Trypanosomiasis induced oxidative stress and hemato-biochemical alteration in cattle

Dr. Rashmi Ranjan Mishra, Dr. SK Senapati, Dr. Sudam Chandra Sahoo, Dr. MR Das, Dr. G Sahoo and Dr. RC Patra

Abstract
In this present study, blood samples were collected from 10 clinically healthy and 20 adult cattle naturally infected with Trypanosomes. Blood samples were evaluated for haematological and biochemical as well as erythrocytic oxidative stress marker for both infected and control groups. The results of the study revealed substantial decrease (%) in levels of Hb (42%), PCV (39%), total protein (46%), albumin (39%), globulin (44.6%), blood glucose (46%), SOD (57.7%), CAT (62%) and increase (%) in LPO (295%), AST (70%) and ALT (4.5%) in infected cattle compared to healthy animals. Oxidative stress marker enzymes like SOD and Catalase decreased two times in infected group whereas Lipid peroxidase (LPO) increased nearly four times in infected group as compared to healthy group. Thus enhanced erythrocytic oxidation and reduction of haematological indices suggests that, trypanosome affected cattle undergoes severe oxidative stress and resultant anaemia.

Keywords: Trypanosomiasis, Oxidative stress, Lipid peroxidation (LPO), Catalase (CAT), superoxide dismutase (SOD), hemato-biochemical alteration

1. Introduction
Trypanosomiasis, is a blood protozoan disease caused by various trypanosome parasites belonging to the order Kinetoplastida, family Trypanosomatidae, and genus Trypanosoma [1, 2]. Disease caused by T. evansi is popularly known as ‘Surra’ and widely prevalent in domestic animals of the Indian subcontinent. Trypanosomes possess zoonotic importance by an ability to infect both humans as well as animals [3]. Human case of T. evansi infection was also reported in India [4]. Among many states of India Trypanosomiasis is also present in Odisha. Trypanosomiasis has the threat to life of cattle, brings economic loss to farmer affecting production and future productivity for a long time after clinical recovery of animals [5].

The trypanosome parasites are mainly found in blood and other tissue fluids and mechanically transmitted by biting flies of the genus: Tabanus [6]. The disease can be acute in young animals and pregnant females, which die within a few weeks, but in endemic areas the usual form is chronic one that lasts for many years and ultimately leads to cachexia and death [7]. The acute form of the disease is noticed with intermittent fever, anemia, emaciation, production losses, dullness with recumbency or staggering gait, labored breathing, lacrimation, bellowing, profuse salivation, twitching of muscles often terminating in convulsions and death [8]. The chronic form lasts for years and is characterized by dullness, anemia, emaciation, recurrent fever, edema in dependent part of body, conjunctivitis, lacrimation, enlargement of the superficial lymph nodes, abortions, infertility, reduced milk yield, progressive emaciation and lowered work out-put [9, 10].

Anaemia in T. evansi infection in ruminants is macrocytic and hypochromic [11]. Dyshemopoiesis and erythrophagocytosis were considered the primary causes of anemia in ruminants infected with T. evansi [12]. Erythrophagocytosis in trypanosomiasis is likely caused by cell damage and increased rate of removal of red cells from the circulation by hemolytic factors released by dying trypanosomes, immune complexes bound to RBCs, together with fever and mechanical damage to RBCs by trypanosomes [13]. Many investigators revealed alterations in blood constituents and tissue lesions in Trypanosomiasis affected animals of different species [14-17]. The infection with trypanosomes results in the production of large amounts of reactive oxygen species (ROS) and free radicals which act as cytotoxic agents [18] damaging vital components of the cell, including proteins and lipids [19].

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Furthermore some reports have shown an important role of free radical induced oxidative stress in the pathogenesis of Trypanosomiasis [20-22]. The erythrocyte, due its role as O₂ and CO₂ transporter, is under constant exposure of free radicals [23]. The RBC antioxidant system primarily consists of superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), GSH-peroxidase, and the antioxidant vitamins A, E and C [24]. Thoroughly synergistic and co-operative interactions of these antioxidants leads to the sequential degradation of peroxides and free radicals as well as on mutual protections of enzymes [18]. Oxidative stress occurs when there is an imbalance between radical-generating and radical-scavenging activity; it may therefore cause an increase in the formation of oxidation products [18]. Lipids especially polyunsaturated fatty acids are sensitive to oxidation, leading to the term lipid peroxidation, of which, malondialdehyde (MDA) is the most abundant [25]. Thus, the evaluation of oxidative stress markers and haemato-biochemical indices helps to determine the degree of damage to hosts tissues caused by the infection [26] and the health status of the infected animals [27].

