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Original article

Histopathological changes and oxidative damage in hepatic tissue of rats experimentally infected with *Babesia bigemina*

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Abstract

The present study was aimed to investigate oxidative stress, DNA damage, and histopathological alterations in hepatic tissues of splenectomized Wistar rats experimentally infected with *Babesia bigemina*. Rats were challenged with $5x10^6$ infected erythrocytes. *Babesia* infection was confirmed both with Giemsa's staining blood smears and nested-PCR amplified region of apical membrane antigen-1 (AMA-1) gene. Parasitemia reached approximately 10 % at day 5 post-infection. Livers of infected rats were enlarged and darker in color, became extremely brittle with marked congestion. Microscopic evaluation showed cytoplasmic clearing of hepatocytes and severe hydropic changes with significantly dilated sinusoids containing macrophages and also intrasinosoidal parasitized erythrocytes. Severe infiltration of lymphoplasma cells was also present throughout the liver parenchyma. Furthermore, Kupffer cells were enlarged and, occasionally, containing *Babesia*-parasitized erythrocytes. The activity of Glutathione (GSH) and catalase (CAT), and total antioxidant capacity (TAC) were also significantly decreased ($p < 0.05$) after infection of rats with *B. bigemina*. *B. bigemina* infection also induced a significant increase ($p < 0.05$) in hepatic malondialdehyde (MDA) and nitric oxide-derived products (NOx) concentrations as well as amount of endogenous hepatocytes DNA damage. Hepatic damage was also reflected through the measurement of lactic acid dehydrogenase (LDH) and protein carbonyl content (PCO) in liver cells. These two indices of liver injury were also significantly elevated ($p < 0.5$) during *B. bigemina* infection. Evaluation of correlation between assayed variables in infected rats revealed that MDA levels were positively correlated with PCO, NOx, LDH and DNA damage in the infected group and negatively correlated with GSH, CAT and TAC. There was also an inverse relationship between the antioxidant enzymes activities of GSH, CAT and TAC with PCO, NOx and DNA damage in infected rats. However, NOx showed positive correlation with PCO and DNA damage in infected rats. On the basis of the above results it can be concluded that the *Babesia* infection increases oxidative stress markers, protein carbonyl content and DNA damage and decreases antioxidant enzymes activities in the liver. These results suggest that *B. bigemina* infection could alter the liver histopathology and causes DNA damage following oxidative stress in hepatic tissue. Further studies are needed to precisely define how hepatic tissue damage takes place in *B. bigemina* infection.

Key Words: liver, oxidative damage, rats, antioxidants, Babesia bigemina

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Introduction

Babesiosis is an ixodid ticks-borne malaria-like illness caused by more than 100 species of the intraerythrocytic protozoan *Babesia* which affects many mammalian species and vertebrate hosts specially cattle and human (Spielman et al. 1985). In tropical and subtropical areas, the main species of *Babesia* that cause bovine babesiosis (BB) are *B. bigemina and B. bovis* which are usually transmitted by feeding of one-host *Rhipicephalus* spp. ticks (Terkawi et al. 2012). *B. bigemina* is reported as the most prevalent and main causative agent of bovine babesiosis worldwide that causes a serious and economically important disease in cattle.The classic presentation is a febrile syndrome with apparent anemia and hemoglobinuria. Without treatment some animals may survive after a long convalescent period, but others may develop shock and/or hepatic, renal and lung failure leading to death (Uilenberg 2006).

The liver is one of the most important organs to be affected during *Babesia* infection since it plays a major role in the clearance and destruction of blood infected by various blood parasites (Sardinha et al. 2010). Although oxidative stress has been reported in many parasitic infections such as malaria (Guha et al. 2006), hepatozoonosis (Kiral et al. 2005), tropical theileriosis (Visser et al. 1995), and babesiosis (Stich et al. 1998), but there are still insufficient data on hepatic oxidative damage and antioxidant defense of host liver during *B. bigemina* infection. The present study was therefore designed to investigate the effects of *B. bigemina* on oxidative stress, DNA damage and histopathological alterations of hepatic tissues in a splenectomized rat model with *B. bigemina* infection.

Materials and methods

All procedures in this study were carried out in accordance with the guidelines of the Animal Ethics Committee of Faculty of Veterinary Medicine, Urumia University (AECVU) and supervised by authority of Urumia University Research Council (UURC).

