EFFECT OF PROTEIN DIET SUPPLEMENTATION IN HUMORAL, CELL MEDIATED IMMUNE RESPONSE AND ANTIOXIDANT STATUS IN *PLASMODIUM YOELII* INFECTED BLOOD OF SWISS MICE (*MUS MUSCULUS*)

Soumitra Banerjee1*, Nirmal Kumar Pradhan2, Rudra Prasanna Banerjee3 and Parantap Sarkar4

1*Department of Biotechnology, Utkal University.
2Department of Physiology, University of Burdwan.
3Department of Molecular Biology & Genetics, Presidency University.
4Department of Zoology, University of Burdwan.

ABSTRACT

*Plasmodium yoelii* like many other parasitic protozoans is deficient of having the advantage of the de novo pathway for purine biosynthesis and starkly acts upon the salvage pathway. It is the causative agent of Malaria. A new approach can be designed by understanding the regulatory mechanism of immune response during the *Plasmodium* infection. *Plasmodium*-specific CD4+ αβ T cells and antibody are mostly important for the immune response during the infection. It has been reported that NK cell-derived IFN-γ that contributes to the early control of *Plasmodium chabaudi* and *Plasmodium yoelii* infections. Protein availability for the metabolic processes, the major reservoir in the lean body mass is represented by muscle. During this disease significant reductions in lean tissue or by extension, the muscle mass will attenuate the immunological response, as well as reduce physical activity. The provision of protein in the diet could meet some of the requirements of amino acids for the immune and restorative response. The influence of infection on protein nutritional status has been extensively reviewed in earlier stage of malaria.

KEYWORDS: CD4+ αβ T cells, *Plasmodium yoelii*, NK cell, Protein diet.
INTRODUCTION
Malaria is one of the most devastating diseases worldwide. About 200-300 millions of new cases and one million deaths cause every year.[21] The responsible microbe for this disease belongs to genus *Plasmodium*. Often uncontrolled parasitemia leads to weak immune response during this disease.[7] A new approach can be designed by understanding the regulation mechanism of immune response during the *Plasmodium* infection. *Plasmodium*-specific CD4\(^+\) \(\alpha\beta\) T cells and antibody are mostly important for the immune response during the infection.[4,16] It has been reported that NK cell-derived IFN-\(\gamma\) that contributes to the early control of *Plasmodium chabaudi* and *Plasmodium yoelii* infections.[3,4] APCs are particularly important to activate T CD4 cells which fight against the parasite by producing inflammatory cytokines which activate other cells such as macrophages and helping B cell activation to produce Abs.[21] A wide variety of host-parasite interactions have addressed malaria immunity since any single rodent model replicates all the features of human malaria.[5] Despite high genetic variability in human populations, most bioassays in mice have used combinations of *Plasmodium* species and inbred mouse strains, which explains the homogeneous outcomes obtained.

In patients the clinical and metabolic changes can be noted significantly. The metabolic pattern, which includes hypermetabolism, a negative nitrogen balance, increased gluconeogenesis and an increased fat oxidation is modulated by hormones, cytokines and other pro-inflammatory mediators,[22] and there is always a reduced food intake. The clinical features include fever and headache. Protein availability for the metabolic processes, the major reservoir in the lean body mass is represented by muscle.[2] Significant reductions in lean tissue or by extension, the muscle mass will attenuate the immunological response, as well as reduce physical activity. The provision of protein in the diet could meet some of the requirements of amino acids for the immune and restorative response. In this work the influence of infection on daily protein and amino acid requirements will be covered. The influence of infection on protein nutritional status has been extensively reviewed in earlier and late stages of malaria.

MATERIAL AND METHOD
1) Animals and parasites
Twenty one female mice, aged 7 weeks were purchased from local distributer and housed at random in airy racks containing and kept under constant standard conditions of light (12h/12h
hr. light-dark cycles), temperature (22–24°C) and humidity (around 50%). All mice were fed a commercial diet (2018 Teklad Global 18% Protein Rodent Diet). All mice were divided into three groups- i) Group-I – 7 mice for control, ii) Group-II – 7 mice for infection with *Plasmodium yoelii*, iii) Group-III – 7 mice for infection and simultaneously feed with high protein diet supplement.

2) Experimental infection
Mice were infected intraperitoneal (i.p.), using a 30 G one-half needle under an approximately 10–15° angle – in the lower quadrant of the abdomen off midline. Parasitemia was monitored sequentially in each mouse by performing Wright's eosin methylene blue solution-stained thin tail blood smears. RBCs were counted sequentially in each mouse using a hemocytometer.

