Original Research

Evaluation of Reactive Oxidative Damage on Erythrocytic Cells Due to Clinical Babesiosis in Lactating Cows

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Abstract

Study has been carried out on the animals suffering from clinical bovine babesiosis in the Patna districts of Bihar, India. A total number of 36 lactating cows of different breed and ages were included in the study for the effect of babesia infection on oxidant marker, antioxidant status, trace mineral status and mean corpuscular fragility of RBC. Erythrocytic LPO in clinically affected cattle were significantly (P<0.05) higher than healthy animals. GSH, SOD and catalase values in ailing animals were significantly (P<0.05) lower than healthy animals. Hemoglobin concentration was significantly (P<0.05) lower in ailing animals. Trace elements Zn, Cu & Se were also significantly reduced whereas serum iron found to be significantly (P<0.05) increased due to hemolysis in infected cows. Erythrocyte fragility at 0.9%, 0.8%,0.4% and 0.2% NaCl concentration was significantly (P<0.05) higher than the control group. However, there was lower erythrocytic fragility in 0.6% concentration of NaCl in infected animals. In conclusion, anemia occurs in Babesia bigemina infection is due to corpuscular oxidative damage as revealed from lipid peroxidation, antioxidant status and associated trace minerals deficiency contributing to RBC fragility and intravascular hemolysis.

Key words: Oxidative Damage, *Babesia bigemina*, Clinical Infection, Lactating Cattle, Piroplasm

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Introduction

Babesiosis is a tick-borne parasitic disease caused by haemotropic protozoa of the genus *Babesia*, family *Babesidae* order *Piroplasmidae* within the phylum *Apicomplexa* (Bock *et al*., 2004). It is a well-recognized disease of veterinary importance in cattle, horses and dogs and has gained attention as an emerging zoonotic disease-causing malaria-like syndrome including fever, haemolysis and haemoglobinuria. Ticks of the *Ixodidae* family are the main vectors and their geographical distribution influences the epidemiology of the disease (OIE, 2008). *Babesia bigemina* and *Babesia bovis* poses great economic threat in Asia, Africa, Central and South America, Southern Europe and Australia (Spicklerand Roth, 2008). Hemoparasites of

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the genus *Babesia* are protozoans which mostly infect ruminants in tropical and subtropical regions (Hashemi-Fesharki, 1997) and impose heavy losses due to high mortality and decreased productivity in affected animals. India suffers losses of about 57.2 million US$ annually due to babesiosis in livestock (Sharma *et al.*, 2013 and McLeod *et al.*, 1999). Babesia likes theileria and malaria parasites, invade red blood cells of infected animals resulting in destruction of parasitized erythrocytes (Otsuka *et al*., 2002). Lysis of erythrocytes leads to severe anemia resulting in anoxia of vital organs and subsequent organ damage. Production of various pro-inflammatory cytokines from mononuclear cells due to hemoparasitic diseases, activates the mononuclear phagocytic cells to release oxygen and reactive nitrogen species (Brown *et al*., 1998; Brunet, 2001 and Woldehiwet *et al*., 2010) leading to oxidative damage in the animal body (Zaidi *et al.*, 2005). Oxidative stress is an imbalance between radical-generating and radical scavenging activity, resulting in oxidant production and tissue damage, when the cellular oxidant concentration is overwhelmed by excess production of reactive oxygen species (ROS), cellular damage occurs through oxidative stress and lipid peroxidation (Saleh *et al.*, 2011). Increased median corpuscular fragility in erythrocytes of affected cattle indicates injury to erythrocytes membrane and consequently altered permeability of these cells. Such alteration can result from oxidative stress and lipid peroxidation. Loss of membrane stability leading to increased RBC osmotic fragility, because of morphological changes in the cell surface of erythrocytes (Wagner *et al*., 1988; Saluja *et al*., 1999).

The present study was envisaged to find out the alteration of oxidant/antioxidant status, trace minerals level and mean corpuscular fragility of RBC in clinical cases of Babesiosis.