Source of parasite

Parasitized red blood cells containing *Babesia bigemina* was originally obtained from naturally infected cattle. This parasite was maintained in our laboratory in Wistar rats (*Rattus norvegicus*) by intraperitoneal injection of infected blood (in 0.2 ml citrated saline/ /animal).

Experimental animals

Fifteen male Wistar rats (200–220 g), aged 6-8 weeks, were used in this study. Animals were kept in individual cages (40×20×10 cm with five animals per cage) at 25 ±2^{0}C temperature with 70% humidity. The animals were provided from animal house of Laboratory of Physiology of Faculty of Veterinary Medicine of Urumia University, Urumia, Iran. They were fed with commercial rat pellets and had continuous access to water. All the animals were screened for prior malaria and/or *Babesia* infections by blood smear examination. The animals were also treated by diphenhydramine before exposure to *B. bigemina* to prevent potential anaphylaxis.

Rats were randomly divided into two groups, a control group with five and an infected group with ten rats. Splenectomy was performed for each rat in infected group as previously described by Phillips *et al*. (1969). Then the rats were infected by intraperitoneal injection of 1 ml of 5x10^6 parasitized RBC with 0.5% sodium citrate, while the control group received normal saline. The presence and degree of parasitemia were determined daily for each animal by blood film examination.

At day 5 all rats in both groups were sacrificed and multiple samples including blood and liver tissue were obtained from each rat.

Molecular confirmation of *B. bigemina* infection in cattle and infected rats

In order to confirm *B. bigemina* infection, blood samples were subjected to nested-PCR analysis after the diagnosis was established by smear microscopy according to methods previously described by Sivakumar et al. (2012).

Histopathologic evaluation

Liver samples from all rats were fixed in 10% neutral buffered formalin and embedded in paraffin blocks. Then 5 μm sections were obtained from each paraffin block and stained with hematoxylin and eosin (H&E) and examined by an expert pathologist.

Tissue preparation

Liver samples were placed in ice-cold 0.15 M NaCl solution and perfused with the same solution to remove blood cells. Next, the samples were blotted on filter papers, weighed and homogenized under standard condition in 9 ml ice-cold 0.15 M NaCl or 0.25 M sucrose containing 6 μl of 250 μM BHT (butylated hydroxytoluene) in ethanol to prevent new peroxides formation then centrifuged at 500×g for 10 min at 4°C. Finally the

supernatant was diluted with Tris-sucrose buffer for biochemical assays.

Biochemical assays

Glutathione (GSH) was determined chemically in liver homogenate using Ellman's reagent. The method is based on the reduction of Ellman's reagent (5, 5 dithiobis (2-nitrobenzoic acid) with GSH to produce a yellow compound. The chromogen is directly proportional to GSH concentration, and its absorbance was measured at 405 nm and express as mmol/g liver.

Catalase (CAT) activity was measured following 30 min preincubation of the post mitochondrial liver homogenate with 1% Triton X-100. Decrease in the absorbance of 10 mM hydrogen peroxide at 25°C (Aebi 1984) was recorded at 240 nm and the activity was expressed in U/g liver.

Total antioxidant capacity (TAC) in the liver homogenate was measured following the method of Koracevic et al. (2001). In brief, the antioxidants in the sample reacted with a defined amount of exogenous H_2O_2, eliminating a certain amount of the provided H_2O_2. The residual H_2O_2 is then determined by a colorimetric method which through a coupled enzymatic reaction 3, 5-dichloro-2- hydroxyl benzensulphonate converted to a colored product that is then immediately measured at 505 nm. TAC was expressed as mM/g liver.

Lipid peroxidation in the liver homogenate was determined according to the method of Ohkawa et al. (1979) by mixing 1 ml of trichloroacetic acid (10%) and 1 ml of thiobarbituric acid (0.67%) with 0.1 ml of the liver homogenate, followed by heating in a boiling water bath for 30 min. Thiobarbituric acid reactive substances were determined by absorbance at 535 nm and expressed as MDA nanomoles per gram of tissue (nmol/g liver).

