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Original Research Article

Influence of MDA and Pro-inflammatory Cytokine levels in the Pathogenesis of Severe Malaria in Experimental Murine Model

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Abstract: Reactive oxygen species (ROS) are reported to be involved in human Cerebral Malaria (CM). To assess the extent of oxidative stress, we have investigated the biomarker of lipid peroxidation, Malondialdehyde (MDA) and its co relation with levels of pro-inflammatory cytokines with malaria severity. The present study was designed to measure Thiobarbituric acid reactive substances (TBARS) for the MDA assay to delineate the oxidative stress in various organs (brain, liver and spleen) of *Plasmodium berghei ANKA* infected *Swiss albino* mice. Further mRNA levels of Tumor Necrosis Factor-α (TNF-α), Interleukin -1β (IL-1β) and Interleukin-6 (IL-6) were measured by qRT-PCR in the organs of mice according to severity of malaria. Result of this study showed that the MDA levels were significantly higher ($P < 0.05$) in the brain, liver and spleen of infected mice compared with the non-infected control group respectively. A study observed highly up regulation of TNF-α between the day 5 and 9 with peak production being detected on the 9th day, and subsequently decreased between the 11th and 13th day. In the brain, spleen and liver IL-1β and IL-6 mRNA were significantly up-regulated ($P<0.0001$) and persisted throughout the course of infection. It might be concluded from the findings of the present study that the oxidative stress response induced by the plasmodium species may trigger the inflammatory cytokine responses in malaria severity and thereby contributes to the pathogenesis of the disease; however the interplay between the oxidative response and inflammatory activity in disease virulence needs further study.
Keywords: Experimental Severe Malaria, *P.berghei ANKA*, Lipid Peroxidation, Malondialdehyde (MDA), Pro-inflammatory cytokine, TNF-α, IL-1β, IL-6

INTRODUCTION:

Malaria is one of the most important global health problem and neglected disease, potentially affecting more than one third of the population. As per WHO estimates, 214 million malaria cases occurred globally in 2014 wherein about 88 percent of these cases were found in African countries and 10 percent in South East Asia region (SEAR) [1]. In Indian scenario, 61 percent of malaria cases were found to be positive along with 41 percent malaria deaths in SEAR region [1,2]. Most of the severe cases of malaria disease are caused by *Plasmodium falciparum* which is endemic in tropical countries. Although the malaria rate has decreased, resistance to drug therapy has increased, especially in patients infected with *Plasmodium falciparum* and *Plasmodium vivax* [3].

In fact, current drugs such as chloroquine and artemisinin have already present resistant strains of *Plasmodium falciparum* [4,5]. Severe infection with *Plasmodium falciparum* may result in severe anemia, hyperparasitemia, hyperpyrexia, prostration, splenomegaly, hepatomegaly, respiratory distress, hemoglobinuria, persistent vomiting and comma [1,2,6]. However, the physio-pathogenic mechanisms of the disease remains poorly understood. Oxidative stress induced damage has been observed in patients with post malaria parasite infection, suggesting that this could play a role in the pathogenesis of severe malaria [7,8]. Earlier studies have demonstrated that cells infected with parasites and human immune deficiency virus exhibit an increased generation of reactive oxygen species (ROS) with decreased antioxidant production, leading to the activation of redox-dependent transcription factors and the production of various cytokines [9-11]. Malondialdehyde (MDA) is a known reactive aldehyde formed by the degradation of polyunsaturated lipids by ROS and a widely used biomarker for the oxidative stress response [12,13]. However, the association of ROS and cytokine

production in malaria infection has not been greatly explored. Previous research and other studies in this area have revealed a significant pro-oxidant state in parasite infection and its association with severe anemia and cerebral cases [8,11]. On the other hand, cytokines are reported to play a significant role in the evolution of severe malaria infection [14,15]. The production and up-regulation of cytokines in response to plasmodium infection are responsible for the development of complications such as cerebral malaria.

The fine mechanisms leading to cerebral complications in human Cerebral Malaria (CM) remains incompletely understood and the understanding of the pathogenesis of CM necessarily relies on the use of experimental animals or *in vitro* models [15]. The full extent of the pathogenic events leading to severe complications in falciparum infection remains largely unresolved since the outcome from post-mortem investigation can only provide the endpoint findings. Therefore, laboratory models are important to elucidate the immunological mechanisms involved in severe malaria and the way of alleviating this serious condition, because of the difficulty in following up human cases with severe malaria and the limited possibility to examine its pathological process.

Since very few reports are available on the correlation between inflammatory and oxidative stress responses in pathogenesis of severe malaria with respect to tissue samples, the present study aimed to evaluate the levels of tissue MDA and the levels of pro-inflammatory cytokines Tumor Necrosis Factor-α (TNF-α), Interleukin -1β (IL-1β) and Interleukin-6 (IL-6) during pathogenesis of malaria infected with murine *Plasmodium berghei ANKA* parasite.