Materials and Methods

This study has been carried out on the animals suffering from clinical bovine babesiosis in the Patna districts of Bihar, India during the period of July 2015 to February 2016. The study region belongs to persistent tick-borne diseases due to high humidity coupled with high dense stocking pattern of animals. A total number of 36 cows of different breed and ages clinically affected with babesiosis and 36 apparently healthy lactating cows as healthy control were included in the study. Clinical manifestations of high fever, anorexia, pale mucus membrane, cachexia, anemia, haemoglobinuria revealed in all the lactating animals suggestive of the infection of Babesia. Thin blood smear was prepared using capillary blood from ear tip and stained with Giemsa for microscopic examination (Shino *et al.*,2003) after appropriate fixation. Conventional PCR were done to confirm the species of *Babesia* using *B. bigemina* specific primer. The PCR conditions were initial denaturation at 95^0C for 3 minutes, denaturation again at95^0C, annealing at55^0C for 45second, extension at35^0C for 1-minute, total cycle 35 and final extension at72^0C for 10 minutes. Blood were drawn from the jugular vein of each animal in heparinized vials of 5 ml capacity for estimation of various parameters. The concentration of oxidative stress markers lipoperoxidase (LPO) as per method Placer *et al.* (1966) in

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haemolysate prepared from the clinically affected animals measured including superoxide dismutase (SOD) as per method Marklund & Marklund (1974), Glutathion peroxidase (GSH) as per method Beutler *et al.*, (1963) and catalase as per method Slaughter and O'Brien (2000). The osmotic fragility tests of erythrocytes were done as per the method described by Chanarin (1989). Hemoglobin concentration (Hb) was measured by "Acid hematin method" (Baker *et al.*, 1965). Minerals copper, selenium, Iron and zinc were measured using an atomic absorption spectrophotometer, AAS (Model No. ECIL4141) manufactured by electronic Corporation of India Hyderabad. The statistical analysis was done using software package for social science (SPSS) version 17.0 (2008) paired t test (Snedecor &Cochran, 1994).

Results

Blood Smear Examination and Oxidative Stress Marker

A total of 500 ticks infested animals screened for babesiosis, among them 36 animals found positive for the presence of piroplasm of *Babesia bigemina* in the red blood cells. The product size of PCR amplicon was found to be 278bp. The oxidant marker and antioxidant parameters were estimated in all 36 infected animal which were compared with healthy control (n=36) and treated animals. Erythrocytic LPO in healthy cattle were 297.00±3.96 and the corresponding values in clinically affected cattle were 452.19±10.3 (Table 1) which was highly significant at (P<0.05).

Table 1: Erythrocytic oxidant marker and antioxidant in terms of mgHb in lactating cows (n=36)

Parameters	Healthy Control	Babesia Positive	Babesia Treated
	Mean±SE	Mean±SE	Mean±SE
LPO (nmol/ml)	293.21±2.162[b]	452.19±10.388[a]	297.26±3.962[b]
GSH (mM/mgHb)	42.45±.462[b]	15.67±.312[a]	40.78±.319[b]
SOD (unit/mgHb)	31.95±.271[b]	23.36±.328[a]	30.09±.162[b]
Catalase(unit/mgHb)	7.24±174[b]	4.72±.191[a]	8.07±.119[b]
Hb(mg/ml)	11.62±.184[b]	3.88±.098[a]	10.03±.190[b]

**Means with different superscript differs significantly at P<0.05.*

Paired t Table

Oxidative Indices	Mean Difference	t- value	df	Are means significant different (<0.05)
LPO	154.9	13.76	35	yes
GSH	-25.11	94.29	35	yes
CATALASE	-3.556	15.09	35	yes
SOD	-6.728	17.43	35	yes
Hb	-6.147	25.43	35	yes

Similarly, GSH values in healthy and ailing cattle were 40.78±0.31 and 15.67±1.8 respectively. SOD value of healthy and infected was 30.09±0.16 and 23.36±0.32 respectively. Catalase was also found 8.07±0.11 and 4.72±0.19 in healthy and infected respectively. The SOD, GSH and Catalase were significantly lower

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in clinical cases than in the corresponding healthy ones (Table 1). The hemoglobin concentration declined significantly at (P<0.05) to 3.88±0.38 in ailing animals from 10.03±0.19 in healthy ones.

