Journal of Equine Veterinary Science 35 (2015) 763–767

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Original Research

Oxidative Damage Inflicted by *Theileria equi* on Horse Erythrocytes When Cultured In Vitro by Microaerophilous Stationary Phase Technique

Arumugam Gopalakrishnan [a,b], Chinmoy Maji [a], Rajesh K. Dahiya [a], Abhinav Suthar [a,b], Rajender Kumar [a], Sanjay Kumar [a,*]

[a] *ICAR-National Research Center on Equines, Hisar, Haryana, India*
[b] *Division of Veterinary Medicine, Indian Veterinary Research Institute, Izzatnagar, Bareilly, Uttra Pradesh, India*

ARTICLE INFO

Article history:
Received 23 February 2015
Received in revised form 22 May 2015
Accepted 27 July 2015
Available online 31 July 2015

Keywords:
Theileria equi
Oxidative stress
Osmotic fragility
MDA
MASP

ABSTRACT

The aim of present study was to assess the osmotic fragility and oxidative stress in horse erythrocytes infected with *Theileria equi* in in vitro culture using microaerophilous stationary phase (MASP) technique. Malondialdehyde (MDA) is biomarker of oxidative damage which is indirectly indicative of lipid peroxidation of erythrocytic membrane. The continuous MASP culture of *T. equi* was established for evaluating the oxidative damage in in vitro condition. *Theileria equi*–infected erythrocytes (*i*RBC) were collected from in vitro cultures at different parasitemia (1%–10%). *Theileria equi*–uninfected erythrocytes (*u*RBC) were also collected for control studies. Malondialdehyde concentrations in *i*RBC and *u*RBC were evaluated. A nonsignificant ($P < .001$) difference in MDA concentration between *u*RBC (339.94 ± 0.80 μM/mL) and 1%–10% of *i*RBC (357.48 ± 0.82 μM/mL) was observed. After 1% of *T. equi* parasitemia, a sequential significant ($P < .001$) increase in MDA levels was observed coinciding with increasing *T. equi* parasitemia. Similarly, osmotic fragility of *i*RBC also increases with rise in *T. equi* parasitemia. Percent hemolysis of *i*RBC increased from 13.89% to 26.40% at 1% to 10% parasitemia in 0.85% of sodium chloride solution. The results of this study demonstrated that horse erythrocytes when infected by *T. equi* in in vitro condition undergo oxidative damage and osmotic fragility, which increased with increasing parasitic load and may be a contributing factor in pathogenesis process of this disease condition.

1. Introduction

Equine piroplasmosis is an intraerythrocytic tickborne hemoprotozoan disease, caused by *Theileria equi* (small form) and/or *Babesia caballi* (large form), affecting equines of all age groups. Both of these parasites are being transmitted by about 14 species of ixodid ticks in the genera *Dermacentor*, *Hyalomma*, and *Rhipicephalus* spp. The infection caused by *T. equi* parasite is more widespread and pathogenic. The animal infected with *T. equi* parasite showed decrease in the performance activity might be due to less tissue oxygenation caused by infecting parasite leading to destruction of red blood cells (RBCs) [1].

In most of the protozoan disease, oxidative stress plays a major role in pathogenesis process of the disease condition. Oxidative stress is a result of imbalance between generated free radical and scavenging activity of cells, leading to unattended oxidation products [2]. These free oxygen radicals cause alteration in cell membrane phospholipids resulting to disruption of the cellular structure and its function [3]. The polyunsaturated fatty acids are important cell membrane phospholipids and are highly sensitive to lipid

* Corresponding author at: Sanjay Kumar, ICAR-National Research Centre on Equines, Sirsa Rd, Hisar 125 001, Haryana, India.
E-mail address: kumarsanjay66@yahoo.com (S. Kumar).

0737-0806/$ – see front matter © 2015 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jevs.2015.07.020

peroxidation (LPO) during oxidative stress condition [4]. Malondialdehyde (MDA) is a most reliable and commonly used indicator for estimation of oxidative damages by LPO [5,6].