MATERIAL AND METHODS:

Mice:

Swiss albino mice were purchased from Advanced Centre for Treatment, Research and Education in Cancer (ACTREC, Navi Mumbai, India). All mice were maintained in specific pathogen-free environment and used at 6-8 weeks of age. Mice were kept in the well sanitized and clean in-house animal facility of Haffkine Institute and maintained at 65-75ºF and 40-60% relative humidity with 10-12 hr light-dark cycle. They were given autoclaved pelleted, non-contaminated and nutritionally adequate feed and fresh potable drinking water. All the mice experiments were approved by the Institutional Animal Ethics Committee (IAEC) of Haffkine Institute under the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) guidelines. (HITRT/IAEC/026/2011)

Infection with *Plasmodium berghei ANKA* parasite:

All *Swiss albino* mice were injected Intraperitoneally (i.p.) with $2x10^6$ *P. berghei ANKA* parasitized RBCs, kindly provided by National Institute of Malaria Research (NIMR), New Delhi, India. For the i.p. injection, mice were sedated through anesthetic chamber for the induction of successful anesthesia. Parasitemia was checked by counting of 400-500 Red blood cells every day from the tail blood smears stained with Giemsa (Sigma Diagnostic, USA). Control mice were injected i.p. with the same volume of sterile normal saline. The infected mice were randomized into five groups of 6 (in separate cages), each group corresponding to selected day post-infection when cytokine levels were determined. Two parallel experiments (n=2) were modeled on this setup. Mice with severe malaria were killed by inhalation of CO2 by Euthanasia method and groups of infected and uninfected mice were sacrificed after respective days according to parasitemia. Brain, liver and spleen of infected and control mice were stored at -80º C for RNA isolation.

Basic illness parameters measurement:

The body weight of mice was measured throughout the study using a Konica digital scale top pan animal balance to the nearest 0.001g. All control and malaria- infected mice were observed visually throughout the experiment for behavioural changes and signs of illness which include Piloerection, and decreased loco motor activity. Any signs of illness were quantified using arbitrary scale and recorded as either absent (-), mild (+), moderate (++) or severe (+++) with respect to parasitemia. Infection in the mice was allowed to continue until all the infected mice died of the infection. Mortality was recorded throughout the experiment. Post mortem examinations were carried out for any observable changes in the appearance of the brain, liver and spleen of both control and infected mice.

Preparation of tissue homogenate:

Tissue homogenates were prepared in phosphate buffer saline (PBS) using a Potter Elvehjem Grinding chambers and were cold centrifuged at 8000g for 10 minutes. The tissues homogenates were stored in -80°C until further use.

Assay for Lipid Peroxidation (TBARS Assay)

Lipid peroxides were estimated by measurement of Thiobarbituric acid reactive substances (TBARS) in tissue by the method of Brown and Kelly [16]. Briefly, 50 µL of tissue homogenate was diluted with 950 µL of TRIS- Base buffer (0.1M, pH-7.4) and added in amber glass vials containing 250 µL of 1.22 M ortho-phosphoric acid, 450 µL distilled water & 250 µL of TBA reagent (0.67% w/v). The mixture was incubated at 95°C in a water-bath for 60 minutes. The samples were then cooled on ice followed by addition of 360 µL methanol and 40 µL of 1M NaOH to neutralize the sample. The pink chromogen produced by the reaction of Thiobarbituric acid with TBARS, a secondary product of lipid peroxidation was measured on a Multimode Reader at 532nm. Results were

estimated as nanomoles of MDA per milligram of protein.

RNA Isolation:

Harvested tissue samples were homogenized and messenger RNA (mRNA) from both infected and uninfected mice were isolated using NucleoSpin® RNA II (Macherey-Nagel) kit according to the manufacturer's instruction. The extraction procedure was carried out at different time points after symptoms of severe malaria, seen by *Plasmodium berghei ANKA* parasite. Extracted mRNA was stored at -80º C until further processing. Three different aliquots of each mRNA samples were made to avoid repeated freeze and thaw conditions.

Gene expression analysis using Real-Time SYBR Green PCR:

Each aliquot of total RNA extracted was used for detection of cytokines namely TNF-α, IL-1β and IL-6 using TaKaRa One Step SYBR® Ex Taq™ qRT-PCR kit as per manufacturer's instructions. Glyceraldehyde-3 Phosphate Dehydrogenase (GAPDH) was used as the housekeeping gene. Primers were used at a concentration of 0.8µM for each of the target gene as well as housekeeping gene. (Table 1) Briefly, qRT-PCR involved initial RT step at 42º C for 5 minutes, hot start at 94 º C for 10 seconds followed by 40 cycles of denaturation 94 º C for 30-60 seconds, annealing for 30-45 seconds of respective gene and extension at 72º C for 60 seconds depending upon the target gene. The melt curve analysis initiated at 95º C for 6- seconds followed by increased in temperature from 60º C to 95º C at R-0.2º C. Amplification, data acquisition and expression analysis were carried out by using ABI StepOne™ instrument (Applied Biosystem, Foster City, CA). Fold change was calculated using ΔΔCt determination using mean ΔCt value method [17,18].

