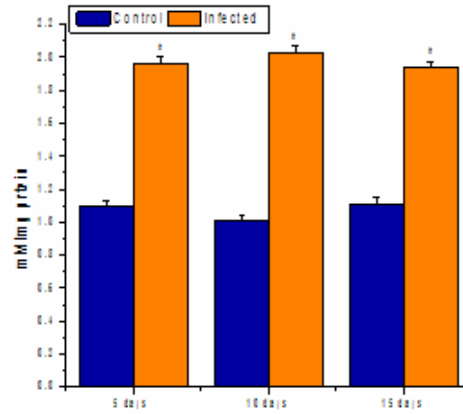
Fig-11: MPO activity in liver of control and *P. berghei* infected groups. Values are expressed as mean±SEM, n=6* Significant difference (P<0.05) compared to control group.
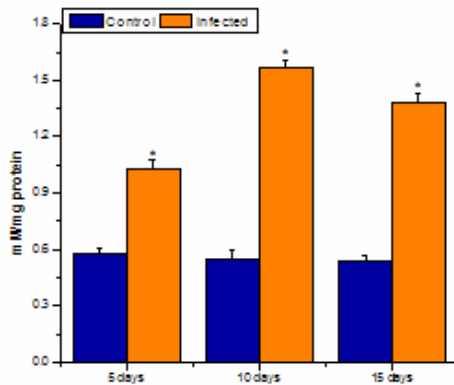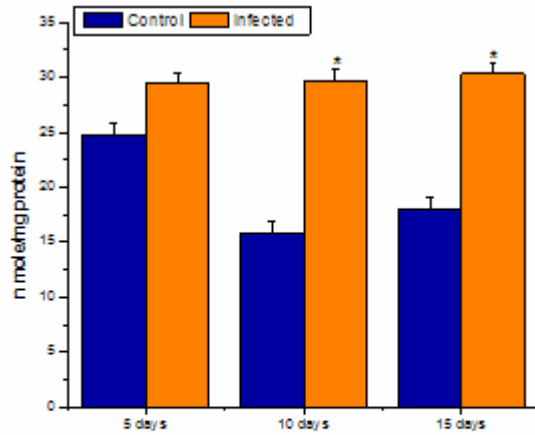

Fig-12: MPO activity in spleen of control and *P. berghei* infected groups. Values are expressed as mean±SEM, n=6* Significant difference (P<0.05) compared to control group.

Fig-13: MPO activity in kidney of control and *P. berghei* infected groups. Values are expressed as mean±SEM, n=6* Significant difference (P<0.05) compared to control.
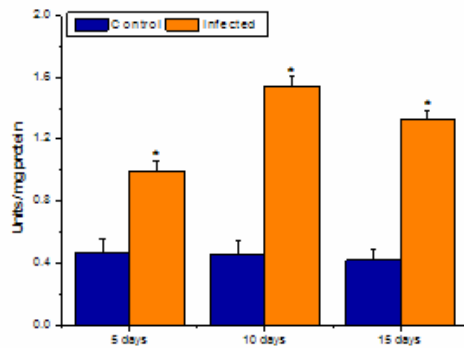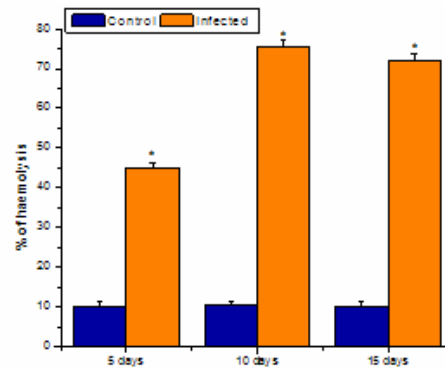
Fig-14: MDA level in RBC of control and *P. berghei* infected groups. Values are expressed as mean±SEM, n=6* Significant difference (P<0.05) compared to control.

Lipid peroxidation in RBC: Lipid peroxidation in RBC after 5, 10, 15 days infection was measured in terms of malondialdehyde. MDA level was significantly ($p < 0.05$) increased in the infected groups by 19.01%, 87.57% and 69.29% respectively, as compared to control (Fig. 14).

Thioredoxin reductase activity and hemolytic activity in RBC: Thioredoxin reductase activity significantly ($p < 0.05$) increased by 111.13%, 237.85% and 214.73% in post 5 days, 10 days and 15 days infection respectively. It was compared to control (Fig. 15). Hemolytic activity in RBC was also found to be increase significantly ($p < 0.05$) by 343.49%, 628.06% and 597.58% from 5, 10 and 15 days infected group respectively, as compared to control (Fig. 16).

Fig-15: TrxR activity in RBC of control and *P. berghei* infected groups. Values are expressed as mean±SEM, n=6* Significant difference (P<0.05) compared to control.