Keeping these in mind the present study was designed to assess the haemato-biochemical and erythrocytic oxidative stress status as indicators of oxidative damage of the erythrocytes and their contributory role in causing cell lyses and anaemia in cattle naturally infected with Trypanosomiasis.

2. Material and Methods
2.1 Study area
Coastal belt of Khurda, Puri and Kendrapara districts of Odisha with hot and humid climate was taken as the area of study for this experiment. This climate may be attributed to the geographical location of Bay of Bengal which is in an average 60 km away from these areas. This study was carried out in the early monsoon period from May 2017 to July 2017. A large population of crossbred as well as indigenous cattle reared in this fly dominated area was taken as the study population. Hot and humid climate and early monsoon showers, which are the prime predisposing factors for fly growth and subsequently the transmission of Trypanosomiasis parasites.

2.2 Animals and Samples for clinical investigation
Detailed clinical examination of cattle from a fly dominated surrounding and showing signs like fever, swelling of superficial lymph nodes (usually the parotid and pre scapular), pale mucous membrane, Edema of dependent parts of body, corneal opacity, watery occculo-nasal discharge, reduced appetite, reduced milk yield, loss of body condition and nervous disorders like wall climbing and back ward walking, sudden falling, head tilt and circling movement were selected for further clinic-pathological investigation for detection of trypanosome parasites in blood. For the present study 34 cattle aged between 1.5 and 5 years with suspected signs of Trypanosomiasis were considered. Smears from first drop of blood collected from the marginal ear vein onto clean grease free glass slides were sent to laboratory of Department of Veterinary Clinical Medicine, Ethics and Jurisprudence, College of Veterinary Science & Animal Husbandry, O.U.A.T. for detection of trypanosome organisms using wet preparations as described by Murray et al. (1977) [28] and Paris [29] et al. (1982). About 10 ml blood was drawn from the jugular vein of animals via hypodermic needle and transferred into the following vials. 3ml into gel clot activator vial for oxidative stress estimation, 3ml into gel clot activator vial for serum separation, and 2 ml each into EDTA vial and Sodium Fluoride vial respectively for hematological parameter and plasma glucose estimation.

Out of the 34 suspected animals, twenty-five were found positive for Trypanosomiasis upon blood smear examination. Twenty cattle were selected from them for further study and ten healthy cattle maintained in nearly identical nutritional and managemental conditions and regularly dewormed and vaccinated against infectious and contagious diseases were selected as control healthy animals.

2.3 Diagnosis of Trypanosomiasis
Thin and thick blood smears were made for each animal on a clean grease-free micro-scope slide for the detection and identification of trypanosome species as described by Murray et al. (1977) [28] and Paris et al. (1982) [29]. The blood smears were prepared from each blood sample, air-dried, fixed in methanol (99%) for 2–3 min, stained in 5% (1:20) Giemsa stain for 30 minutes and rinsed twice in distilled water (pH 7.2). The smears were examined at 100x magnification (oil immersion) under microscope (Hund Wetzlar H600) for the presence of Trypanosomes at least 50 fields were searched per slide and each slide was examined twice before being considered as negative.

2.4 Estimation of haematological parameters
The blood samples collected in EDTA vials from selected cattle were analyzed for Hb%, PCV, TEC, TLC and DLC following standard protocols. Differential Leucocytes Count (DLC), the packed cell volume (PCV) and haemoglobin concentration (Hb) were determined by microhematoctrit and cyanometaemoglobin methods, respectively [30]. Blood glucose was estimated based on Trinder’s method. In brief glucose is oxidized by Glucose oxidase and produces gluconate and hydrogen peroxide. The hydrogen peroxide is oxidatively coupled with 4-aminoantipyrine and phenol. The intensity of the coloured complex (quinoneimine) is proportional to the glucose concentration in the sample and can be measured photometrically at 505nm (500-540nm).

2.5 Estimation of oxidative and biochemical parameters
For estimating the oxidative parameters the blood samples were centrifuged at 2000 rpm for 10 min. Plasma and buffy coat were removed. The resulting erythrocyte pellet was washed thrice with PBS and washed packed erythrocyte pellets were diluted in EDTA- mercapto ethanol stabilizing solution at a 1: 9 (V/V) ratio and kept at 4°C until further analysis. This 10% packed erythrocytes were used for the estimation of lipid per-oxidation (LPO), Superoxide Dismutase (SOD) and Catalase (CAT) activity. All analyses of oxidative parameters were done within 2 h after sample collection.