Table-1: Primers sequence of target genes and housekeeping gene with annealing temperature

Gene	Direction (5'-3')	Sequence	Annealing temperature used (°C)	Amplified product length
TNF-α	Forward Reverse	TTGACCTCAGCGCTGAGTTG CCTGTAGCCCACGTCGTAGC	50	374
IL-6	Forward Reverse	GTACTCCAGAAGACCAGAGG TGCTGGTGACAACCACGGCC	55	308
IL-1β	Forward Reverse	CAGGATGAGGACATGAGCACC CTCTGCAGACTCAAACTGCAC	60	447
GAPDH	Forward Reverse	GGAGAAGCTGCCAATGGATA GTGGTCTTCACGTTCGCATT	54	218

STATISTICAL ANALYSIS:

All the presented data were statistically analyzed using GRAPHPAD PRISM™ 5 software. (GraphPad software, Inc. La Jolla, CA). The results obtained in this work were performed in triplicate by identical methods. Results were expressed as Mean ± Standard Deviation (SD) for each group of mice. Group differences were assessed using ANOVA-Bonferroni multiple comparison tests. In all cases $P < 0.05$ was considered as significant (as indicated).

RESULTS AND DISCUSSION:

Complications of severe malaria are the major determinant of maternal and children morbidity and mortality in all over the malaria endemic countries. A number of diverse etiological pathways and mediators have been implicated in its pathogenesis. The role of oxidative stress during malaria infection is still unclear. Some authors suggest a protective role, whereas others claim a relation to the physiopathology of the disease [19]. However, recent studies suggest that the generation of reactive oxygen and nitrogen species (ROS and RNS) associated with oxidative stress, plays a crucial role in the development of systemic complications caused by malaria [3,19]. Furthermore, oxidative stress markers in infected humans are found in high levels compared to uninfected controls [8, 10, 20 and 21]. In such cases oxidative stress seems to result from increased production of free radicals, a fact suggested by increased MDA, an important lipid peroxidation marker, and not from a decrease in levels of antioxidants, reinforcing the suggestion that oxidative stress is an important mechanism in parasite infection [3, 21]. The present study was planned to assess the role of MDA, IL-1β, IL-6 and TNF-α as prognostic determinants.

Survival rate and parasitemia levels in *Swiss albino* mice:

Parasitemia levels were taken as the percentage of parasitized red blood cells measured in the blood smear. None of the control or uninfected mice showed any sign and symptoms as were seen in infected mice. In the malarial mice, an increase in parasitemia with increasing days after inoculation were observed with peak parasitemia occurred on day 13 (Figure1). This model of malaria infection is lethal and death would follow the peak parasitemia. 40% mortality was recorded on day 7 post infection, followed by 90% mortality on day 13 were observed at peak parasitemia.

The parasitemia rose rapidly reaching upto 83.14±2.16 (mean ± standard deviation) at day 13 with only 5.55±9.61 of the mice surviving and dying at day 13 post infection due to overwhelming hyperparasitemia. A red blood cell infected with multiple parasites was counted as one parasitized red cell.