The assay of nitrite/nitrate products (NOx) in the liver homogenate was performed according to Green et al. (1982) method. The method was based on reaction of liver homogenate with the Griess reagent. The Griess reagent is based on a two-step diazotization reaction in which acidified nitrite produces a nitrosating agent, which reacts with sulphanilic acid to produce diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine dihydrochloride to form red colored chromophoric azo-derivative at 540 nm. Nitrite value was express as μM/g liver.

Lactate dehydrogenase (LDH) assay was performed in liver homogenate by using a commercial kit based on transformation of pyruvate to lactate by LDH. The final activity was expressed as U/g protein.

Protein carbonyls were measured using Levine et al. method with some modifications. The absorbance of the sample was measured at 365 nm. The carbonyl content was calculated based on molar extinction coefficient of DNPH (ε=2.2×104 cm/M). Protein carbonyl content was express as nmol/g protein.

The hepatocyte DNA damage was analyzed by alkaline comet assay using a similar method described by Kalantari et al. (2012). Comets were scored visually and classified according to the amounts of DNA in tails (Azqueta et al. 2011). Total scores were between 0 and 400 arbitrary units (AU).

Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Statistical evaluation of data was performed using analysis of variances (ANOVA). Normality of data was evaluated with the Kolmogorov-Smirnov test. P-values <0.05 were considered to be statistically significant.

Results

Blood smears prepared from the 10 infected rats showed presence of piroplasms that were morphologically compatible with *B. bigemina*. An expected 211 bp fragment of apical membrane antigen-1 (AMA-1) gene of *B. bigemina* was amplified from all of the infected rats by nested-PCR. On the contrary, there was no amplification of *B. bigemina* DNA from the control rats. None of the rats survived more than 5 days after infections. Daily examination of Giemsa-stained blood films revealed that the maximal parasitemia was about 10 %.

The comparison between control and *B. bigemina* - infected rats showed that liver of infected rats were severely damaged: The organ was enlarged and darker in color as well as became extremely brittle. Microscopic evaluation showed cytoplasmic clearing of hepatocytes and severe hydropic changes with significantly dilated sinusoids containing macrophages and also with marked intrasinosoidal congestion along with parasitized erythrocytes. The lobular inflammation characterized by severe infiltration of lymphoplasma cells was also present throughout the liver, localized in parenchyma. Furthermore, Kupffer cells were enlarged and, occasionally, containing *Babesia*-parasitized erythrocytes (Fig. 1).

The activity of GSH and CAT, and TAC were also significantly decreased ($p<0.05$) after infection of rats with *B. bigemina* (Tables 1). *B. bigemina* infection also induced a significant increase ($p<0.05$) in hepatic MDA and NOx concentrations as well as amount of endogenous hepatocytes DNA damage (Table 1, 2).