For the estimation of lipid per-oxidation (LPO) described protocol measures the erythrocytic Malonyldialdehyde (MDA) or more precisely thiobarbituric acid reactive substances (TBARS) using the spectrophotometric method developed by Placer et al. (1966) [31]. Briefly, 1.3 ml Tris-KCl buffer was added to 0.2ml of 10% haemolysate taken in sterile test tube. Further 1.5ml TBA reagent was to the mixture and placed in boiling water bath for 10 min followed by cooling under tap water. Then 3 ml freshly prepared pyridine-butanol (3:1 V/V) and 1 ml 1N NaOH was added to the solution. Then it was mixed well to get a grey colour in the solution. A blank was run containing 0.2 ml distilled water.
in place of haemolysate. Finally absorbance was recorded at 548 nm against blank. The values of lipid peroxidation were expressed as (nm) Nano moles of MDA produced/ mg of Hb. The plasma CAT activity was measured as per method described by Cohen et al., (1970)\textsuperscript{32}. In brief, 0.1ml of 10% erythrocyte lysate was incubated in 2.9 ml of 10 mM H\textsubscript{2}O\textsubscript{2} at 37°C and O.D. was recorded at 240 nm in a UV spectrophotometer (ECI, India). Time required for the Abs\textsubscript{240} nm to decrease from 0.45 to 0.40 was recorded and reading was taken against phosphate buffer as blank.

The activity of SOD in 10% erythrocyte lysate was determined by using nitro blue tetrazolium as a substrate. After suitable dilution according to the method of Marklund and Marklund (1974)\textsuperscript{33} with certain modifications suggested by Minami and Yoshikawa (1979)\textsuperscript{34} the estimation can be done. Assay the 3ml mixture consisting of 50 mM of Tris-cacodylic acid buffer (PH 8.2), enzyme preparation (sample) after suitable dilution and 0.2 mM of pyrogallol was examined at 420nm using spectrophotometer. In the blank enzyme was substituted by equal quantity of distilled water. The increase in absorbance due to auto oxidation of pyrogallole was recorded at 420nm using spectrophotometer. The serum Aspartate transaminase (AST), Alanine transaminase (ALT) concentration (U/L) was determined as per the method given by Reitman and Frankel (1957)\textsuperscript{35} with a spectrophotometer (Systronics UV-VIS spectrophotometer-118).

3. Statistical analysis
The results are reported as means ± SE for both the pre and post therapy values of infected and control groups. Data were analysed statistically using independent and paired t-tests. P < 0.05 was considered as statistically significant\textsuperscript{36}.

4. Results
In the present investigation influence of Trypanosomiasis on haemato-biochemical and oxidative status of cattle were studied. The thick and thin blood smear (Giemsa stained) of infected animals examined for parasitological identification revealed slender and flagellated trypomastigote forms morphologically comparable with T. evansi (Fig. 1)\textsuperscript{37}. The mean (±SE) values of haemato-biochemical and oxidative parameters of naturally infected with Trypanosomes and non-infected control cattle are presented in Table 1. The hematological parameters exhibited significant decrease in hemoglobin and PCV concentration in infected animals compared to healthy animals. However other hematological indices did not show significant variations but slight decrease in lymphocytes, monocytes and basophils and rise in neutrophils and eosinophils were observed in infected compared to control healthy animals. The results of oxidative stress markers observed in the present study revealed significant increase in LPO, but decreased in SOD and CAT activity in Trypanosomiasis affected animals compared to healthy control animals. The biochemical indices showed higher AST and ALT activity in infected animals. Beside this, total protein, serum albumin, serum globulin and glucose concentration decreases drastically in infected animal compared to healthy one(Table1).

