Diminazene aceturate attenuates oxidative and nitrosative imbalance in rats experimentally infected with *Trypanosoma evansi* Steel

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Trypanosomosis is an endemic disease in many parts of the world especially in tropical countries like India. Anaemia, thrombocytopenia and free radical mediated tissue damage is thought to play critical role in the pathogenesis of trypanosomosis, but its exact mechanism is not clearly understood till now. Hence, the present study was designed to access the correlation between oxidative stress indices and anaemia, and also with thrombocytopenia in trypanosomosis which can reveal the additional information regarding pathogenesis of the disease as well as to assess the ameliorating effect of diminazene aceturate in reducing the oxidative stress and its correlation with anaemia and thrombocytopenia in rat model. Twenty four rats were randomly divided in to four groups (n=6). Group C and Group D rats were intraarterially inoculated with 10⁷ *Trypanosoma evansi* Steel on day 0 and were checked regularly for onset of parasitaemia. At the onset of streaming parasitaemia (day 3), the Group D rats were treated with diminazine aceturate intra muscularily but Group C rats did not receive any treatment. Group B rats were injected with the same dose of diminazine aceturate on day 3 and acted as treatment control while Group A played as healthy control. Blood was collected from individual rats on day 0, 3 and 7 for evaluation of haematological parameters, oxidative stress indices (LPO, GSH, SOD and CAT) and nitric oxide (NO). At the end of study (day 7) brain sample was collected from each rat for PCR and histopathology. A negative correlation of haemoglobin and platelet count with LPO and a positive correlation with GSH, SOD and CAT were observed which implicates their role in the pathogenesis of anaemia and thrombocytopenia. The significant increase in haematological values and antioxidant indices (GSH, SOD and CAT) as well as the significant decrease in LPO and NO observed after diminazene therapy (Group D), indicate that diminazene aceturate is highly effective in combating oxidative as well as nitrosative damage caused by *T. evansi* and also significantly check anaemia and thrombocytopenia.

**Keywords:** Livestock, Nitrosative stress, Oxidative stress, Surra, Thrombocytopenia, Trypanosomosis

*Trypanosoma evansi* is one of the important parasites in the tropics and subtropics, especially in developing countries such as India1. Trypanosomosis, popularly known as surra, is considered as an endemic disease in northern, eastern and arid regions of India2. This hemoprotozoan parasite infects a diversified range of mammalian hosts, such as equine, bovine, canine, feline, their wild counterparts and recently a case of human trypanosomosis has also been reported in India3,4,5. It is mechanically transmitted by biting of hematophagous flies and most outbreaks are reported in rainy season due to explosion of vector population6. The disease in livestock is characterized by progressive anaemia, pyrexia, anorexia, loss of condition, nervous signs, relapsing parasitaemia and death in some cases.

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Diminazene aceturate has been widely used as a chemotherapeutic agent for trypanosomosis in livestock since 1955. The trypanocidal action of diminazene is thought to be by binding to kinetoplast DNA and thereby inducing complete and irreversible loss of kDNA in certain strains of trypanosomes. Earlier studies have also shown the role of diminazene to modulate the host immune response. Despite its use for more than 50 years, the exact mechanism of diminazene is not clearly understood until now.

The reactive oxygen species (ROS) and reactive nitrogen species (RNS) play significant role in pathogenesis of trypanosome induced tissue damage and anaemia. ROS/RNS production or compromised antioxidant system would result in inefficient removal of free radicals, such as hydrogen peroxide (H₂O₂), superoxide anion (O₂⁻), singlet oxygen, etc. which leads to oxidative/nitrosative damage. Also, *T. evansi* infection in rats is reported to cause induction of
Materials and Methods