Oxidative stress has been documented in parasitic infection conditions such as babesiosis in dogs [7], *Leishmania chagasi* infection [8], and *Thieleria annulata* infections in calves [9]. The parasite-infected erythrocytes produce reactive oxygen free radicals impeding the biochemistry of RBCs and may contribute to the pathogenesis process of the disease condition. Determination of MDA allows detection of the degree of LPO and the level of free oxygen radicals indirectly [10]. Further oxidation of the erythrocytes causes alteration in its membrane rendering to osmotic fragility and cell destruction [11]. So far, no reports are available about the estimation of oxidative damage in *T. equi*–infected erythrocytes cultured in vitro by microaerophilous stationary phase culture system (MASP) technique. The present study was carried out to evaluate indicator of oxidative damage and LPO to erythrocytes (i.e., MDA) and their contributory role for causing osmotic fragility during in vitro developmental cycle of the *T. equi* in MASP system at different parasitemia.

2. Materials and Methods

2.1. Parasites

A *T. equi* [12] latently infected pony has been maintained at the large animal house facility of the National Research Centre on Equines, Hisar. The whole blood from this pony was collected in a vacutainer and processed for in vitro cultivation of *T. equi* by MASP techniques.

2.2. In Vitro Cultivation of T. equi and Evaluation of Parasite Growth

The in vitro cultivation medium for *T. equi* parasite consisted of Medium M199 (Sigma-Aldrich) supplemented with 40% defibrinated horse serum, antibiotic solution (containing 60 U/mL penicillin and 60 mg/mL streptomycin), and 200 μM hypoxanthine [13,14]. Packed RBCs were washed three times with Vega Y Martinez buffer, and 100 μL was dispensed in wells of 24-well plate containing 1.0 mL of cultivation medium. The 24-well plate was incubated in a double gas incubator that maintains inner chamber temperature at 37°C with microaerophilic atmosphere of 5% CO_2, 3% O_2, and 95% N. The overlaid supernatant medium in the wells of 24-well culture plate was replaced daily with cultivation medium until live *T. equi* parasites start appearing on blood smear examination. The seed cultures were subcultured further once *T. equi* parasitemia reached 2% to 3%. Subculturing was done by splitting the *T. equi*–infected erythrocytes in other fresh wells of 24-well culture plate having uninfected naive horse erythrocytes suspended in 1.0 mL of cultivation medium. *Theileria equi*–infected erythrocytes and uninfected naive horse erythrocytes final volume were kept at 100 μL.

Primary evaluation of parasite growth in the MASP culture system blood smears was prepared from each well on different days of culture showing increasing level of *T. equi*. These blood smears were Giemsa stained and examined under oil immersion lens of microscope at ×1,000 magnification. Qualitative and quantitative counting of *T. equi*–infected erythrocytes was performed, and for this purpose, at least 1,000 erythrocytes were counted in different field. *Theileria equi*–infected erythrocytes were collected at different level of parasitemia (1%–10%); the washed and pelleted erythrocytes were used for analysis of osmotic fragility assay and for estimation of oxidative damage by evaluating MDA levels.

2.3. Osmotic Fragility Assay

This assay was performed on *T. equi*–infected erythrocytes (*i*RBC) collected at different parasitemia (1%–10%) from MASP in vitro culture system. Different concentrations of sodium chloride solutions (NaCl, from 0.00% to 1.0%) were used in this assay. Osmotic fragility assay was performed as per method described by Tietz [15]. Briefly, 200 μL of each concentration of NaCl was added to the duplicate wells of enzyme-linked immunosorbent assay (ELISA) plate, and 2 μL of each *i*RBC representing respective *T. equi* parasitemia (1%–10%) was added to each separate concentration of NaCl, as above. Uninfected normal horse erythrocytes (*u*RBC) were taken as control, and 2 μL of these erythrocytes were also added separately to the 200 μL of each duplicate concentration of NaCl in ELISA plate. These ELISA plates were incubated at room temperature for 30 minutes and thereafter were centrifuged (×2,500 rpm for 10 minutes). The respective 100-μL supernatant from each well of ELISA plate was transferred to a fresh ELISA plate. The optical density of this ELISA plate was read in ELISA plate reader at 540 nm. Percent hemolysis in *i*RBC collected at different *T. equi* parasitemia against respective concentration of NaCl was calculated as below:

$$\%\ Hemolysis = 100 \times \frac{Average\ OD\ of\ wells\ representing\ individual\ T.\ equi\ parasitaemic\ RBC\ against\ respective\ concentration\ of\ NaCl}{Average\ OD\ of\ the\ wells\ representing\ 100\%\ lysis\ with\ respective\ T.\ equi\ parasitaemic\ RBC}$$