![Trypanosome parasite in Giemsa stained blood smear under 100X and digitally magnified view](image)

**Fig 1: Trypanosome** parasite in Giemsa stained blood smear under 100X and digitally magnified view

**Table 1:** Influence of Trypanosomiasis on oxidative and haemato-biochemical indices of Cattle

<table>
<thead>
<tr>
<th>S. No</th>
<th>Parameter with units</th>
<th>Infected Cattle (n=20)</th>
<th>Healthy Cattle (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Haemoglobin (g/100ml)</td>
<td>6.9±0.10\textsuperscript{a}</td>
<td>12.0±0.27</td>
</tr>
<tr>
<td>2</td>
<td>PCV (%)</td>
<td>21.89±1.28\textsuperscript{a}</td>
<td>35.9±0.89</td>
</tr>
<tr>
<td>3</td>
<td>Mean TEC (10\textsuperscript{6}/ mm\textsuperscript{3})</td>
<td>3.63±0.11\textsuperscript{a}</td>
<td>6.12±0.13</td>
</tr>
<tr>
<td>4</td>
<td>Mean TLC (10\textsuperscript{3}/ mm\textsuperscript{3})</td>
<td>3.73±0.13\textsuperscript{a}</td>
<td>5.03±0.19</td>
</tr>
<tr>
<td>5</td>
<td>Neutrophil (%)</td>
<td>49.99±0.93</td>
<td>38.59±0.96</td>
</tr>
<tr>
<td>6</td>
<td>Lymphocyte (%)</td>
<td>36.93±0.65</td>
<td>58.03±0.94</td>
</tr>
<tr>
<td>7</td>
<td>Monocyte (%)</td>
<td>2.00±0.21</td>
<td>2.40±0.24</td>
</tr>
<tr>
<td>8</td>
<td>Basophil (%)</td>
<td>1.00±0.25</td>
<td>1.20±0.37</td>
</tr>
<tr>
<td>9</td>
<td>Eosinophil (%)</td>
<td>7.49±0.93</td>
<td>2.80±0.27</td>
</tr>
<tr>
<td>10</td>
<td>Blood Glucose (mg/dl)</td>
<td>31.04±0.96\textsuperscript{a}</td>
<td>58.10±2.07</td>
</tr>
<tr>
<td>11</td>
<td>LPO (n Mol MDA/ mg Hb)</td>
<td>4.27±0.11\textsuperscript{a}</td>
<td>1.08±0.05</td>
</tr>
<tr>
<td>12</td>
<td>Superoxide dismutase (Units/mg Hb)</td>
<td>1.89±0.06\textsuperscript{a}</td>
<td>4.47±0.14</td>
</tr>
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</table>
5. Discussion

The changes in haematological and biochemical parameters are marker of various disease conditions such as Trypanosomiasis [16]. The decrease in Hb and PCV is a characteristic feature reported in Trypanosomiasis in different species of animals [14, 16, 38]. Hemolytic associated with bovine Trypanosomiasis may be due to decrease in the life span of erythrocytes and extensive erythrophagocytosis [39]. The erythrophagocytosis occur mainly due to the activation of mononuclear phagocytic system that reduces half-life of the erythrocytes [40]. Therefore hemoglobin (Hb) concentration (g/dl), total erythrocyte count (TEC), packed cell volume (PCV), Total Leucocyte count (TLC), in affected group were significantly at lower range than healthy control group which indicates there is trypanosome induced reduction in Hb concentration which is in agreement with the previous findings [39, 41-43]. Furthermore lower in Mean PCV mean TEC and mean TLC concentration in Trypanosomiasis affected group is in accordance with the findings of Adenike et al., (2010) [14]; Sivajothi et al., (2014) [45]; Hussain et al., (2016) [46] and Oparah et al., (2017) [47]. This may be due to the immunosuppressive action of trypanosomes as well as exhaustion of immune system [48], besides this leucopenia is always seen at terminal stage of the infection (Ansoa, 1988) [43] usually due to wax and wear syndrome on the animal immune system caused by the ever changing variable surface glycoprotein of the infecting trypanosomes [49]. However the Trypanosomiasis affected cattle show higher mean neutrophil (%), higher mean Eosinophil (%) and lower Mean Lymphocyte (% than the healthy control.This is in agreement with the findings of Chaudhary et al., (2000) [50] and Hussain et al., (2016) [46] which may be due to initial enhanced immunological response followed by immunosuppressive effect of trypanosome, influenced with the ever changing variable surface glycoprotein of the infecting trypanosomes [51, 48]. Along with the eosinophilia condition may be due to the feature of parasitic infections and is associated with immediate-type hypersensitivity reactions [45].