**Preparation of blood sample**
In all the enzyme assays, the blood sample is initially washed with 1X phosphate buffer saline (PBS) pH-7.5 repeatedly and then subjected to cell lyses by freeze and thaw method. After cell lyses, the sample was centrifuged. The pellet was discarded and the supernatant was used as the enzyme source.\[^{15}\]

3) Maintenance of parasite
A rodent malarial parasite *P.yoelii*, which is maintained by serial blood passages, is used for the experimental studies. Swiss mice are generally inoculated with inocula of 1X10^6 – parasitized erythrocyte intraperitoneally. Blood containing the parasite was diluted in equal amount of citrate buffer and injected into the number of fresh mice.\[^{6}\]

4) Superoxide dismutase assay
Discovered by Irwin Fridovich and Joe McCord, Superoxide dismutase (SOD) are a class of enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. As such, they are an important antioxidant defense in nearly all cells exposed to oxygen. There are three major types of superoxide dismutase, depending on the metal cofactor: Cu/Zn (which binds both copper and zinc), Fe and Mn types (which bind either iron or manganese), and finally the Ni types which binds either nickel. In this assay, nitroblue terazolium (NBT) is converted into formazone in the presence of NADH and phenazine methosulphate (PMS). However the rate of formation of formazone is inhibited in the presence of SOD. The formazone has maximum absorbance at 560nm and therefore its formation can be followed
using a spectrophotometer. The sample was stirred for 10 min on a magnetic stirrer in cold with chloroform (15% v/v) and ethanol (25% v/v). It was then centrifuged at 10000 rpm for 0 min at 4°C. The supernatant is dialyzed against 1 liter 10 mM Tris-Cl (pH-8.1) in cold for minimum 3 hrs. This dialyzed supernatant was used for SOD enzyme assay.\[14\]

Table1: Reaction mixture for Super oxide dismutase assay was set as follows:

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M sodium pyrophosphate buffer (pH-8.3)</td>
<td>0.10 ml</td>
<td>0.10 ml</td>
</tr>
<tr>
<td>TDW</td>
<td>2.54 ml</td>
<td>2.44 ml</td>
</tr>
<tr>
<td>0.3 mM NBT</td>
<td>0.16 ml</td>
<td>0.16 ml</td>
</tr>
<tr>
<td>0.47 mM NADH</td>
<td>0.10 ml</td>
<td>0.10 ml</td>
</tr>
<tr>
<td>Enzyme</td>
<td>----</td>
<td>0.10 ml</td>
</tr>
<tr>
<td>0.93 µM PMS</td>
<td>0.10 ml</td>
<td>0.10 ml</td>
</tr>
</tbody>
</table>

5) Catalase Assay

Catalase is a common enzyme found in nearly all living organisms which are exposed to oxygen, where it functions to catalyze the decomposition of hydrogen peroxide to water and oxygen. The sample was prepared in the similar manner as explained above. The reaction volume was 3 ml. The OD was measured at 240nm for 2 minutes at 30 seconds interval. The blank was set with buffer.\[12\]

Table2: Reaction mixture for Catalase assay was set as follows:

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 M Sodium phosphate buffer (pH-7.0)</td>
<td>0.65 ml</td>
<td>0.65 ml</td>
</tr>
<tr>
<td>TDW</td>
<td>2.00 ml</td>
<td>1.95 ml</td>
</tr>
<tr>
<td>H₂O₂ (10mM)</td>
<td>0.35 ml</td>
<td>0.35 ml</td>
</tr>
<tr>
<td>Enzyme</td>
<td>-</td>
<td>0.05 ml</td>
</tr>
</tbody>
</table>

6) Assay for reducing power

Different concentration of the sample were mixed with potassium phosphate buffer (2.5 ml,0.2M, pH 6.6) and potassium ferriccyanide [k₃Fe(CN)₆] (2.5 ml 1%). The mixture was incubated at 50°C for 20 min. Aliquots (2.5 ml) of trichloroacetic acid (10%) were added to the mixture. The upper layer of solution (2.5ml) was mixed with distilled water (2.5ml) and FeCl₃ (0.5 ml, 0.1%) and absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicates an increase of reduction capability.\[11\]
7) Glutathione reductase assay

Glutathione reductase (GR) is an enzyme which reduces glutathione disulfide (GSSG) to the sulphydryl from GSH, which is an important cellular antioxidant. For every mole of oxidized glutathione (GSSG) one mole of NADPH is required to reduce GSSG to GSH. NADPH shows maximum absorbance at 340 nm; therefore its disappearance can be followed using a spectrophotometer. The sample was prepared in the similar manner as explained above. The reaction volume was 3 ml. The OD of the reaction was measured at 340 nm for 2 minutes at 30 seconds interval.\[^{17}\]