Hemolysis was expressed as percentage, and 100% hemolysis was determined from the absorbance of the distilled water (0.0% NaCl). A scattered line graph was plotted between percent hemolysis in *i*RBC at respective *T. equi* percent and respective NaCl concentration.

2.4. Estimation of MDA

Malondialdehyde, which is a biomarker of LPO and its concentrations, was measured in *T. equi*–infected erythrocytes (*i*RBC) as per Okhawa et al [16]. Briefly, 200 μL of *i*RBCs (collected at different *T. equi* parasitemia) or *u*RBC collected from MASP in vitro culture and 1.8 mL of 1.15% potassium chloride was added to each test sample. Each sample was applied in triplicate for the MDA assay; 0.2 mL from these samples was transferred to fresh tubes, and 0.2 mL of potassium chloride (1.15%) was also taken in separate tubes as blank. These tubes were processed further by sequential addition of 0.2 mL of sodium dodecyl sulfate solution (8.1%), 1.5 mL acetic acid solution (20%, pH 3.5), and finally 1.5 mL of 2-thiobarbituric acid (0.8% aqueous solution). The contents were thoroughly mixed, and the final volume was made to 4.0 mL with distilled water. These test tubes were kept in water bath (95°C) for 60 minutes and cooled immediately after incubation; 1.0 mL of distilled water was added to each tube, and thereafter, 5 mL of n-butanol plus pyridine (15:1 vol/vol) solution was added to each tube and tubes were shaken vigorously. The tubes were centrifuged at 4,000*g* for 10 m, and upper organic pink color layer was collected from each tube and absorbance was read at 532 nm against blank.

Different concentrations (5, 10, 15, 20, 25, and 30 nM/mL) of 1,1,3,3-tetramethoxy propane (TMP) solution were made in 1.15% of potassium chloride. 1,1,3,3-Tetramethoxy propane concentrations were used as a standard for estimation of MDA levels in the samples; 0.2 mL of these concentrations were processed separately as described previously, and optical density (OD) of the final upper organic pink color layer was taken at 532 nm (OD_{532}) and used for derivation of regression linear line and equation ($y = a + bx$, where y, concentration of MDA (μM); x, OD of the sample; a, the constant; and b, the regression coefficient) for calculation of concentration of MDA in *i*RBC or *u*RBC.

2.5. Statistical Analysis

The one-way analysis of variance followed by the Newman Keuls test was computed to know the concentration of MDA in uninfected cultures and those infected with *T. equi* with different parasitemia rates. The P values $<.001$ were considered statistically significant differences between the different parasitemia rates and control uninfected cultures.

3. Results

3.1. In Vitro Cultivation of T. equi

Different developmental stage of the *T. equi* parasites were observed in in vitro culture system which included—pair pear shaped, trophozoites (oval, round, elliptical, or spindle shaped), and tetrad or Maltese cross form (Fig. 1A). The maximum parasitemia observed was 9% to 10%. *Theileria equi*–infected erythrocytes were collected from these cultures at different level of parasitemia—1%, 2%, 5%, 7%, and 10%—and processed for osmotic fragility assay and MDA estimation.

3.2. Osmotic Fragility Assay

Percent hemolysis curve at high *T. equi* parasitemia shifted to the right, indicating hemolysis of the erythrocytes even at high NaCl concentration (Fig. 1B). This curve sequentially shifted to left with the decrease in the *T. equi* parasitemia. At high *T. equi* parasitemia (10%), the percent hemolysis at 0.85% NaCl concentration was 26.40%, which sequentially decreased to 13.89% at 1% *T. equi* parasitemia, whereas it was 9.05% in the control *u*RBC (Fig. 1). These observations have indicated that at high *T. equi* parasitemia, infected erythrocytes were more fragile, as compared with low parasitemic-infected erythrocytes.