In the present study various erythrocytic oxidative stress markers like catalase (CAT) and Super Oxide Dismutase (SOD) have been estimated. The measurement of antioxidant enzyme activities like SOD and CAT are appropriate indirect ways to assess the status of antioxidant defense [51]. Increase in erythrocytic LPO and decrease in antioxidant enzymes as in present study have been reported by many workers in Trypanosomiasis involving various species [52, 53, 15]. In this regard Trypanosomiasis affected cattle showed significantly (P<0.05) lower mean erythrocytic Catalase concentration and SOD concentration in infected group compared to that of the healthy control of present study which is agreement with the findings of Ranjithkumar et al., (2011) [53] and Eze et al., (2016) [54]. Bovine trypanosomosis induced oxidative stress was measured as mean lipid peroxides (LPO) in form of malonyl dialdehyde (MDA) (n mol MDA/ mgHb). It was documented that mean LPO values in infected group is significantly higher than control group which is in agreement with the findings of Ranjithkumar et al., (2011) [53]; Wolkmer et al. (2009) [55]; Ogunsanni and Taiwo (2007) [20] and Yusuf et al. (2012) [38]. T. evansi infection produces oxidative stress in erythrocyte and also reduces the antioxidant capacity of RBC [54]. Further T.evansi infection produces superoxide (O2-), hydrogen peroxide (H2O2), peroxyl radicals (ROO*), and hydroxyl radicals (OH) results in oxidative stress and tissue damage [15]. Trypanosome protozoa releases sialidase and phospholipase that damages the erythrocyte membrane leading to formation of insulted RBCs which produces Reactive Oxygen Species (ROS) causing oxidative stress and enhanced lipid peroxidation in RBC [56]. Albumin is an important extra-cellular antioxidant by virtue of its thiol groups, which protects vital targets such as erythrocytes from copper ion-induced Fenton reaction and inhibits iron-dependent free radical production[57][58]. Trypanosomiasis affected cattle showed significantly (P<0.05) lower mean serum Albumin, total protein and globulin compared to that of the healthy control which is in agreement with previous findings [59][60][61][62][63] which may be due to increase hepatocellular damage arising in Trypanosomiasis infection [64]. Trypanosomiasis affected cattle showed higher mean serum ALT and AST concentration (Units/L) in infected group compared to that of the healthy control which is in accordance with the findings of Takeet et al., (2009) [67]; Yusuf et al., (2012) [38]; Umar et al (2000) [61]; Hussain et al (2016) [45] which indicative of increased damage to liver, heart and brain [65] associated with Trypanosomiasis [64].

Besides this, Trypanosomiasis infected cattle showed significantly (P<0.05) lower mean blood Glucose (g/dl) compared to that of the healthy control that is in agreement with findings of Sazmand et al., (2011) [66]; Takeet et al., (2009) [67]. This hypoglycaemic condition occurs as trypanosomes are voracious consumers of host glucose utilizing for their metabolism [15] and also may be due to increased metabolic rate due to fever and hepatocellular damage [67].

6. Conclusion

The cattle suffering from Trypanosomiasis undergo oxidative stress as their erythrocytic lipid peroxide (LPO) value increased more than three times than healthy control group. The antioxidant enzyme system of the body is not capable enough to counter the bovine Trypanosomiasis induced oxidative stress. Persistent anemia with low haematocrit values including leucopenia, lymphocytopenia and neutrophilia in all trypanosoma affected cattle were noticed indicating exhaustion of immune system. In this study all trypanosome affected cattle showed hypoglycaemia with altered liver function as the serum AST values rises and total protein serum albumin and serum globulin concentration decreases. Further study regarding effect of restoration of oxidative stress markers and altered hematology parameters to promote quicker recovery in production and

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<th>Catalase (Units/mg Hb)</th>
<th>AST(U/L)</th>
<th>ALT(U/L)</th>
<th>Total Protein(g/100ml)</th>
<th>Serum albumin(g/100ml)</th>
<th>Serum Globulin(g/100ml)</th>
<th>Albumin-Globulin Ratio</th>
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<tbody>
<tr>
<td>13</td>
<td>1.02±0.04*</td>
<td>168.29±2.25*</td>
<td>34.51±1.3</td>
<td>3.69±0.11*</td>
<td>1.93±0.12*</td>
<td>1.77±0.16*</td>
<td>1.2±0.15</td>
</tr>
<tr>
<td>14</td>
<td>2.71±0.12</td>
<td>99.02±1.9</td>
<td>33.01±1.07</td>
<td>6.96±0.19</td>
<td>3.17±0.18</td>
<td>3.24±0.14</td>
<td>1.92±0.1</td>
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<td>15</td>
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<td></td>
<td>*superscript Indicates significant difference between groups.</td>
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7. Acknowledgement
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