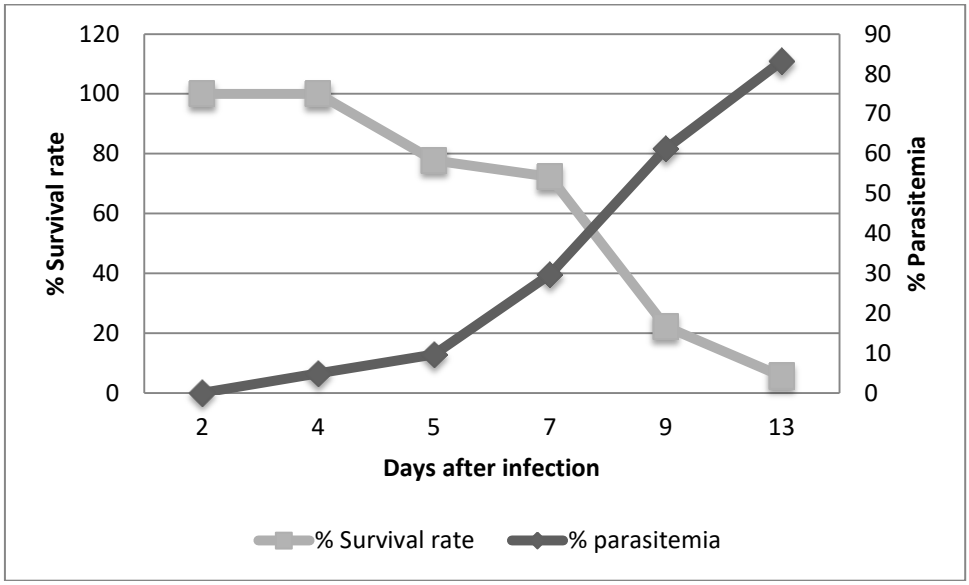

Fig 1: Measured parasitemia in malaria infected mice after days post infection: Comparison of parasitemia levels (% Mean±S.D.) and survival rate (% Mean±S.D.) for *Swiss albino* mice during progression of severe malaria (n=6). (In both cases P<0.0001)

As per previous report, the deformability of erythrocytes is also affected by oxidative stress. Excessive ROS in erythrocytes causes damage to the cytoplasmic membrane and associated cytoskeleton in the mature red cell, effects that manifest as decreased deformability of RBCs and splenic sequestration [3,22,23]. In our study, total count on the normal red blood cells (RBCs) also showed a tremendous decrease in the number with more than 75% reduction in the total number of parasitized RBC (pRBCs) during severe malaria infection (Figure 2).

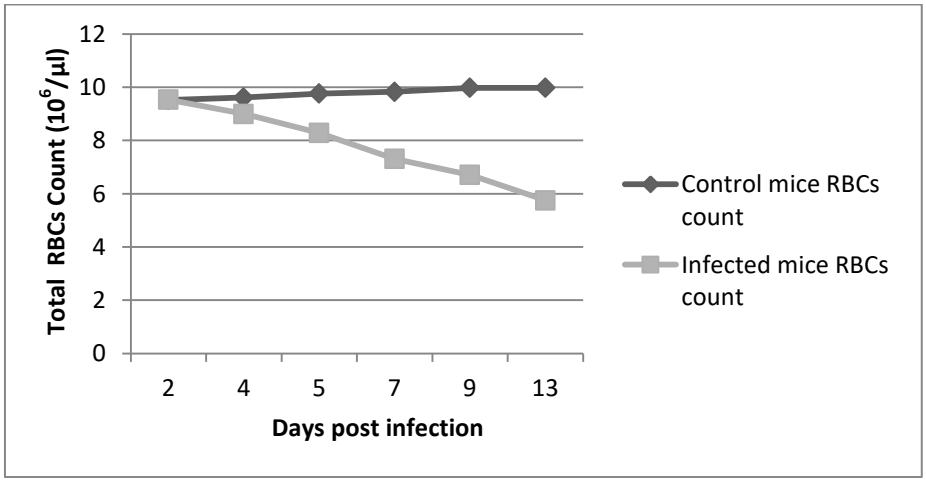

Fig 2: Total RBCs counted in the control and malaria infected mice: Results are expressed in Mean±S.D. (P<0.0001).

Symptoms of malaria occurred as early as on day 4, following inoculation with the *P. berghei ANKA* parasite in *Swiss albino* mice. Hyperparasitemia with ultimate death suggests the severe degree of infection in this model. In case of human malaria infected with *P. falciparum*, death is also the ultimate complication of severe cases. Reduction in the total number of normal RBC in this model showed a characteristic sign of severe anemia which is one the major clinical manifestation of severe malaria in human. The pathogenenesis of severe anemia during malaria infection is complex and involves multiple processes relating to both the destruction and decreased production of erythrocytes [22]. During *P. falciparum* infection, low levels of reticulocytes indicating the suppression of erythropoietin synthesis were also recorded [22,24]. Severe anemia has been identified as one of the main mechanisms of severe morbidity and

mortality in *P. falciparum* infection since it can lead to profound hypoxia and congestive cardiac failure [22,24-26]. Our study supports the previous findings that massive destruction of RBCs is associated with severe malaria cases with complications of anemia (Figure 2).

Visual observation and basic parameters of severe malaria:

Symptoms of malaria including ruffled fur, shivering, retinal whitening, change in gait, passage of dark urine were noticed in all mice infected with *P. berghei ANKA* parasite (Table 2). Physical signs of illness observed in this model were Piloerection, which may be related to the hypo- thermic state during the infection as the homeostatic mechanism is adapting to cerebral malaria. The dark urine passage in this model may be related with the black water fever normally occur in falciparum malaria [27-29]. Our study supports the complication of black water fever during severe malaria, but we can conclude in details with these visual and basic findings.