Osmotic Fragility of Erythrocytes

The maximum erythrocytic fragility was 92.11±0.18 at 0.2% NaCl concentration, while it was 4.66±0.10 at 0.9%NaCl concentration in ailing cattle as compared to post treated as well as healthy control animals. The erythrocyte fragility at 0.9%, 0.8%, 0.4% and 0.2% NaCl concentration was significantly (P<0.05) higher than the control groups (Table 2). In contrast, MCF in clinical cases was less but insignificant in comparison to healthy and treated groups of animals at NaCl 0.6% concentration.

Table 2: Erythrocyte fragility test in lactating cows with clinical Babesiosis at various concentration of NaCl (n=36)

NaCl Conc. (%)	Healthy Control	Group-I (Infected)	Group-II (Treated)
	Mean ±SE	Mean± SE	Mean ±SE
0.9	0.035±0.002[b]	4.66±0.10[a]	0.044±0.001[b]
0.8	2.43±0.03[b]	13.71±0.14[a]	2.85±0.07[b]
0.6	13.12±0.06[b]	30.9±0.13[b]	15.42±0.09[b]
0.4	84.62±0.24[b]	81.67±0. 15[a]	85.24±0.15[b]
0.2	93.51±0.26[b]	92.11±0.18[a]	95.11±0.15[b]

** Means with different superscript differs significantly at P<0.05.*

Paired t Table

NaCl Conc.%	Mean Difference	t- value	df	Are means significant different (<0.05)
0.9	4.26	44.28	35	yes
0.8	10.87	82.01	35	yes
0.6	102.3	1.178	35	No
0.4	-3.571	14.55	35	yes
0.2	-2.987	13.37	35	yes

Trace Minerals Status

Mean± SE of all the four trace minerals concentration in serum of clinically affected, healthy (n=36) as well as treated animals are shown in Table 3. The copper concentration in infected cattle was significantly (P<0.05) lower (0.31±0.01 µg/ml) than the treated group (0.85±0.20 µg/ml) and healthy group (0.81±0.03 µg/ml). Similarly, Zn concentrations were 0.40±0.02, 0.98±0.02 and1.03±0.06 in infected, treated and healthy groups of animals respectively and significantly lower in infected than healthy and infected groups at (P<0.05). Se concentrations were also found to show the similar trend 0.16±0.01, 0.58±0.03 and0.80±0.08 in infected treated and healthy groups of animals respectively and significantly lower in infected than healthy and infected groups at (P<0.05). The serum concentration of iron (Fe) was 8.83±0.18

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in infected animals which was significantly (P<0.05) higher than the treated group 5.66±0.15 and healthy control 5.0±0.36 µg/ml.

Table 3: Trace mineral concentration in serum of lactating cows with clinical Babesiosis (n=36)

Parameters	Healthy Control	Babesia Infected	Babesia Treated
	Mean±SE	Mean±SE	Mean±SE
Copper(µg/ml)	0.81±0.039[a]	0.31±0.01[b]	0.85±0.20[a]
Zinc(µg/ml)	1.03±0.06[a]	0.40±0.02[b]	0.98±0.02[a]
Selenium(µg/ml)	0.80±0.08[a]	0.16±0.01[b]	0.58±0.03[a]
Iron(µg/ml)	5.0±0.36[a]	8.83±0.18[b]	5.66±0.15[a]

**Means with different superscript differs significantly at P<0.05.*

Paired t Table

Trace Minerals	Mean Difference	t- value	df	Are means significant different (<0.05)
Copper	-0.55	46.05	35	yes
Zinc	-0.678	16.57	35	yes
Selenium	-0.402	28.23	35	yes
Iron	2.665	23.06	35	yes

Discussion

Free radicals are generated continuously by normal metabolic processes, but the rate of production increases during certain inflammatory or other disease conditions (Bernabucci *et al.*, 2005). Oxidative stress occurs either due to more production of free radicals or inadequate presence of antioxidants or a combination of both. Lipid peroxidation is a basic deteriorating change in unsaturated fatty acids of the cellular membranes induced by excessive free radicals (Haliwell and Gutteridge, 1999). Estimation of malonyldialdehyde continues to be a reliable method to assess the degree of oxidative damage to cell membrane, as it is the principal aldehyde formed as a by-product during this process (Gurer *et al.*, 1998). Erythrocytes are highly susceptible to peroxidative damage due to abundance of fatty acids and presence of powerful transition-metal catalyst (Ranjan *et al.*, 2005).