3.3. MDA Estimation in iRBC and uRBC

A regression equation was derived between different concentrations of TMP (MDA standard) and their respective OD_{532}, which is $Y = 8.0333 + 660.76x$. The concentrations of MDA in uninfected and infected cultures with *T. equi* with different percent parasitemia are presented in Table 1. The results of this study indicated that the MDA concentration in uninfected culture erythrocytes (339.94 ± 0.80 μM/mL) differs significantly from the erythrocytes collected at different *T. equi* parasitemia. A significant rise in MDA level was observed coinciding with increasing *T. equi* parasitemia. At 10% parasitemia, *i*RBCs showed significant rise ($P < .001$) in MDA (639.99 ± 0.92 μM/mL) as compared with 1%, 2%, 5%, and 7% parasitemia and uninfected RBC cultures MDA as 357.48 ± 0.82, 389.91 ± 0.66, 470.91 ± 0.54, 537.15 ± 0.54, and 339.94 ± 0.80 μM/mL, respectively (Table 1).

4. Discussion

Theileria equi–infected horses stay lifelong carrier to infection without demonstrating any clinical manifestation of the disease condition and act as nidus to other healthy equids. Disease condition caused by *T. equi* parasite is generally more pathogenic than by *B. caballi*, and during acute clinical infection in natural host, a significant increase in erythrocyte membrane protein, lipid, and plasma MDA has been observed, indicating marked alteration in biochemical composition of the infected erythrocytic membrane [17]. Lipid peroxidation contributes to pathogenesis of many parasitic diseases [18,19]. Polyunsaturated fatty acid, an integral component of erythrocyte membrane, is highly sensitive to oxidative damage rendering the infected erythrocytes for further biochemical alteration [6,17,19]. Malondialdehyde is the end product of LPO and indicative biomarker. Increasing *T. equi* parasitemia enhances the accumulation of oxidative ions in erythrocytes, resulting LPO and increased concentration of MDA. So, the present study was designed to assay the oxidative damage of erythrocytes infected with *T. equi* in in vitro MASP study model.

We successfully established in vitro cultivation system for *T. equi* parasite, and all the merozoite developmental stages were observed (Fig. 1A). The MDA concentration in *u*RBC (339.94 μM/mL) was