Table 3: Reaction mixture for Glutathione reductase assay was set as follows:

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M Sodium phosphate butter (pH-7.4)</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>TDW</td>
<td>1.35</td>
<td>1.25</td>
</tr>
<tr>
<td>4 mM GSSG</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Enzyme</td>
<td>-</td>
<td>0.1</td>
</tr>
<tr>
<td>2.4 nM NADPH</td>
<td>0.05</td>
<td>0.05</td>
</tr>
</tbody>
</table>

8) ELISA assay

Total IgM, IgG, IgE were quantified by Ab isotype-specific ELISA. Total IgM, IgG, IgE were quantified using anti-mouse IgM, IgG, IgE as the capture Ab. Coating Ags were incubated for 2 h at RT and subsequently overnight at 4°C. From this step onwards, the manufacturer's protocol was followed (abcam). Briefly, plates were blocked with 1% BSA in Tris-buffered saline solution and duplicate diluted serum samples were added for 1 h at RT (1×5000 for IgM, 1×50000 for IgG, 1×5000 for IgE). Total IgM, IgG, IgE Abs were detected with HRP-labeled goat anti-mouse IgM or IgG or IgE at a 1×75000 dilution. The enzyme reaction was developed using 3,3′,5,5′tetramethyl benzidine (TBM) as the enzyme substrate. Samples were read at 450 nm in a Varian Cary 50 Bio spectrophotometer. Sera from uninfected mice were used as negative controls. Purified myeloma-derived mouse IgG, IgM, IgE were used to generate a logistic four-parameter sigmoidal standard. The lower limit of positivity (cut-off) was determined by the mean of negative healthy controls + 2 SD.

9) Cytokine antibody assay

To determine cytokines in the mouse sera, the Mouse Cytokine Ab Assay II kit was used according to the manufacturer's protocol. Pooled serum samples from each group of mice (7 days infected) were applied to the membranes and, after incubation with the detection Ab, membranes were developed with streptavidin-HRP followed by a chemiluminescence reagent.
Membranes were then exposed to X-ray film. Pixel densities were calculated for each spot of the array using Quantity One software (Bio-Rad Laboratories) and mean values for duplicate spots were compared. Arbitrary cut-off expression values higher than 1.5-fold were set to consider changes between groups or differences within a group.

RESULT

Antioxydant status

The Activity of superoxide dismutase in normal blood is 10645.16 µg/ml/min whereas the value is 21290.25 µg/ml/min/mg proteins for the same in infected blood (7 Days) and 10987.45 µg/ml/min/mg proteins (7 Days) in protein diet supplemented blood. The specific activity of superoxide dismutase increased almost double i.e., 1085.96 µg/ml/min/mg protein in infected blood with respect to normal blood i.e., 517.81 µg/ml/min/mg protein and most importantly in protein supplemented blood i.e., 622.76 µg/ml/min/mg. Catalase activity is 7360.40 µmole H₂O₂ dissipated/min in normal blood but the activity is 7741.11 µmole H₂O₂ dissipated/min for the infected blood and 7410.87 dissipated/min/mg in protein supplemented blood. Here the specific activity increases in some fractional value in case of infected blood i.e., 394.85 µmole H₂O₂ dissipated/min/mg protein. The initial value for the same in case of normal blood was 358.03 µmole H₂O₂ dissipated/min/mg protein. In protein supplemented blood 392.90 µmole H₂O₂ dissipated/min/mg. The reducing powder has greater reductive capability which is 4.89 and the normal blood has the 0.3494 as the reductive capability value. The activity of Glutathione reductase is 0.2015 in normal blood and it has a little small value in infected blood i.e., 0.3137. The specific activity has inverse result as it has higher value in infected blood i.e., 0.016 and a smaller value in normal blood i.e., 0.0098. In protein supplemented blood the activity is 0.0587 and specific activity is 0.0106.

Humoral immune response
Serum IgE starts increasing from 3rd day of infection and ultimately attends peak on 30th day of infection. The protein diet supplemented infected mice attends a good height at 15th day and continues up to 30th day [Fig1]. Serumic IgG in infected mice attends pick on 30th [Fig2]. Malaria infected blood of mice serum IgM starts inclination from 3rd day. Protein diet supplemented infected mice shows highest pick in 30th day of infection [fig3]. IgE and IgG shows a gradual increment after 3rd day [Fig1, Fi2]. IgM maintains more or less same concentration up to 30th days of infection [Fig3]. Humoral immune response also triggered by the pathogen. In 7th day of infection IL2 significantly rises with respect to the TNFα and IFx.