Table-2: Comparison of visual observations between control and malaria infected mice

Behavior changes	Days post infection	Control mice	*P. berghei* infected mice
Piloerection, Ruffled fur and shivering	5	-	-
	7	-	++
	9	-	++
	13	-	+++
Retinal whitening	5	-	+
	7	-	+
	9	-	++
	13	-	+++
Change in gait	5	-	-
	7	-	+
	9	-	++
	13	-	+++
Dark urine passage	5	-	-
	7	-	-
	9	-	+
	13	-	+++
(-) absent; (+) mild; (++) moderate; (+++) severe			

In our study, to monitor the effects of infection on factors such as food and water intake, gut function and metabolism, we measured the body weight of infected mice. The decrease in body weight of malarial mice was clearly evident from the fifth day of infection and presumably due in part to the decrease in food intake. Decrease in body weight may also be the consequences of disturbed metabolic function and hypoglycemia that has been reported to be associated with severe malaria infection [30]. In our study, the overall mean values of body weight in the malaria infected mice were significantly decreased as compared to the control uninfected group (Figure 3).

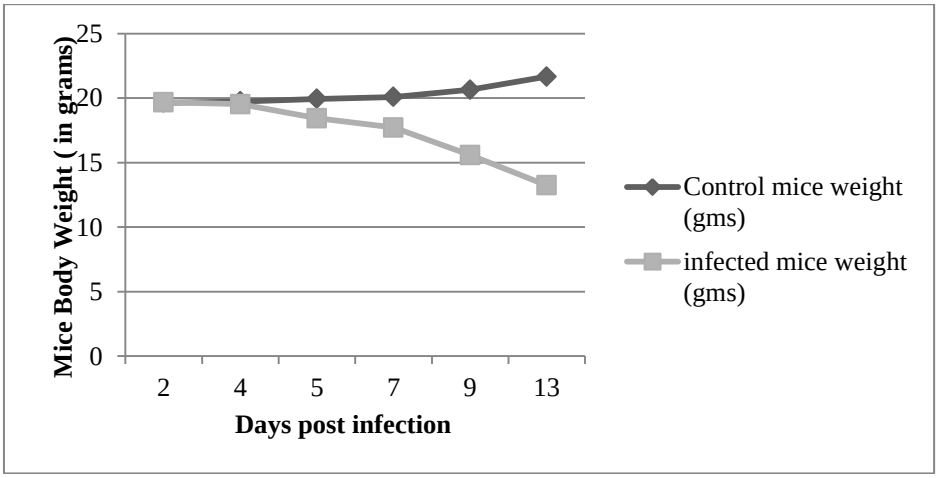

Fig 3: Body weight of control and malaria infected mice: Results are expressed in Mean±S.D. (P<0.0001)

During severe malaria infection, changes in the physical appearances of vital organs in the body are common. Splenomegaly and hepatomegaly are among the common phenomena [27-29]. Post mortem

examinations on the internal organs revealed the darkening of the liver and spleen of the malaria-infected mice. Malarial mice also showed enlarged spleen and liver indicating splenomegaly and hepatomegaly respectively. Both organs are congested and swollen from the accumulation of the malarial pigment, haemozoin, which led to discoloration. The weights of all the liver and spleen were recorded gradually increased towards the late stages of the infection as compared to the controls (Table 3). In case of brain, paleness of the brain was also observed in the infected group as compared to the control indicating complications of severe anemia may lead to cerebral malaria. The weights of infected brain samples were increased as compared to control samples may be because of the swelling in the brain tissues. However, histo-pathology of these vital organs including kidney and lungs need to be study for concluding further findings.

Table-3: Comparison of weight of organs between control and malaria infected mice

Name of the mice organ	Control (Mean ±S.D. weight in mg; P<0.0001)	Malaria infected mice (Mean ±S.D. weight in mg; P<0.0001)			
		Day 5	Day 7	Day 9	Day 13
Brain	273.7±1.69	276.1± 0.89	279.5±1.01	287.8±1.90	295.8±0.56
Liver	834.1±4.42	894±3.98	945.5±4.51	1112±8.92	1231±4.51
Spleen	91.63± 1.80	96.82± 1.51	106.8± 4.19	127.3± 2.41	152.6± 5.31

Lipid peroxidation end product- TBARS estimation:

As per previous reports, Reactive Oxygen Species (ROS) and antioxidants remain in balance in normal physiology and in case any disruption towards an overabundance of ROS results in oxidative stress leads to damage of tissues, organs and cells [31]. Furthermore, during malarial infection, both the host and parasite are under oxidative stress due to increased amount of ROS, produced by activated monocytes and neutrophils and degradation of hemoglobin by the parasite [32]. ROS play a dual role in malaria, besides their protective effect against the malaria parasite; ROS produced in post capillary venules in various organs may contribute to pathology in malarial infection [33]. In our study, to observe if malaria infection can induce oxidative stress, the levels of MDA were measured as an indicator of the redox status. We have observed enhanced levels of MDA in the liver, spleen and brain of infected mice may be due to the accumulative effect of severity of the malaria parasite antigen.