Intraerythrocytic invasion of parasites produce hemolytic effects by increasing lipid peroxidation (Deger *et al*., 2001; Ginsburg *et al*., 1993). Lipid peroxidation is general mechanisms whereby free radicals induced tissue damages occur and implicated under several diverse pathological conditions (Halliwell and Chirico, 1993). The reactive oxidative process in erythrocytes due to the presence of free radicals affects cell structure, hemoglobin and membrane of erythrocytes. The membrane of erythrocytes is rich in polyunsaturated fatty acids, a primary target for reactions involving free radicals, and may allow the erythrocytes vulnerable to oxidative damage (May *et al*., 1998). Bovine babesiosis caused by *B. bovis* involves production of interleukin-1b, interleukin-12, gamma interferon (IFN-γ), tumor necrosis factor-α (TNF-α). These mediators activate mononuclear phagocytes or macrophages to release reactive nitrogen

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intermediates (Shoda *et al*., 2000 and Goff *et al*., 2002). Stich *et al*. (1998) concluded that *B. bovis* infected erythrocytes and a membrane enriched fraction of merozoites stimulate inducible nitric oxide synthase (iNOS) transcription and nitric oxide production by the activated macrophages of cattle. The significant negative correlation between erythrocytic MDA and the anemia indices (including RBC, Hb and PCV values) revealed that membrane lipid peroxidation is main attribute for the reduction of these values and accordingly anaemia, one of the most important cardinal signs of *B. bigemina* infection in cattle (Wright, 1973 and Mahoneye *et al.,* 1977).

In the present study we found marked increase in the values of oxidative marker LPO in the affected cattle by *Babesia* and ticks if compared with healthy control animals (Table 1) which is in agreement with Siemieniuk *et al.* (2008). Decrease in activity of SOD, GSH-PX, and CAT in cattle infected with *Babesia bigemina* in the present study are in agreement with the findings of Hamid *et al.* (2014) in buffaloes and Esmaeilnejad *et al.* (2012) who found a decrease in activity of SOD, GSH-PX, CAT and TAC in sheep infected with *Babesia*.

Oxidative stress is an imbalance between radical-generating and radical-scavenging activity leading to oxidative tissue damage (Nabile, 2003) as a result of increase production of antioxidants owing to increased synthesis or turnover (Celi, 2010 and Ozbilge *et al.*, 2005). Oxidative stress is a general mechanism whereby free radicals induce oxidative damage and reduce the antioxidant defense of biological system (Tskahara, 2007). It protects –SH groups of lipoproteins present in the cellular membrane and haemoglobin from oxidative damages (Kosower *et al.,* 1977). GSH can react with various electrophilic compounds and can effectively scavenge free radicals either directly or indirectly through enzymatic reactions (Fang *et al.*, 2002). Lower levels of GSH in present case might be due to its enhanced utilization for neutralization of excess free radicals generated in these conditions.

SOD and catalase are principal antioxidant enzymes present in mammalian cells. SOD augments the formation of O_2 from reactive oxygen species. A co-product of SOD activity is H_2O_2, which is converted to H_2O by catalase (Fang *et al.*, 2002). In the present study SOD activity was found significantly ($P < 0.05$) lower than control animals. Significant decrease in blood zinc and copper concentration also supported this hypothesis, since it may be due to enhanced utilization of these elements for synthesis of SOD. It is well established that when the risk of oxidative damage increases, endogenous antioxidant protection correspondingly increases (Basha and Rani, 2003). Increased activity of SOD might have resulted in augmentation of H_2O_2 production and thereby increased utilization of catalase for conversion of H_2O_2 into H_2O. This may be the reason behind lower catalase activity in cattle with babesiosis. Increase in SOD activity and decrease in catalase and glutathione peroxidase activities were also reported (Hanzneci *et al.*, 2005).