Fig-16: Haemolytice activity in RBC of control and *P. berghei* infected groups. Values are expressed as mean±SEM, n=6* Significant difference (P<0.05) compared to control.

Discussion

In our present study, it has been evaluated that parasite infection after first inoculation gradually increased and parasitemia became the maximum on 10 days infection (Fig. 1). There was provided evidence that protective immunity to a particular strain can be said to have developed when parasite numbers drop to subclinical levels. Rarely if infection eliminated quickly; usually it persists for some time as a low-level parasitemia but the fatality of malaria infection usually begins with a phase of high parasitemia [26].

The results, we have described above show that superoxide (O_2^-) generation have potent mediation to develop *P. berghei* infection in Swiss mice over the 10 days observation period (Fig. 2). These O_2^- leads to oxidative damage of macromolecules including lipid, protein, DNA and antioxidant enzymes. The NADPH oxidase transports electrons from NADPH on the cytoplasm side of the membrane to oxygen in the extracellular fluid to form O_2^- [27]. Here maximum significant ($p < 0.05$) activity of NADPH oxidase found in 10 days infected mice (Fig. 3).

Our experiment demonstrated NO generation of lymphocytes significantly ($p < 0.05$) increased and maximally on 10 days infection (Fig. 4). It also found that the maximum NO release from serum occurred from 10 days infected group (Fig. 5). Nitric oxide reacts with the O_2^- to generate peroxynitrite which is a selective oxidant and nitrating agent that interacts with numerous biological molecules, thereby damaging them [28]. In addition to NO, C-reactive protein (CRP) is a major acute phase protein, present in normal serum, which increases significantly after most forms of tissue injuries and infections as a non-specific innate defense mechanism of the host.

In the experiment, significantly ($p < 0.05$) increased of CRP level was found on 10 days infection (Fig. 6) and CRP is reported to be a critical element during malaria infection and there is a strong association of elevated CRP levels during the acute phase of severe [29] and clinical forms of malaria [30].

LDH activity (Fig. 7) was observed in serum of *P. berghei* infected group but maximum LDH activity were calculated on 10 days infection. Serum level of both SGOT and SGPT become elevated whenever disease processes affect liver cells. SGPT is the more liver specific enzyme [31]. In this experiment SGOT and SGPT level in serum significantly ($p < 0.05$) increased and it was also been found that both SGOT and SGPT level was the maximum on 10 days infection (Fig. 8, 9).

Here the result also showed that MPO activity of the infected serum, liver, spleen and kidney significantly ($p < 0.05$) increased. The highest MPO activity found after 10 days infection (Fig. 10, 11, 12, 13). Here superoxide may potentiate oxidant damage at inflammatory sites by optimizing the MPO-dependent production of HOCl [32].

This study demonstrated that lipid peroxidation (MDA level) of RBC significantly ($p < 0.05$) increased as well as parasite infection decreased lipid deposition in infected erythrocyte. The maximum increased amount of MDA level was found after 10 days infection (Fig. 14).

In this experiment we proofed that TrxR significantly ($p < 0.05$) increased during maximum infection (Fig. 15). During infection, TrxR is responsible for transferring of electrons from NADPH to thioredoxin to contribute the antioxidant capacity of the cell. Plasmodium TrxR differs significantly from mammalian counterpart. Thioredoxin functions as redox messenger in parasite maintaining reduced intracellular environment [15].

Haemoglobin proteolysis in intraerythrocytic malaria parasites is a biochemical event. The oxidant haeme group is separated from globin chains, a process in which Fe^{+2} is oxidized to Fe^{+3} and electrons produced react with molecular oxygen to form oxygen radicals [33-34]. Our data suggested that haemolytic activity on 10 days of *P. berghei* infection markedly increased (Fig. 16).

In this experiment it was found that *Plasmodium berghei* NK65 infection in Swiss mice was maximally developed on 10 days infection through immune-modulator activity and mediating the superoxide radical generation. However, given the small sample size used in this study, further follow-up investigation on this phenomenon are necessary.

Acknowledgement

Abbreviations

DTNB   : 5', 5'-dithio (bis)-2-nitrobenzoic acid
EDTA   : Ethylene diamine tetra acetate
LDH   : Lactate Dehydrogenase
CRP   : C-reactive proteinase
TrxR   : Thioredoxine reductase
SGOT   : Serum glutamate oxalate transaminase
SGPT   : Serum glutamate pyruvate transaminase
MPO   : Myeloperoxidase
MDA   : Malondialdehyde
NADPH   : Nicotinamide adenine dinucleotide phosphate
PBS   : Phosphate buffer saline
ROS   : Reactive oxygen species
TBA   : Thiobutiric acid
TBARS   : Thiobutiric acid reactive substance
TCA   : Trichloro acetic acid
OPD   : O-diamisidine
H_2O_2 : Hydrogen peroxide

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