Malaria, a reemerging disease caused by *Plasmodium* is one of the most important parasitic diseases. More than 500 million people suffer from this disease and at least 1 million, mostly children below the age of 5 years die every year. The situation is becoming even grimmer over the last few years with growing drug resistance to parasites and appearance of insecticide-resistant anopheline mosquitoes that serve as the vector for the disease.

Erythrocytic stages of *Plasmodium* are considered sensitive to oxidative stress [1], which is caused by the production of reactive oxygen species (ROS) from parasite’s metabolism [2], parasitized red blood cells, and host immune responses [3]. ROS, nitric oxide, superoxide, and peroxynitrite kill intraerythrocytic parasites by production of IFN-γ by Th1 cells, which activates macrophages to secrete parasiticidal nitric oxide and ROS [4]. Oxidative stress is combated by the parasite with antioxidant enzymes, like superoxide dismutase, glutathione reductase, glutathione peroxidases, and thioredoxin reductase.

*Plasmodium* possesses 2 functional redox systems involving the low molecular weight thiol glutathione and thioredoxin that are heavily involved in the redox regulation of the cell and contribute greatly to the antioxidant capacity of the cell [5]. Glutathione metabolism is an important metabolism in malaria parasites. Glutathione has been shown to be the main non-enzymatic antioxidant defense in *Plasmodium* [6] and helps in maintaining the reduced environment of the cytosol. Glutathione reductase (GR; E.C. 1.8.1.7) is an ubiquitous flavoenzyme of disulphide reductase family catalyzing the nicotinamide adenine di-nucleotide phosphate reduced (NADPH) dependent reduction of oxidized glutathione (GSSG) to reduced glutathione, which permits glutathione to function as an intracellular reducing agent. Thioredoxin system composed of small peptide thioredoxin (Trx), thioredoxin reductase (TrxR; E.C. 1.8.1.9), and NADPH as reducing cofactors is involved in maintenance of crucial redox state. TrxR transfers electrons from NADPH to Trx, which itself acts as a reductant for disulphide containing process, such as ribonucleotide reductase [7].

Antioxidant enzymes are essential for the survival of parasites for combating the intraerythrocytic oxidative stress. Disruption of these enzymes is a feasible way to interfere with the erythrocytic development of malaria parasites. The present study was undertaken to demonstrate the activities of GR and TrxR in *P. berghei*-infected host erythrocytes and their fractions. Activities of glutathione reductase and thioredoxin reductase in *P. berghei*-infected host erythrocytes were found to be higher than those in normal host cells. These enzymes were mainly confined to the cytosolic part of cell-free *P. berghei*. Full characterization and understanding of these enzymes may promise advances in chemotherapy of malaria.

**Key words:** *Plasmodium berghei*, glutathione reductase, thioredoxin reductase, antioxidant, antibiotics
an equal volume of PBS (0.01 M), pH 7.2, and loaded onto a CF-11 cellulose (Whatmann) column to remove the leukocytes [8]. The erythrocytes were centrifuged at 1,000 g for 10 min at 4 ℃. A small part of the pellet was separated and used as total erythrocytes and to the remaining part an equal volume of saponin (0.2% w/v) in 0.01 M PBS, pH 7.2, was added and incubated for 30 min at 4 ℃ with intermittent mixing. The suspension was centrifuged at 15,000 g for 30 min at 4 ℃ to obtain various fractions. In the case of normal erythrocyte preparations, hemolysate and erythrocyte membranes were obtained. These membranes were washed thrice with 0.01 M PBS, pH 7.2. In the case of P. berghei-infected erythrocyte preparations, 3 fractions, i.e., hemolysate, erythrocyte membranes, and cell free parasites, were obtained, aspirated separately, and then the last 2 fractions were washed thrice with 0.01 M PBS, pH 7.2 [9].

All the above collected fractions were suspended in an appropriate volume of 0.01 M PBS, pH 7.2, homogenized in Potter-Elvehjem homogenizer (Genscript, Piscataway, New Jersey, USA) at 4 ℃, centrifuged at 1,000 g for 10 min and the supernatant of each fraction was used as the enzymes in assay. Results tabulated are the mean data of 3 experiments each done in duplicate.

Cell-free P. berghei was homogenized at 4 ℃ in pre-chilled 0.25 M sucrose solution and subjected to differential centrifugation according to Banyal et al. [10] and different fractions were obtained.

GR activity was determined according to the modified method of Worthington and Rosemeyer [11] in a double beam spectrophotometer (Perkin-Elmer, Waltham, Massachusetts, USA) at room temperature. The 2.0 ml assay system contained 0.1 M potassium phosphate buffer, pH 7.2, 0.2 mM EDTA, 0.1 mM NADPH and appropriate volume of enzyme extract, and the reaction was initiated by the addition of 0.1 mM GSSG at room temperature. The oxidation of 1 µmol of NADPH resulting in decrease in absorbance was monitored at 340 nm.

