RESEARCH ARTICLE

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Effect of treatment with trypanocides on *Trypanosoma brucei* induced oxidative stress and antioxidant enzyme activities in dogs

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Abstract

Oxidative stress and alteration of endogenous antioxidant enzymes play roles in the pathophysiological mechanism of trypanosomosis. The oxidative stress marker: malondialdehyde- MDA and antioxidative stress markers: Serum catalase- CAT, Serum Reduced Glutathione -GSH-Rd and Serum Superoxide dismutase- SOD of Nigerian local dogs (NLD) experimentally infected with *Trypanosoma brucei* were evaluated after treatments with diminazene aceturate (DA) and isometamidium chloride (IMC). Twenty dogs of age 3 – 4 months were assigned to any of the four groups of five dogs each as follows: 1 = infected and treated with DA (7.0 mg/kg); 2 = uninfected untreated; 3 = infected and untreated; 4 = infected and treated with IMC (0.5 mg/kg). DA and IMC cleared the parasites from the blood, following treatment of the dogs. Relapse was recorded in two dogs in group 1 and one dog in group 4 on days 35 and 56 post-infection (PI) respectively. No dog died except one in group 1. The levels of malondialdehyde- MDA increased significantly by day 7 post-infection in all the infected groups. However, by day 14 post-infection the malondialdehyde levels in group 4 became similar with group 2. The MDA level in group 1 remained significantly higher than in group 2. As from days 7 – 14 post-infection Catalase, Reducedg glutathionend superoxide dismutase levels in the infected groups were significantly lower than group 2. Nevertheless, both trypanocides did not return the levels of CAT, GSH, and SOD to pre-infection values before the termination of the experiment. The findings suggested that the two trypanocides could neither reverse the induced oxidative stress nor normalize the antioxidant capacity of the dogs infected with *T. brucei.*

Introduction

The effects of free radical and oxidative stress on tissues and cells have been leading topic in a significant number of studies in human medicine for over 40 years (Salvador & HendrqueHendriquwes, 2004). More recently, this subject has been gaining ground in veterinary medicine and there are many possibilities for research on the correlation of oxidative stress to diseases. It is a vast field yet to be explored in veterinary sciences.

There are currently lines of scientific evidence showing that many diseases are related to oxidative stress caused by reactive oxygen species (ROS) or reactive nitrogen species (RNS). The most severe damages are the ones affecting the DNA or RNA of the cells. Oxidative stress and alteration of endogenous antioxidant enzymes play roles in the pathophysiological mechanism of trypanosomosis.

Oxidative stress is detected through malondialdehyde (MDA) and isoprostane quantification in addition to the determination of the concentrations of antioxidant agents such as vitamin E and glutathione peroxidase (Winter *et al.*, 2009). Some diseases of dogs that are related to oxidative stress and antioxidative stress marker quantifications include canine lymphoma, mammary tumors (Szuzzubial *et al*., 2004; Neto *et al*., 2005; Ascar *et al.,* 2009; Galvao, 2009), kidney disease and canine cardiopathies (Freeman *et al*., 2005). Infectious conditions associated with oxidative stress in dogs include cutaneous conditions such as demodicosis (Romanucci *et al.,* 2011). In reproduction, oxidative stress has been associated with ovariohysterectomy.

Few studies have associated oxidative stress with canine trypanosomosis (Omobowale *et al*., 2015), but none have demonstrated the ameliorative effect of diminazene aceturate (DA) or isometamidium chloride (IMC) on the oxidative stress induced in trypanosomosis. Diminazene aceturate and Isometamidium chlorideare the two commonest drugs used in managing trypanosomosis. Hence, this work was therefore aimed at comparing the ameliorative effect of these two drugs on experimental *T. brucei* induced oxidative stress in dogs.

Materials and Methods

Experimental animals

Twenty growing mongrel dogs aged between 3 and 4 months were purchased from Orie Orba market in Nsukka area of Enugu State, Nigeria for the study. They were housed in a fly proof – kennel at the animal house of the Department of Veterinary Parasitology and Entomology of the University of Nigeria, Nsukka. They were acclimatized for four weeks during which they were dewormed and deticked with Ivermectin (ivomec, Merck sharp and dohmes; BV. Hearlem, Holland) at the dose of 0.2 mg/kg subcutaneously. They were fed once daily with food procured from the restaurant but supplemented with a meal of garri and okro soup or stew rice cooked with beans prepared by the attendant. Water was provided *ad libitum* throughout the experiment. They were screened and confirmed negative for trypanosome by buffy coat method (Murray *et al*., 1977) before the commencement of the experiment. They were also vaccinated against rabies with anti-rabies vaccine and polyvalent vaccine against parvovirus, leptospirosis, canine distemper, infection canine hepatitis diseases using DHLPP vaccine (Forte Dodge Company USA). They were tagged for identification.