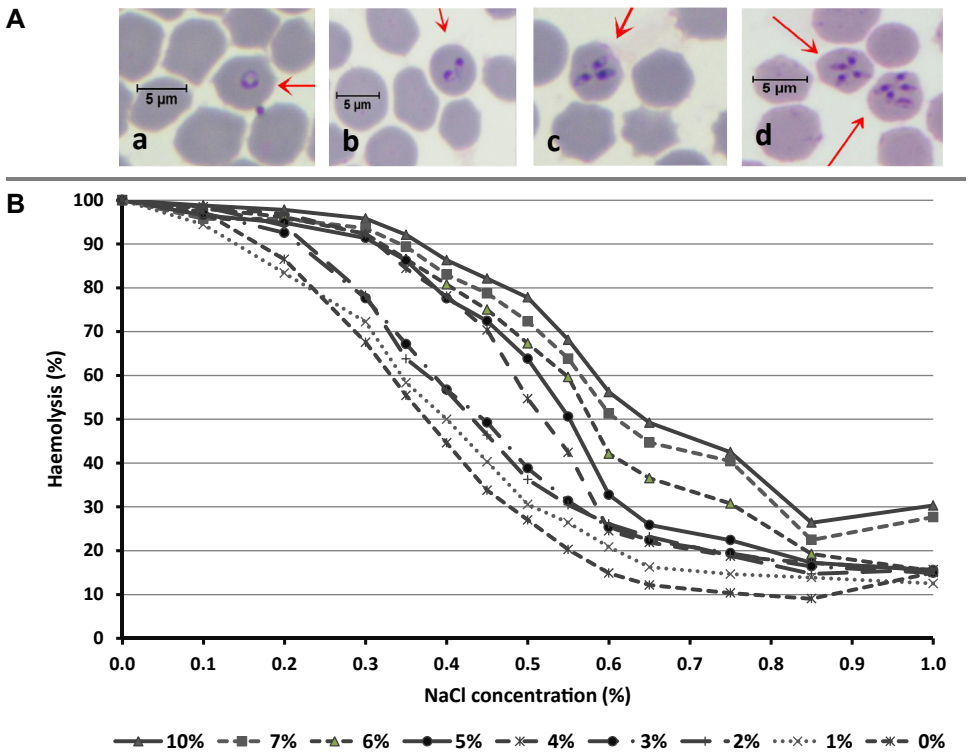

Fig. 1. (A) Microphotographs showing *Theileria equi*–infected erythrocytes collected at different parasitemia from in vitro cultures. Different forms of *T. equi* parasites are shown—single (a) or pair pear-shaped trophozoites (b) and tetrad or Maltese cross form (c, d). (B) Osmotic fragility assay curve showing percent hemolysis in in vitro cultured *T. equi*–infected erythrocytes at different parasitemia against respective sodium chloride (NaCl) concentrations. Each point represents mean value of respective percent hemolysis.

significantly lower than observed in 1% *i*RBC (357.48 µM/mL). Malondialdehyde levels increased significantly ($P < .001$) onward rising to maximum concentration (639.99 ± 0.92 µM/mL) at 10% *T. equi* parasitemia in *i*RBC. Mohan et al [20] also observed significant increased MDA levels in in vitro cultured *Plasmodium falciparum* in human erythrocytes.

Observations on osmotic fragility assay also indicated that at higher *T. equi* parasitemia, infected erythrocytes became more fragile entailing to high percent hemolysis (Fig. 1B). Results of osmotic fragility assay further supported the findings of increased MDA levels with the rise in *T. equi* parasitemia in in vitro cultured erythrocytes. Increased percent hemolysis values evidently indicated that high *T. equi* parasitemic erythrocytes are under osmotic stress leading to oxidative alterations. Saleh [21] observed high corpuscular osmotic fragility in erythrocytes infected with *Babesia bigemina* collected from cattle. Similarly, Ambawat et al [17] also observed increased in plasma MDA levels in donkeys experimentally infected with *T. equi*, whereas Shiono et al [22] and Saleh [21] demonstrated increased MDA concentrations in erythrocytes infected with *T. sergenti* and *B. bigemina*, respectively, collected from diseased cattle. There was no report on analysis of oxidative stress and osmotic fragility on the *T. equi*–infected erythrocytes, and this study has successfully demonstrated existence of oxidative stress on the *T. equi*–infected erythrocytes and its assessment by measuring MDA concentration and osmotic fragility. This study may contribute in assessment of oxidative damage in *T. equi* naturally infected equids by analyzing MDA levels and hence would help in evaluating severity of disease condition.

It can be inferred from this study that normal horse erythrocytes when infected by *T. equi* in in vitro condition undergo oxidative damage and osmotic fragility, which increases with increasing parasitic load and may be a contributing factor in pathogenesis process of this disease condition.

Table 1
Concentration of malondialdehyde (MDA) in uninfected and *Theileria equi*–infected erythrocytes at different parasitemia cultured in vitro by MASP technique.

Theileria equi Parasitemia as Observed in Erythrocytes (%)	MDA (µM/mL) (Mean ± SE)
0 (Uninfected)	339.94 ± 0.80[a]
1	357.48 ± 0.82[b]
2	389.91 ± 0.66[c]
5	470.91 ± 0.54[d]
7	537.15 ± 0.54[e]
10	639.99 ± 0.92[f]

Abbreviations: MASP, microaerophilous stationary phase; SE, standard error.
[a,b,c,d,e,f]Values with different superscript letters indicate significant differences ($P < .001$) between different parasitemia and control uninfected cultures.

Acknowledgments

The authors wish to acknowledge their gratitude to the Director, ICAR-National Research Centre on Equines (Indian Council of Agricultural Research), Hisar, Haryana, India, for providing all the necessary facilities for conducting this study and to the Head, Division of Veterinary Medicine, ICAR-Indian Veterinary Research Institute, Izatnagar, for managing the administrative matters of the first author as a student of the division. The authors declare that they have no conflict of interest.

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