Animals

The present study was conducted in the Division of Veterinary Medicine, Indian Veterinary Research Institute. Twenty four male Wister rats weighing 100-150 g, 6-8 weeks old were brought from the University Laboratory Animal Research Division after approval from Institutional animal ethical committee and maintained in clean polypropylene cages with ideal condition of temperature (25±2°C), humidity (45-55%) and 12 h light, 12 h dark cycle throughout the experimental period. They were provided with ad lib standard laboratory animal feed, and water. After acclimatization in experimental animal house for 7 days, they were randomly divided into 4 groups (n=6) Group A, B, C and D. Group C and D rats were inoculated with 10^4 Trypanosoma evansi intraperitoneally on day 0, and were checked regularly for onset of parasitaemia. At the onset of streaming parasitaemia (day 3), the Group D rats were given dimenazene aceturate deep intramuscularly at 14 mg/kg body wt. and it served as treatment group but Group C rats did not receive any treatment. Group B rats were injected with the same dose of dimenazene aceturate on day 3 which served as treatment control, and Group A acted as healthy control without receiving any treatment or infection.

Estimation of parasitaemia

The presence and degree of parasitaemia was accessed daily for each animal by blood film examination. A drop of blood was collected from the tail, placed on a clean glass slide and a thin blood smear was prepared manually. The blood films were stained with Giemsa staining and were examined under microscope, counting 10 fields at 1000X magnification.

Blood sampling

About 1.5 mL blood was collected from all the rats on day 0 (before inoculation of parasite), day 3 (onset of streaming parasitaemia) and day 7 (death or recovery), from the orbital plexus using microhaematocrit capillaries piercing through the outer canthus from each animal in two separate vials one with EDTA as anticoagulant for estimation of haematology and other with heparin as anticoagulant for estimation of oxidative stress (LPO, GSH, SOD and CAT). Plasma was harvested from heparinised blood for evaluation of nitric oxide (NO) concentration.

Tissue

At the end of the study (day 7), all the rats were sacrificed, half of brain tissue was collected in sterile polythene pack without any preservatives and stored at –20°C for isolation of DNA and conduction of PCR and other half with 10% formalin solution to conduct histopathology.

Haematological evaluation

Haemoglobin, total erythrocytic count (TEC), total leucocytic count (TLC), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and platelet count was estimated by autoanalyzer (Nihon Kohen, Celltac α, MEK-6450K).

Estimation of oxidative/nitrosative stress indices

Lipid peroxidases (LPO) level in 10% RBC haemolysate was estimated spectrophotometrically following the method of Placer et al. Superoxide dismutase (SOD) was measured in the supernatant of 10% RBC haemolysate following the method of Marklund & Marklund with certain modifications suggested by Menami & Yoshikawa. Each unit of SOD activity is defined as the quantity of enzyme that inhibits auto oxidation of pyrogallol by 50% under suitable experimental conditions. Catalase (CAT) activity in 10% RBC haemolysate was estimated spectrophotometrically at wave length of 240 nm after peroxidative injury to erythrocytes in T. evansi has already been reported by various researchers in different species of animals.8-10

Anaemia, thrombocytopenia, free radical mediated tissue damage and immunosuppression play key roles in the pathogenesis of trypanosomosis. In spite of substantial progress in the study of pathophysiology of anaemia in T. evansi infection, its detail mechanism is still not clear. Hence, in the present study, we explored the correlation between oxidative stress indices and anaemia, and also with thrombocytopenia in trypanosomosis which can reveal additional information regarding pathogenesis of the disease and thereby assess the ameliorating effect of dimenazene aceturate in reducing the oxidative stress in rat model.

pro-inflammatory cytokines like tumor necrosis factor alpha (TNF-α), gamma interferon, etc.7. RBCs are particularly susceptible to oxidative damage due to high concentrations of polyunsaturated fatty acids (in the membrane), and of oxygen and haemoglobin8. These factors may contribute to haemolysis and consequent anaemia. Peroxidative injury to erythrocytes in T. evansi has already been reported by various researchers in different species of animals.8-10
appropriate dilution following the method of Cohen et al.\textsuperscript{15} and the values were expressed in units per milligram of haemoglobin. Glutathione (GSH) was estimated in packed RBC following DTNB (di-thiobis2-nitro benzoic acid) method\textsuperscript{16}. Nitric oxide (NO) level of blood plasma was measured by nitrate reduction on copper cadmium alloy (Cu–Cd alloy) followed by colour development with Griess reagent (0.1% naphthalene diamine dihydrochloride in 3 N hydrochloric acid and 1% sulphanilamide 1:1)\textsuperscript{17}. Extraction of genomic DNA and polymerase chain reaction