TrxR activity was spectrophotometrically measured by the reduction of di-thionitrobenzene (DTNB) in the presence of NADPH [12]. A 1.0 ml of assay mixture contained 100 mM potassium phosphate buffer, pH 7.5, 2 mM EDTA, 3 mM DTNB, 0.2 mM NADPH, and an appropriate volume of enzyme extract. After initiation of reaction with NADPH, the increase in absorbance was monitored at 412 nm at room temperature. The protein content was determined according to Lowry et al. [13], and the specific activity was evaluated as units of enzyme per mg protein.

Cell-free P. berghei exhibited significant activities of GR and TrxR. Both of these enzymes reacted with the substrate resulting in change in the absorbance. The host erythrocytes from normal and parasitized hosts also contained these enzymes. The activities of GR and TrxR were higher in parasitized hemolysate compared to the normal hemolysate. The red cell membranes did not contain any enzyme activity (Table 1).

When the cell-free parasites were subjected to differential centrifugation, the maximum activity of each enzyme was found in organelle-free cytosolic fractions. There was 4.6 times higher GR and 5.8 times more TrxR activities in the cytosolic fractions compared to the parasite homogenate (Table 2). The other sediments obtained contained no or very less activity of these enzymes compared to the supernatant (cytosolic) fraction.

During malaria infection, ROS plays an important role in the

Table 1. Activity of glutathione reductase (GR) and thioredoxin reductase (TrxR) in normal and Plasmodium berghei-infected erythrocytes and their fractions

<table>
<thead>
<tr>
<th>Erythrocyte fraction</th>
<th>GR (U/mg)</th>
<th>P. berghei-infected (U/mg)</th>
<th>TnR (U/mg)</th>
<th>P. berghei-infected (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>0.181 ± 0.02</td>
<td>0.186 ± 0.02</td>
<td>0.660 ± 0.51</td>
<td>0.787 ± 0.61</td>
</tr>
<tr>
<td>Hemolysate</td>
<td>0.170 ± 0.02</td>
<td>0.342 ± 0.04</td>
<td>0.715 ± 0.55</td>
<td>0.969 ± 0.75</td>
</tr>
<tr>
<td>Erythrocyte membranes</td>
<td>N/A</td>
<td>Nil</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Cell-free parasite</td>
<td>-</td>
<td>0.617 ± 0.08</td>
<td>N/A</td>
<td>0.128 ± 0.10</td>
</tr>
</tbody>
</table>

Results are mean ± SD of 3 experiments.
pathology of the disease. Parasites are highly sensitive to such stress being inimical for their growth and survival. Oxidative stress is generated by the ROS produced by parasite’s metabolism, parasitized host red blood cells, and host immune responses. Antioxidant enzymes provide an important defense mechanism against the oxidative agents, like free radicals and ROS that cause undesirable biological effects. GR and TrxR are important antioxidant enzymes that help the parasite to tide over the oxidative stress. Multiplicity of antioxidants is beneficial as specific antioxidant molecules are effective for neutralizing specific ROS and reactive nitrogen species (RNS) and also different antioxidants tend to locate preferentially in different areas of the cells [14].

In our study, P. berghei parasitized host erythrocytes contained higher GR and TrxR activities compared to normal mice erythrocytes, whereas the erythrocyte membranes lack these enzymes. Increased activity of these antioxidant enzymes in P. berghei infection indicates their role in oxidative stress. Increased GR activity in P. berghei-infected erythrocytes indicates that glutathione is not taken up from the host, but there is glutathione synthesis due to glutathione cycle in parasites. It has been also reported that Plasmodium falciparum-infected erythrocytes contain higher GR activity compared to normal erythrocytes [15]. TrxR helps maintenance of redox homeostasis in Plasmodium infection. TrxR is essential for the survival of erythrocytic stages of parasites. TrxR knocked out parasites are non-viable [16].

Maximum activities of GR and TrxR were observed in the cytosolic part of the parasite. Decomposition of toxic FP IX requiring glutathione occurs in the cytosol, hence maximum GR activity in the cytosol part may be due to such requirement. Human TmrR and bovine TrxR are also reported to be cytosolic [17]. A linked thioredoxin-glutathione system in the cytosolic and mitochondrial compartments has been characterized in helminths also [18].

In the dismal scenario where the parasite become resistant to antimalarials and vectors become resistant to insecticides, there is a direct need to identify and validate a new potential antimalarial drug which targets the parasite metabolism. The deficiency of 2 important antioxidant enzymes in malaria parasites, i.e., catalase that detoxifies hydrogen peroxide and glutathione peroxidase that reduces lipid hydroperoxides to their alcohols, further underscores the role of GR and TrxR. The function of these enzymes in malarial infection is not well characterized and a full understanding of the host and parasite enzymes would promise advances in malarial treatment. Mechanistic and structural work on these antioxidant enzymes needs to be done further for the development of new antimalarials targeting specifically the parasites.

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