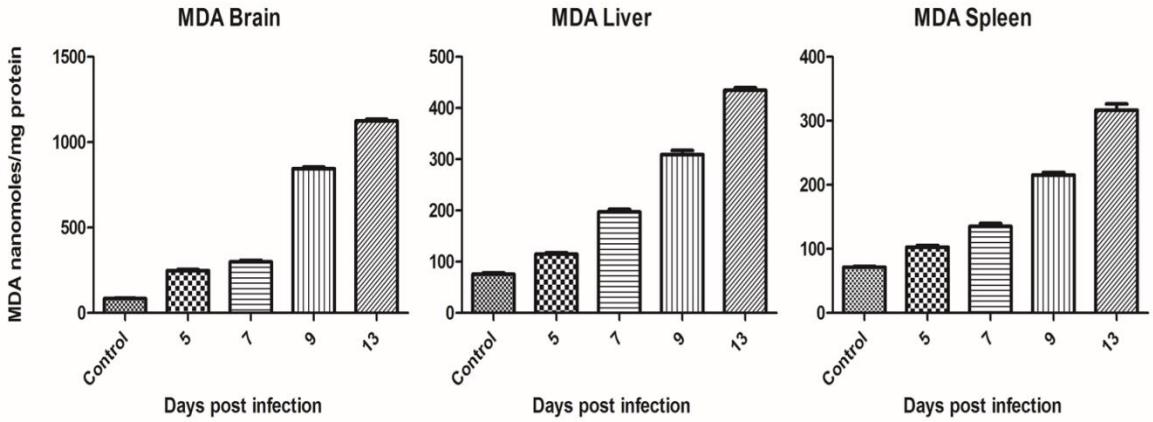

Fig 4: Levels of MDA: *Swiss albino* mice were injected i.p. with pRBCs and Brain, Liver and Spleen were harvested at the indicated time point. Tissue lipid peroxides were estimated by TBARS assay. Levels of MDA were measured in nanomoles/mg protein and values were expressed by Bonferroni multiple comparison tests. (P<0.05 significant)

The levels of MDA were measured as extent of lipid peroxidation and found to be significantly higher ($P < 0.05$) in the brain, liver and spleen of infected mice compared with the non-infected control group respectively (Figure 4, Table 4). These results indicate that *P. berghei ANKA* infection can induce oxidative stress and the response is higher in severe cases.

Table 4: ANOVA- Bonferroni multiple comparison tests' results for levels of MDA (nanomoles/mg protein) in brain, liver and spleen

Name of the mice organ	Control (Mean ±S.D.) (P<0.05)	Malaria infected mice (Mean ±S.D.) (P<0.05)			
		Day 5	Day 7	Day 9	Day 13
Brain	84.09±2.88	248.3±5.51	299.8±7.91	846±8.65	1124±10.32
Liver	75.47±3.09	114.7±2.99	197.2±4.93	309.4±8.02	435±4.95
Spleen	71.43±1.24	102.9±2.54	135.3±4.48	215.4±4.20	316.8±9.72

Pro-inflammatory cytokine levels:

Further we studied Inflammatory cytokines namely TNF-α, IL-6 and IL-1β that have long been implicated in severe malaria. The result of qRT-PCR analysis revealed that mRNA expression of TNF-α was significantly up regulated (P<0.0001) up to day 9 in brain, spleen and liver of infected mice as compared to control mice (Figure 5).