Genomic DNA was extracted from formalized brain tissue using commercial DNA extraction kit (Promega, WI, USA). The PCR reactions were performed using Trypanozoon sub genus specific diagnostic primers \textit{i.e.} 21mer forward primer (5'TGCAGACGACCTGACGC TACT3') and 22mer reverse primer (5'TCTTAGAG CTTCGTTGTCCT3') targeting repetitive sequence probe pMuTec6.248 in genomic DNA of \textit{T. evansi} given that \textit{T. evansi} is the only reported member of the subgenus prevalent in the region\textsuperscript{18}. Histopathological analysis

Brain tissue samples was collect after end of study from all rats, fixed with 10% solution of buffered formalin (pH 7.4), sectioned and stained using standard technique for histopathological analysis.

Statistical analysis

Data were analyzed as mean values ± standard deviation. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey test and \( P <0.05 \) was considered statistically significant\textsuperscript{19}. Pearson’s correlation coefficient (r) and linear regression (\( R^2 \)) analysis was calculated using paired data from individual animals. All analyses were performed using the statistical package for the social science software (SPSS-12 Inc., Chicago, IL, USA).

Results

Parasitaemia

The onset of parasitaemia was observed on day 3 after inoculating \textit{T. evansi} infection in both Groups C and D rats. The parasitaemia was increased significantly on day 7 in Group C, but decreased day by day in Group D rats and ‘nil’ by day 7.

Haematology

The detailed haematological profile was given in Table 1. Haematology revealed a significant (\( P <0.05 \)) decrease in Hb, TEC, PCV and platelet count in both the infected grouped rats at the onset of parasitaemia (day 3). The above values were again decreased significantly on day 7 in Group C but increased non-significantly on Group D with respect to day 0 values. ‘Between groups comparison’ also revealed a significantly increased concentration of the above parameter in Group D compared to Group C on day 7.

Oxidative and nitrosative stress indices

The mean LPO and NO activity increased significantly (\( P <0.05 \)) on day 7 in trypanosome infected rats (Group C) in comparison to day 0 activity and also with respect to mean activity of rest of the groups. Treatment with diminazene acetate could able to reduce the mean LPO and NO activity significantly on day 7 in Group D as compared to Group C. The mean GSH, SOD and CAT activity decreased significantly (\( P <0.05 \)) on day 7 in infected animals without any treatment (Gr. C) in comparison to their respective day 0 activities. On the other hand, the above activities were increased significantly after treatment in Group D and statistically similar to that of healthy control rats on day 7. All the four oxidative stress indices were statistically similar in treatment control group (Gr. B) as compared to healthy control rats (Gr. A) after end of the study (Table 2).

Correlation and regression analysis

Pearson’s correlation (r) and linear regression (\( R^2 \)) analysis of the paired data obtained by individual infected rats without treatment (Gr. C) and individual infected rats with diminazene treatment was presented on Figs 1-3. There was a negative correlation between erythrocytic LPO activity and haemoglobin (\( r = -0.755, R^2=0.569 \) and \( P=0.001 \)) in trypanosome infected animals (Gr. C), on the other hand a positive correlation among LPO and GSH (\( r =0.568, R^2=0.323 \) and \( P=0.024 \)), SOD (\( r =0.503, R^2=0.253 \) and \( P=0.032 \)) as well as CAT (\( r =0.540, R^2=0.291 \) and \( P=0.006 \)) in Group C animals (Fig 1). The LPO (\( r = -0.222, R^2=0.049 \) and \( P=0.342 \)) and GSH (\( r = -0.163, R^2=0.026 \) and \( P=0.518 \)) activities were negatively correlated with haemoglobin concentration in diminazene treated rats (Gr. D), on contrary SOD (\( r =0.314, R^2=0.098 \) and \( P=0.204 \)) and CAT (\( r =0.266, R^2=0.071 \) and \( P=0.287 \)) were positively correlated with haemoglobin (Fig 1).