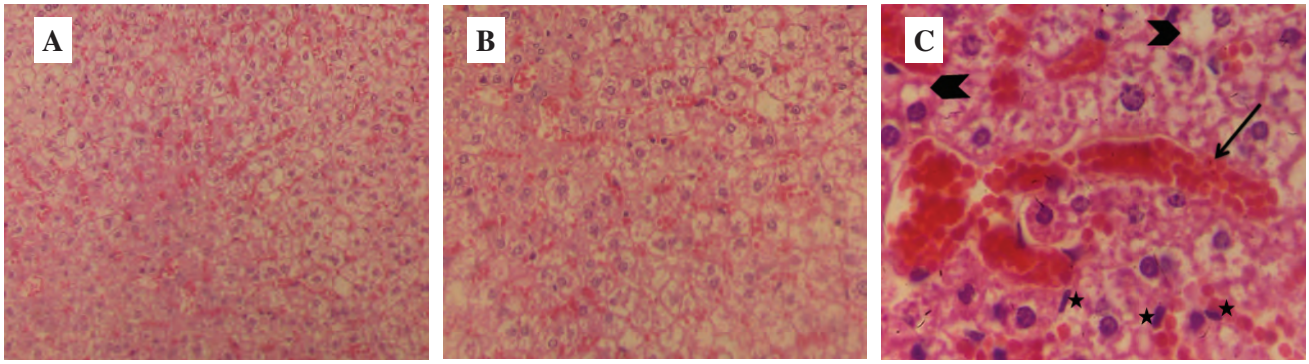

Fig. 1. Liver of rats experimentally infected with *B. bigemina* showing: A) Low power view of liver tissue with severe hydropic change with marked congestion. Liver sinusoidal spaces are filled with blood and RBCs (congestion) (H&E, x10), B) Higher power of view for previous figure showing liver tissue with severe hydropic change, cytoplasmic clearing and liver sinusoidal spaces filled with blood and RBCs (congestion) (H&E, x20) and C) Liver tissue with focus on a sinusoidal space surrounded by deformed Kupffer cells (star) and hepatocytes with marked hydropic changes. The sinusoidal space is congested, filled by RBCs. There are small brown dot-like structures within intrasinusoidal RBCs (*Babesia* elements) (arrow) are also present vacuolation of some hepatic cells (arrow head) (H&E, x 40).

Table 1. Values for glutathione, malondialdehyde, nitrite/nitrate, and total antioxidant capacity in hepatic tissues of normal and *Babesia bigemina*.

	GSH(mmol/g)	CAT(U/g)	MDA(nmol/g)	NOx(μM/g)	TAC(mM/g)
Uninfected	13.9 ± 0.46	5.18 ± 0.01	12.7 ± 0.3	13.1 ± 0.2	2.1 ± 0.4
Infected	9.1 ± 0.52*	1.37 ± 0.01*	19.1 ± 0.1*	21.3 ± 0.1*	0.83 ± 0.3*

Table 2. Changes of lactic acid dehydrogenase activity, protein carbonyl content and DNA damage in hepatic tissue of rats infected with *Babesia bigemina.*

	LDH(U/g protein)	PCO(nmol/g protein)	DNA Damage
Uninfected	165 ± 3.8	7.2 ± 0.12	0.5 ± 0.2
Infected	257 ± 1.2*	12.4 ± 0.22*	2.2 ± 0.9*

Hepatic damage was also assessed through the measurement of LDH and protein carbonyl content in liver cells. These two indices of liver injury were also significantly elevated ($p<0.5$) during *B. bigemina* infection (Table 2).

he correlation coefficients obtained between biochemical parameters in the infected rats are presented in Table 3. Evaluation of correlation between assayed variables in infected rats revealed that MDA levels were positively correlated with PCO, NOx, LDH and DNA damage in the infected group ($r= 0.751$, $p<0.05$; $r=0.986$, $p<0.05$; $r=0.641$, $p<0.05$ and $r=0.766$, $p<0.05$; respectively) and negatively correlated with GSH, CAT and TAC ($r=-0.211$, $p<0.05$; $r=-0.219$, $p<0.05$; $r=-0.217$, $p<0.05$; respectively). There was also an inverse relationship between the antioxidant enzymes activities of GSH, CAT and TAC with PCO, NOx and DNA damage in infected rats. However, NOx showed positive correlation with PCO and DNA damage in infected rats ($r=0.811$, $p<0.05$ and $r=0.873$, $p<0.05$; respectively).

Discussion

Our findings have shown that intraperitoneal inoculation of rats with 5×10^6 *B. bigemina* - parasitized erythrocytes, caused parasetemia and death of rats up to five days from infection. *B. bigemina* infection induces clinical state of fever, shivering and shock in the rat, but unlike the bovine natural host, the course and acuity of infection observed here were more rapid and mostly fatal. These observations were in accordance with the findings by Hamoda et al. (2014).

Animals with *Babesia* spp. infection presented with acute febrile hemolytic anemia. The anemia following *Babesia* infection was attributed to destruction by immunomediated phenomena by autoantibodies directed against component of membrane of infected and uninfected erythrocytes, production of toxic hemolytic factors of the parasite, mechanical damage by trophozoite intra-erythrocytic binary fission, erythrophagocytosis, through the release vasoactive molecules such as kallikrein and increase in host erythrocyte permeability (Esmaeilnejad et al. 2014).

Table 3. Correlation coefficients obtained between biochemical parameters in the infected rats.