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The increased mean corpuscular fragility of RBC in infected group provides evidence that erythrocytes of infected cows are under osmotic stress. The median corpuscular fragility (MCF) in affected cattle was insignificantly ($P < 0.05$) lower than those of healthy cattle in 0.6% NaCl concentration and showed significantly ($P< 0.05$) higher erythrocytes fragility in comparison with healthy and treated cattle in 0.9%,0.8%,0.4% and 0.2% NaCl concentration (Table 2). This finding shows that erythrocytes from *Babesia* infected cattle are more susceptible to hemolysis. Increased median corpuscular fragility in erythrocytes of affected cattle indicates injury to red blood cell membrane and consequent alteration in permeability of these cells. Such alteration can result from oxidative stress and lipid peroxidation. The present findings are in accordance with the findings of Saleh (2009), who reported increased erythrocytic fragility in crossbred cattle infected with *Babesia bigemina.* Wright (1973) observed the increased erythrocytic fragility in calves infected with *Babesia bigemina*. Rezaei *et al.* (2006) and Yagi (1989) also reported the same in calves infected with *T. sergenti*. Loss of membrane stability leads to increased RBC osmotic fragility due morphological changes in the cell surface of erythrocytes (Saluja *et al.*, 1999).

Trace elements, such as Cu, Zn and Se are essential components of the body's antioxidant defense that play an important role in preventing free radical mediated damage (Evans and Halliwell, 2001). The Concentrations of Zn, Cu, and Se in plasma/serum are affected by infection, stress, pregnancy, and erythrocyte hemolysis (Kincaid, R. L. 1999). Because erythrocytes in cattle have a 160 days life span, concentrations of trace minerals in whole blood change more slowly than those in plasma in response to changes in intakes of trace minerals. Since, the hemolysis is the major outcome of babesiosis, thus trace minerals have important role in the mitigation of oxidative stress by inducing the synthesis of antioxidant enzymes *viz.* Cu and Zn for SOD and Se for GSH. The Cu and Zn are essential component of SOD which is located in the cell cytosol (McCordand Fridovich, 1969) and contribute to the first line of antioxidant pathway by catalyzing the conversion of O_2 into H_2O_2. Se containing GPX catalyzes the conversion of H_2O_2 to H_2O through the oxidation of R-GSH (Chance *et al.*, 1979). In clinical case of bovine babesiosis, the trace mineral status is expected to vary (Esmaeilnejad *et al.*, 2014) and reported that serum concentrations of copper, zinc, manganese, and selenium showed a marked decrease in infected sheep. In contrast Chaudhari *et al.* (2008) reported low levels of blood iron, zinc and copper in babesiosis in dogs, due to *B. gibsoni*. As per results of this study, low levels of serum trace minerals including copper, zinc and Selenium along with the increased levels of iron in the infected cattle implicate another alternative aspect of oxidative shock.

Thus, it is speculated that the decreased level of trace minerals in the serum of infected cattle proved that Babesia species can pose a marked effect on the mineral nutrients status and also represents their coordinated antioxidant role accompanied by antioxidant enzyme activities during the infection with these parasites. Esmaeilnejad *et al.* (2014) also reported the same pattern of variation in trace minerals in sheep

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infected with *Theileria*. The elevated serum iron in the infected animals can probably be described as hemolytic anemia. In intravascular hemolysis, infected RBCs are directly ruptured inside vasculature and so hemoglobin is degraded to globin, heme and iron, therefore serum iron is increased (Stockham and Scott, 2002).

Conclusion

The present study revealed that clinical symptoms in babesiosis occur as a result of parasitemia as well as oxidative stress caused by piroplasm in RBC. However, possibility of oxidative stress might have predisposed the cattle for developing clinical manifestations which cannot be ruled out at this stage. It may also be concluded that the significant decrease in the level of antioxidant enzymes activities and some associated trace elements accompanied by a significant increase in the lipid peroxidation (LPO) imply that the *Babesia bigemina* can interfere with the protective antioxidant mechanism of RBCs against oxidative injuries and subsequently leads to increased erythrocyte fragility due to oxidative membrane damage resulting in haemolytic anaemia. Further research is needed to explicit the role of oxidative stress in the pathogenesis in bovine babesiosis and possible use of antioxidants for therapeutic or preventive purposes.

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