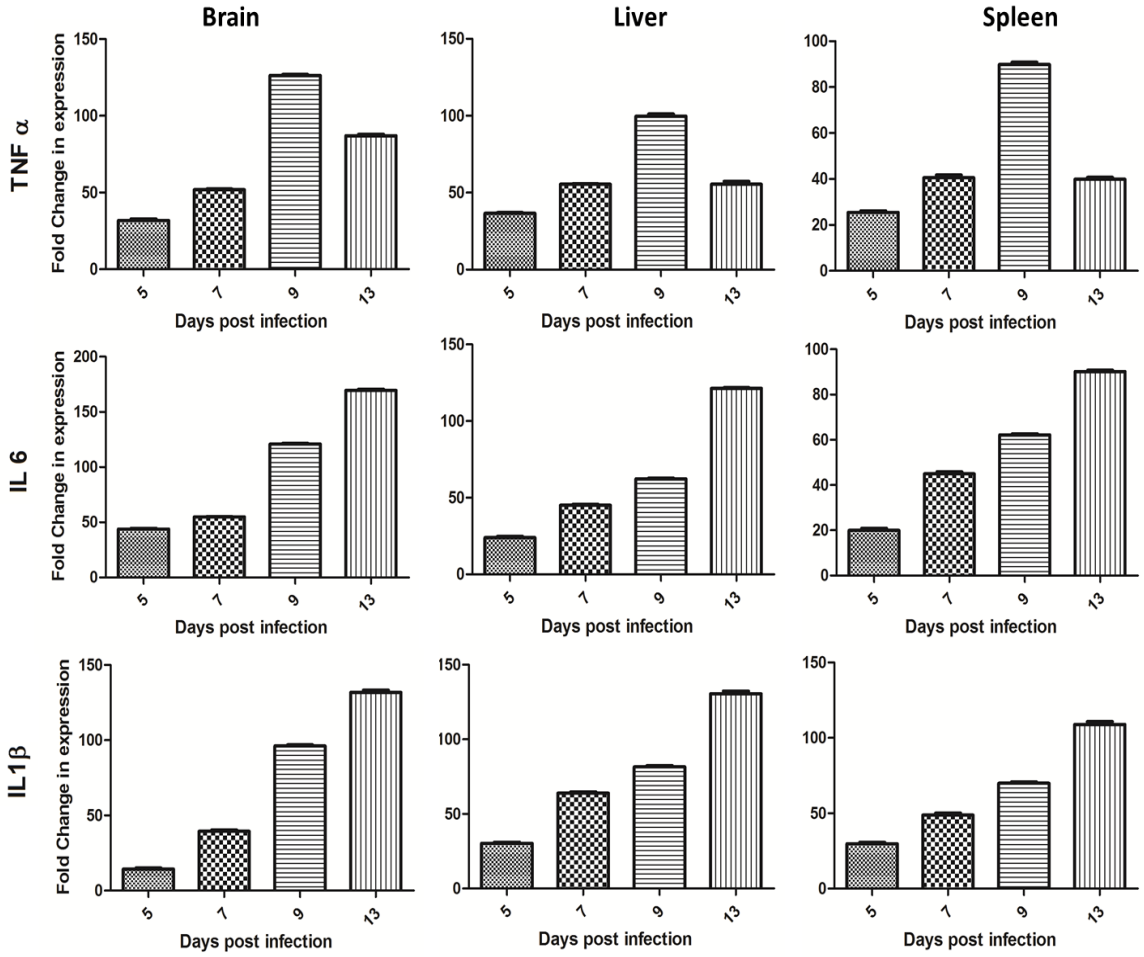

Fig-5: Pro-inflammatory cytokines mRNA expression: TNF-α, IL-6 and IL-1β cytokines' mRNA expression were measured in brain, liver and spleen at the indicated time point in malaria infected mice with respect to control. Group difference were assessed by Bonferroni multiple comparison tests. (P<0.0001 significant)

TNF-α for the test group from day 5 post infection was significantly different as compared to that of day wise parasitemia level (Table 5). The levels remained significantly higher (P<0.0001) up to day 9 in all tested organs. At day 13 post infection, the levels of the experimental group declined significantly (87.121±0.77 in brain, 55.41±1.92 in liver and 39.84±0.90 in spleen) as compared to the day 9. (126.3± 0.63, 99.71± 1.34 and 89.88± 0.89 respectively). However, highly up regulation of TNF-α between the $5^{th}$and 9^{th} day with peak production being detected on the 9^{th} day, and subsequently decreased between the 11^{th} and 13^{th} day (Figure 5).

Previous findings from the group of researchers shown the role of TNF- α in parasite clearance in early phase of infection [34]. At physiological concentrations, recombinant TNF- α is antiparasitic, synergizing with IFN-γ to induce production of NO and other toxic radicals for the early

protection from *P.falciparum* malaria [35]. In contrast, TNF- α may mediate the two major causes of mortality associated with malaria anemia and cerebral malaria [34,35]. However, repeated injections of recombinant TNF- α into malaria- infected mice have been shown to reduce parasitemia and protect against lethal infection [35]. Our study on mice with TNF- α supports the previous results assuming that the protective as well as pathogenic role of this cytokine during malaria depends on the quantity of TNF- α produced and the time period over which the production is sustained, the tissue where it is produced and the presence of other cytokines for the complications of severe malaria.

Table-5: ANOVA- Bonferroni multiple comparison tests' results for TNF-α, IL-1β and IL-6 fold change in expression in brain, liver and spleen

Name of the mice organ	Malaria infected mice (Mean ±S.D. fold change in expression- with respect to control) (P<0.0001)				
	Cytokine	Day 5	Day 7	Day 9	Day 13
Brain	TNF-α	31.80± 1.03	51.80± 0.47	126.3± 0.63	87.12± 0.77
	IL-1β	14.32± 0.69	39.40± 0.93	96.22± 0.71	131.8± 1.48
	IL-6	43.95± 0.58	54.93± 0.38	120.9± 0.62	169.5±1.06
Liver	TNF-α	36.59± 0.53	55.46± 0.41	99.71± 1.34	55.41± 1.92
	IL-1β	30.33± 0.74	63.93± 0.89	81.55± 0.85	130.5± 1.77
	IL-6	23.94±0.89	45.02± 0.63	62.27± 0.49	121.4± 0.44
Spleen	TNF-α	25.44± 0.67	40.57± 1.16	89.88± 0.89	39.84± 0.90
	IL-1β	29.76± 0.89	48.77± 1.31	70.00± 0.83	109± 1.82
	IL-6	19.98±0.92	44.98±0.92	62.18±0.52	90.17±0.63