Parameter	MDA	GSH	CAT	TAC	PCO	NOx	LDH	DNA damage
MDA PC[a]	____	-0.211	-0.219	-0.217	0.751	0.986	0.641	0.766
GSH PC	-0.713	____	0.878	-0.883	-0.794	-0.771	-0.851	-0.786
CAT PC	-0.763	0.786	____	0.787	-0.991	-0.857	-0.797	-0.779
TAC PC	0.793	0.784	0.795	____	-0.881	-0.887	-0.778	-0.891
PCo PC	0.714	-0.695	-0.700	-0.715	____	0.916	0.837	0.766
NOx PC	0.767	-0.656	-0.618	-0.697	0.811	____	0.916	0.873
LDH PC	0.864	-0.631	-0.653	-0.714	0.797	0.868	____	0.914
DNA damage PC	0.826	-0.643	-0.678	-0.698	0.803	0.786	0.903	____

[a] Pearson Correlation

All together, *Babesia* spp. parasites destructed the parasitized erythrocytes by the invasion mechanism, including attachment, re-orientation, tight junction, invasion, internalization, lysis of parasitophorous vacuoles (PV) of the *Babesia* species and eventually destruction of infected erythrocyte (Yokoyama et al. 2006). Our structural observations showed pathological changes characterized by severe congestion of hepatic blood vessels and sinusoids and inflammation responses. Moreover, hepatic tissue showed cytoplasmic vacuolation, which is mainly a consequence of the considerable disturbances in lipid inclusions and fat metabolism occurring during pathological stress (Zhang et al. 1984, Abd-Elmaleck et al. 2015). These pathological changes observed in examined livers in this study are in agreement with those reported earlier (Dkhil et al. 2010, Okla et al. 2014). According to the present study, perivascular as well as parenchymal mononuclear leukocytic cellular infiltrations are mainly eosinophils, lymphocytes, plasma cells and histiocytes. Enlargement of Kupffer cells was also observed in the liver sections of the infected rats. This situation is probably due to increased activity of liver mononuclear phagocyte system and implementation of phagocytosis as a protective mechanism in order to remove pathogens during of infection. Similar findings have been reported by previous investigations which studied the immunity and histopatological changes of *B. divergens*, *B. mocroti*, *B. bigemina* and *B. Cameli* in the liver of rats (Ben Musa and Dawoud 2004, Hamoda et al. 2014, Okla et al. 2014, Abd-Elmaleck et al. 2015). Studies of molecular immunology done on tissue collected from mice and rats infected with *B.microti* corroborated an increase in pro-inflammatory cytokine mRNA expression (Shimamoto *et al.* 2012, Okla et al. 2014). Furthermore, hepatocytes manifested with severe cytoplasmic hydropic degeneration, which is mainly outcome of ischemia and considerable disturbances in lipid inclusions and fat metabolism occurring during pathological stress (Zhang and Wang 1984, Ebaid et al. 2007).

Over-production of oxygen and nitrogen free radicals and subsequently failure of normal defense mechanisms and decreased antioxidant levels have a role in the pathogenesis of various parasitic infections including *Babesia*, *Leishmania*, *Hepatozoon*, *Ehrlichia*, *Theileria,* and *Plasmodium* (Thurnhann et al. 1988, Asri-Rezaei and Dalir-Naghadeh et al. 2006, Kumar et al. 2006, Esmaeilnejad et al. 2012). Reactive oxygen and nitrogen species (ROS and RNS) may adversely affect cell survival because of membrane damage through the oxidative damage of lipid, protein and irreversible DNA modification, thus, leading to worsening of the liver function (Váli et al. 2006). The results shown in this study indicated that infection of rats with *B. bigemina* is accompanied by induced oxidative and nitrosative stress by production of ROS and RNS in

liver as evidenced by the decreased GSH, CAT and TAC concentration as well as increased production of MDA and nitrite/nitrate and hepatocyte DNA damage.

GSH is a potent endogenous and nonenzymatic antioxidant which protects the important cellular components from oxidative damage by scavenging various types of reactive radicals. As a consequence of GSH deficiency, a number of related functions may be impaired greatly reducing ability to detoxify, increasing DNA damage and inability to its repair, weakened cell membranes, protein biosynthesis and immune function (Hayes and McLellan 1999, Annuk 2001). This result is in agreement with the results reported that the GSH concentration fell in animals infected with parasite species (Hayes and McLellan 1999, El-Sokkary et al. 2002, Bicek et al. 2005, Kolodziejczyk et al. 2005, Ince et al. 2010) and suggests that *Babesia* infection causes a significant depletion of the antioxidant reserve of the host (Esmaeilnejad et al. 2014).