**Cell mediated immune response**

Serum cytokine profiles during the 1st wk of infection in control, infected and protein diet supplemented infected mice were compared by protein microarrays. In infected mice IL2 has a little higher value than IFx and TNFα. In normal mice the values of 3 cytokines are almost
half in this case IFɤ has highest value. Cytokine increases in protein diet supplemented mice. IFɤ shows significant increment.

DISCUSSION
During malarial infection the activity of superoxide dismutase increases probably due to the over expression of the enzyme. Hence, there are increased oxidative stress conditions due to the production of hydrogen peroxide during the infection. This in turn increases the activity of lipid peroxidase. In case of malarial infection, the release of Fe from hemoglobin takes place. However, the activity of catalase remains unchanged during the infection. This further increases the oxidative stress due to the accumulation of hydrogen peroxide. It has a critical role in higher oxidative tension during malarial infection. Increased activity of glutathione reductase implies that it may be involved in decreasing the oxidative stress by reducing glutathione (using NADPH) which is used by glutathione peroxidase to detoxify hydrogen peroxide. However, this increase in glutathione reductase activity during infection is not very significant to relieve the oxidative stress completely. During pregnancy, malarial parasite *P. falciparum* also infects human placenta causes placental malaria. Trophozoite infected subject has a risk in raising the level of H₂O₂ which ultimately increases the oxidative stress. Glutathione reductase can play a protective role in oxidative stress. Glutathione reductase has got the ability to play significant role in other diseases. This enzyme can successfully be able to decrease the ROS (Reactive Oxygen Species) in stable transfection. In acute respiratory distress syndrome (ARDS), oxidative stress phenomenon is well evinced. Antioxidants exhibit potential positive result. Evidence is sufficiently compelling to suggest that antioxidants have got the potentiality for therapeutic purposes at least in some cases. On the other hand the protein diet supplementation reduces the oxidative stress. So protein diet supplement can be used along with medicine for treatment of *Plasmodium* infection. The requirement of protein and amino acid is reported from similar study. Protein diet supplementation creates a good result in humoral immune response as well as cell mediated immune response. IgG and IgM rises significantly after giving protein supplement and in cytokine IFNɤ level raises at decent level. Injuries or infections lead to an increased nitrogen loss from the body. The specific response to bacterial, viral or protozoan infections, in terms of the N₂ balance has been reviewed, and the catabolic response of adults to infections with different organisms like bacteria, rickettsia and virus was prospectively evaluated by metabolic balance studies, during exposure as well as during overt infection. The infections were treated by antibiotics in the more severe cases. However, given
the anorexia that exists during the acute phase of the infection it is unlikely that efforts to increase protein intake would be successful. The striking feature of these data is the dependence of the negative nitrogen balance on the duration of fever, and negative nitrogen balance was not observed until the febrile response began, although the catabolic response can be observed in the absence of fever or other clinical symptoms as well.\[3\] Wilmore\[22\] has summarized the pattern of the catabolic response to infection with the following observations: 

(i) The increased nitrogen loss occurs via the urine, mainly as urea, although it is possible that with fever and sweating (significant losses could occur through sweat,\[19\]) (ii) there is a dose response in that the greater the infection (in terms of the degree and duration of the fever), the more extensive the nitrogen loss; (iii) more nitrogen is lost from a well-nourished individual than a depleted patient following a comparable insult; and (iv) the response is not constant, and follows a time course, increasing to a peak and then gradually returning to equilibrium.

CONCLUSION

Protein dietary supplementation could be an alternative way in designing the treatment of Malarial prophylaxis in near future.

REFERENCE


6. David P. Fan And Mary M. Beckman (1972); New Centrifugation Technique for Isolating Enzymes from Large Cell Structures: Isolation and Characterization of Two Bacillus subtilis Autolysins; Journal Of Bacteriology, 1258-1265 Copyright 1972 American Society for Microbiology.
12. Joe M. McCord and Irwin Fridovich (1969); Superoxide Dismutase An Enzymic Function For Erythrocuprein (Hemocuprein); Received June 23,. JBC 1969, by the American Society of Biological Chemists.
20. Sh tiyong ifoue1,2, c teugwa mofo1, i gouado3, g teto1,2, t asonganyi2 and p h amvam
zoollo1; evaluation of oxidative stress and antioxidant status of pregnant women suffering from malaria in cameroon, indian journal of clinical biochemistry, 2009; 288-293.