There was a negative correlation between erythrocytic LPO activity and platelet (\( r= -0.104, \) ...
<table>
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<tr>
<th>Parameter</th>
<th>Group</th>
<th>Day-0</th>
<th>Day-3</th>
<th>Day-7</th>
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<td>Hb (g/dL)</td>
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<td>C</td>
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<td>TLC (10&lt;sup&gt;3&lt;/sup&gt;/μL)</td>
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<td>PCV (%)</td>
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<td>31.48±1.49&lt;sup&gt;C&lt;/sup&gt;</td>
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<td>Platelet count (10&lt;sup&gt;3&lt;/sup&gt;/μL)</td>
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<tr>
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[Group A, healthy control; Group B, treatment control; Group C, infected without any treatment; and Group D, infected + diminazene treatment rats. Mean±SE values within same column for a particular parameter (capital letters) and in same row (small letter) bearing similar superscript do not differ at P <0.05]
R²=0.111 and P=0.680) in trypanosome infected animals (Group C), on the other hand a positive correlation among haemoglobin and GSH (r=0.393, R²=0.154 and P=0.106), SOD (r=0.875, R²=0.765 and P=0.001) as well as CAT (r=0.589, R²=0.346 and P=0.010) in Group C animals. The LPO (r=−0.278, R²=0.078 and P=0.236) and GSH (r=−0.071, R²=0.005 and P=0.778) activities were negatively correlated with platelet in dimenazene treated rats (Group D), on contrary SOD (r=0.145, R²=0.021 and P=0.567) and CAT (r=0.182, R²=0.033 and P=0.469) were positively correlated with platelet (Fig. 2). A strongly positive correlation was observed between erythrocytic LPO and NO (r =0.806, R²=0.650 and P=0.001) in group C or T. evansi infected rats without having any treatment (Fig. 3A), but it was merely positive (r =0.085, R²=0.007 and P=0.737) in diminazene treated rats (Fig. 3B).

Polymerase chain reaction (PCR)

PCR amplification of single 227 bp fragment specific for T. evansi repetitive nucleotide sequence was observed in brain sample from Group C rats, but not in brain of dimenazene treated rats (Fig. 4).

Histopathology

The histopathological examinations of brain tissue sample of healthy rats possess normal architecture
of brain (Fig. 5A). However, brain sample of trypanosome infected rats without any treatment (Group C) revealed neuronal loss in Purkinje layer of cerebellum (blue arrow) and presence of Mott cells (black arrow); and (C) T. evansi infected rats indicating focal gliosis (glial cell proliferation) in cerebral cortex (Fig. 5C).

Discussion

Anaemia is the hallmark of pathology of T. evansi infection. A gradual decrease in haemoglobin, TEC and PCV were observed in infected animals without having any treatment, in our study. The analysis of erythrocyte indices revealed marked anaemia in the infected rats, first normocytic-normochromic followed by macrocytic-hypochromic. Anisocytosis and reticulocytosis were also observed in the blood smear examination of Group C rats. These results are in agreement with previous studied done by Wolkmer et al.\textsuperscript{8}. The mean platelet count decreased significantly in Group C rats on day 7 with respect to that of day 0 values. Thrombocytopenia might have occured due to increased destruction of platelets due to disseminated intravascular coagulopathy (DIC) or immune mediated destruction\textsuperscript{20}. Shehu \textit{et al.}\textsuperscript{21} also revealed that the production of enzyme neuraminidase by circulating trypanosomes is one of the main causes of red cell destruction resulting in anaemia. However, the exact mechanism of anaemia is still not completely understood. The significant improvement in anaemia and thrombocytopenia by dimazinazene might be due to its trypanocidal effect which was in agreement with Da Silva \textit{et al.}\textsuperscript{5,22}.