TNF- α does not act alone in the induction of severe malaria which leads to cerebral malaria. There is increasing evidence that it acts in concert with other pro inflammatory cytokines such as IL-1β and IL-6. In the brain, spleen and liver IL-1β mRNA increased and persisted throughout the course of infection, (Figure 5). In the brain and liver, IL-1β was highly up regulated from day 9 to day 13. No significant induction of message for IL-1β was detected in the brain, liver or spleen of these animals (Table 5). Since encephalitis is defined as "inflammation of the brain" [4,36], these results demonstrate that murine CM is an encephalitis characterized by sequestration of parasites and production of message for pro inflammatory cytokines in the brain. Mice die of the encephalitis while parasitemia is at levels that produce no detectable symptoms in CM-resistant mice [37,38].

IL-6 is a cytokine with pleiotropic effects that can be produced by a variety of cell types including monocytes, T cells, and endothelial cells [37], which are important in the protection and patho-physiology of severe malaria.. Also, it has been shown that the synthesis and release of IL-6 can be induced by TNF-α [34,35,37]. Therefore, we have analyzed the possible contribution of IL-6 to the pathogenesis of severe malaria in our model. In this study, mRNA level of IL-6 was highly up-regulated as per the progression of the severity of malaria after 9 days (Table 5). It was highest in the brain samples (169.5±1.06) at 13^{th} day suggesting possible role of the IL-6 in cerebral malaria. As per the previous work in human malaria, IL-6 appeared to correlate with disease severity since elevated levels were noted in the severe-malaria patients compared to the matched uncomplicated malaria cases and, similarly, in uncomplicated malaria cases compared to healthy control [39]. Our data supports the role of IL-6 in severity of the disease.

Another striking finding in our study is the increasing correlation between the level of MDA and pro-inflammatory cytokines. The free radical release during malaria infection is reported to be up-regulate the production of pro-inflammatory cytokines [7]. We have observed a significant increase in oxidative markers of protein and lipid injury during the early phase of malaria infection. MDA levels were found to be significantly increased in all the test groups compared to the uninfected control group. Similar to the expression pattern of IL-1β and IL-6, the level of MDA appeared to be higher in the severe disease groups compared to the control mice. Thus, a significant positive association was observed between these biomarkers of lipid oxidation tissue injury and the IL-1β, IL-6 level in severe disease. In the study of Medana *et al.;* significantly elevated levels of TNF-α were observed in malaria infected mice, whereas reduced TNF-α level were obtained in the presence of IFNγ [10]. Another study conducted by Percario *et al.;* showed, during the erythrocyte stage of malaria, RBCs lysis and release of haemozoin occurs, which consist primarily of monomers and methemoglobin in plasmodium proteins. Haemozoin is able to induce cytokine release through cells of the monocytes and macrophage system [3]. This hemolysis or extensive cell damage can lead to increased concentration of free heme causing oxidative stress and inflammation. This shows that the presence of TNF-α could enhance the release of other pro-inflammatory cytokines by inhibiting the parasite-induced oxidative stress response and associated oxidative injury. The results of the present study support this concept by showing elevated levels of MDA and the pro-inflammatory cytokines

upon *P.berghei ANKA* infection. Thus a significant positive association between the marker of lipid oxidation injury and TNF-α level in cases with severe disease has been observed in the current study. The ratio of TNF-α to IL-1β and IL-6 was found to be positively correlated with MDA in all the infected mice which leads to the understanding that pro-inflammatory cytokines are associated with oxidative stress responses in malaria infection. However further studies are needed to determine the exact mechanism of the interactions between the inflammatory response and the oxidative stress markers.

CONCLUSION:

In conclusion, the present study suggests a possible interplay of pro-inflammatory cytokines and oxidative stress in the immunopathogenesis of severe malaria disease. We propose that plasmodium parasite may induce an oxidative stress response, which in turn activates the release of pro-inflammatory cytokines, leading to severe malaria in mice. Since supplemental antioxidants could inhibit oxidative stress and reduce the pro-inflammatory cytokine release, we suggest that antioxidants may be useful as adjuvant therapy for malaria. We further conclude that the ratio of TNF-α to IL-1β and IL-6 around defervescence may serve as an indicator to predict disease severity in malaria individuals. A better insight into the pathophysiology of severe malaria will help in early detection and better management of this disorder.

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