According to the results of this study, catalase activity in hepatic tissue of infected rats significantly decreased. It has been reported that catalase is important in the defense of liver and spleen tissue in *B. divergence* - infected gerbils against H_2O_2 generating reactions (Dkhil et al. 2013). The results of our study indicated that catalase might be acting to scavenge H_2O_2 for the protection of liver infected by *Babesia*. Depleted level of TAC in the infected rats may probably be ascribed to generation of large quantities of ROS during *B. bigemina* infection then subsequently consumption of antioxidant enzymes as free radical scavengers during the oxidative process.

In this study, higher levels of MDA as well as a significant rise in PCO content of liver occurred in infected rats. In accordance of our findings, high level of MDA and significantly increased formation of protein carbonyls has been reported in *B. divergens*-infected gerbils (Dkhil et al. 2013). Our results demonstrated that, *Babesia* infection causes lipid peroxidation and alterations in structure and function of proteins induced by free radicals (Marks et al. 1996). Increased levels of MDA and PCO content have also been reported in *B. gibsoni*, *B. ovis* and *B. bigemina* infection (Murase et al. 1995, Chaudhuri et al. 2008, Kucukkurt et al. 2014) and in *Theileria equi* infection (Gopalakrishnan et al. 2015, Radakovic et al. 2016). During experimental infection with blood parasites and liver fluke, there is an increase in the production of hepatic alanine aminotransferase (ALT) and aspartate aminotransferase (AST), indicating hepatocyte lesions (El Hag et al. 1994, Kolodziejczyk et al. 2005, Dkhil et al. 2010). Furthermore, the lactic acid dehydrogenase (LDH) enzyme is considered to be a specific marker of liver damage (Kikkawa et al. 2005), its level is increased in case of tissue oxidative stress (Wright et al. 1981, Yeruham et al. 1998, Jovanovic et al. 2010, Radakovic et al. 2016) reported the elevation of LDH in cattle, sheep and horses due to infection with *B. bovis*, *B. ovis* and *T. equi*, respectively.

Infection with some pathogens could act as genotoxic stress to cells (Murray et al. 1982, Scory et al. 2007). Numerous studies have reported increased DNA damage in animals infected with parasites (El Sayed and Aly 2014). To our knowledge, there are no reports regarding induced DNA damage of hepatocyte in rat with babesiosis. In the present study, rats infected with *B. bigemina* showed higher degreeof hepatocyte DNA damage compared to non-infected rats. The results of this study are in conformity with the results of most other studies, yet it also showed that *Babesia* causes significant damage to DNA (Dkhil et al. 2013, Kucukkurt et al. 2014). DNA damage we observed in rats hepatocyte can be related to hydroxyl radicals (OH•) produced in parasitic infections (Jackson and Loeb 2001) which are capable of reacting with DNA nitrogen bases and forming critical biomarkers of oxidative stress, such as 8- hydroxyguanine (8-OHG) (Ciftci et al. 2014).

In this study, the negative correlation between MDA with GSH, CAT and TAC suggests that the increased lipid peroxidation leads to inactivation of the enzymes by crosses linking with MDA; this will cause an increased accumulation of superoxide, H_2O_2 and hydroxyl radicals which could further stimulate lipid peroxidation (Ji 1995, Grewal et al. 2005, Hafize et al. 2007).

Our correlation study indicated that there is negative correlation between antioxidant enzymes activities with PCO, NOx and DNA damage in the liver of infected rats. This correlation finding conformed and supported the rapid consumption and exhaustion of stores of this enzyme in fighting free radicals generated during *B. bigemina* infection (Delic et al. 2010).

According to our result, NOx concentration was positively correlated with PCO and DNA strand breaks in infected rats. This observed relationship could be attributed to modification of proteins by formation of carbonyls (aldehydes, ketones) on the side chains of amino acids e.g. Lys, Arg, Pro, Thr. Also, nitric oxide--induces DNA damage via the N_2O_3 pathway (Burney et al. 1999, Aruna Kulkarni and Narayan Madrasi 2008).

In conclusion**,** *B. bigemina* - induced hepatic tissue damage which caused a state of oxidative shock leading to the alteration of liver histopathology as well as DNA and protein structure that may play an imperative role in the pathogenesis of hepatic failure in babesiosis caused by *B. bigemina*. However, further studies are needed to precisely define how hepatic tissue damage develops in *B. bigemina* infection.

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