\textit{T. evansi} can be diagnosed by microscopic examination of blood smear but polymerase chain reaction (PCR) has been considered as more sensitive and specific marker\textsuperscript{13}. In the present study, brain sample was taken for diagnosis of trypanosome by PCR to prove migration of parasite to brain tissue by crossing blood brain barrier during heavy parasitaemia.
PCR amplification of single 227-bp fragment specific for *T. evansi* repetitive nucleotide sequence was observed in brain sample from infected Group C rats, but not from diminazene treated (Group D) rats indicating the trypanocidal effect of diminazene aceturate in nervous system in rats. The histopathological examination of brain tissue sample revealed focal gliosis in cerebral cortex, neuronal loss in Purkienjee layer of cerebellum as well as concretion of blood vessel and haemorrhages in trypanosome infected animal without any treatment indicating the inflammatory changes and free radical related tissue damages caused by trypanosome after crossing blood brain barer and related oxidative damaged.

Oxidant/antioxidant equilibrium disturbance play a critical role in pathogenesis of trypanosome induced tissue damage and anaemia. The erythrocytes are highly susceptible to peroxidative damage due to presence of high concentration of polyunsaturated fatty acids, continuous exposure to high concentration of oxygen and the presence of iron, a powerful metal catalyst. Thus, higher production of peroxyl radicals and consequent elevated LPO concentration renders the RBCs more fragile and prone to lyses leading to anaemia. It is also considered that, the oxidative burst products from neutrophils and activated macrophages produced during trypanosome infections have been shown to infiltrate a self propagating reaction of oxidative damage to the polyunsaturated fatty acid components of erythrocyte plasma membranes, leading to cell destruction. NO may react with O₂⁻ leading to production of peroxinitrite anion (ONOO⁻) or by Fenton reaction to produce hydroxyl radical (OH⁻). Both ONOO⁻ and OH⁻ are the most potent oxidising agents which have the ability to initiate lipid peroxidation by interacting with polyunsaturated fatty acids in the cell membranes of RBCs. Mabbott & Sternberg demonstrated direct correlation of NO production with development of anaemia in *T. brucei* infected mice, and that treatment with NO blockers significantly reduces the anaemia. There is negative correlation between LPO and Hb as well as Platelet count with LPO and a positive correlation with GSH, SOD and CAT recorded in trypanosome infected rats indicating oxidant/antioxidant imbalance. A negative correlation of haemoglobin and platelet count with LPO and a positive correlation with GSH, SOD and CAT were observed, which implicates their role in the pathogenesis of anaemia and thrombocytopenia in *T. evansi* infected rats. There was no significant difference of oxidative or nitrosative stress indices between treatment control rats and healthy control rats, indicating diminazene itself does not induce oxidative damage. After treatment with diminazene aceturate, there was complete absence of trypanosome in brain sample after day 7. A significant increase in haematological values and antioxidant indices as well as significant decrease in LPO and NO also observed after diminazene therapy, indicating diminazene aceturate is an effective trypanocidal drugs and antioxidant might be supplement in therapeutic regimen of trypanosomosis.

**Conclusion**

In the current study, there was increased level of oxidative and nitrosative stress indices (LPO and NO) and decreased level of antioxidant enzymes (GSH, SOD and CAT) recorded in trypanosome infected rats indicating oxidant/antioxidant imbalance. But, to our knowledge, there was no published literature to compare the correlation between LPO and Hb or Platelet, or LPO with NO after diminazene treatment.

GSH, SOD and CAT act as important antioxidant enzymes in mammalian erythrocytes. The catalytic activity of these enzymes allows transformation of superoxide anion (O₂⁻) into hydrogen peroxide (H₂O₂) and water, thereby reducing formation of free radicals and related oxidative stress. A significant decrease in antioxidant enzymatic activities was observed in the current study in *T. evansi* rats without having any treatments (Group C). Similar observations were also reported in *T. evansi* infected buffalo, horse and dog. Saleh et al. reported significant decrease in SOD level in *T. evansi* infected camels, but CAT level was not decreased. In the current study, the SOD, CAT and GSH levels increased significantly in diminazene treated rats (Group D) as compared to infected rats without any treatment (Group C) which was in agreement with Ranjithkumar et al. in *T. evansi* infected horse with quinpyramidine sulphate treatment. But, to our knowledge, there was no published literature to compare the correlation between SOD and CAT with Hb or Platelet, after diminazene treatment.
References