Experimental design

They were assigned into four (4) groups of 5 dogs each as follows:

Group 1: infected, treated with DA (7.0 mg/kg)
Group 2: uninfected, untreated (negative control)
Group 3: infected, untreated (positive control)
Group 4: infected, treated with IMC (0.5 mg/kg).
Treatment was scheduled to commence when all the dogs are parasitaemic.

Trypanosome infection

The *Trypanosoma brucei* used in this study was obtained from Nigerian Institute of Trypanosomiasis Research (NITR) Vom, Plateau State Nigeria. The parasites were maintained in rats before infection of the experimental animals intraperitoneally (i.p) at 10^6 trypanosomes/ml of saline diluted infected blood. The number of parasites was estimated using the rapid matching method of Herbert and Lumsden (1976).

Serum collection

Four (4) mL of whole blood was put into clean tubes and allowed to stay for some time to enhance serum yield. The serum was decanted and centrifuged at 2000 rpm for 10 minutes to separate the sera from the cells. The clear serum was used to assay for the oxidative stress and antioxidant markers.

Drug administration

Diminazene aceturate (Veriben CEVA SANTE ANIMALS, CEDEX France), reconstituted by dissolving

1.05g of the drug per sachet in 12.5 mL of sterile water to make and 7.0mg/kg was administered to the dogs.

Isometamidium chloride (Trypanidium-Samorinmerial, Lyon France). Reconstituted by dissolving 1 g of the sachet in 50 ml of sterile water and 0.5 mg/kg was given to the dogs.

Estimation of parasitaemia
The parasitaemia level was estimated by the rapid matching method as described by Herbert and Lumsden (1976).

Determination of malondialdehyde:
Serum malondialdehyde level, a product of lipid peroxidation, was measured based on the method of Stocks & Dormandy (1971).

Determination of (Serum Catalase-(CAT), Serum Superoxide Dismutase-(SOD), Serum Reduced Glutathione-(GSH-Rd)
The serum catalase activities were determined according to the method described by Goth (1991). A serum superoxide dismutase activity was determined according to the method developed by Misra & Fridovich (1972). Serum reduced glutathione activities was determined according to the method described by Moron *et al*. (1979).

Handling of experimental animals during the study
Manuscript complied with current animal welfare and ethical guidelines. Of University of Nigeria Nsukka, Faculty of Veterinary Institutional Animal Care and Use Committee. Reference No. FVM-UNN-IACUC-2019-1131.

Statistical Analysis
Data generated were presented as means with standard deviation. The data was subjected to analysis of variance (ANOVA) and the means separated using Duncan's multiple range tests. Means were considered significant at P< 0.05.

Results
Parasitaemia/clinical manifestation
During the course of infection, clinical signs of dullness, depression, pale mucous membrane, ocular discharges and corneal opacity were observed in the infected untreated (group 3) until the dogs died before the termination of the experiment after showing signs of staggering and aggression. These clinical signs in the treated groups (groups 1 and 4) gradually disappeared following treatment with the trypanocides. The treated groups never showed any sign of the central nervous system. The signs of depression and anorexia were observed again in three dogs in group 1 (DA treated) following a relapse infection.

The *Trypanosoma brucei*, infected dogs (groups 1, 3, and 4) were positive for *T. brucei* by day 6-7 post-infection (PI). Treatment for groups 1 and 4 commenced by day 7 post-infectionhen all the infected dogs became parasitaemic. The treated dogs (groups 1 and 4) became a parasitaemic by day 14 post-infection. The parasitaemia was progressive in the untreated control (group 3). By day 28 post-infection 4 members of group 3 died and they all died by day 56 post-infection. By day 35 post-infection relapse infection occurred in two members of group 1 dog (Diminazene aceturate- DA treated), and by day 63 post-infection one member of group 1 (DA treated) died. Relapse of infection also occurred in one member of group 4(IMC treated) by day 56 PI. No mortality was recorded in this group till the termination of the experiment (Table 1).

The infection caused a significant increase (P< 0.05) in the concentration of serum malondialdehyde when compared to the normal control by day 7 post-infection. The isometamidium chloride treated group became similar with the normal control starting from day 28 unlike the diminazene aceturate treated group. The infected untreated group remained significantly higher (P<0.05) than the normal control and beyond (Table2).

Following infection, the Serum Catalase of the infected groups lowered significantly (P<0.05) when compared with the normal control by day 7 post-infection. From day 14PI the IMC treated group became similar with the normal control except from day 49 post-infection unlike the diminazene aceturate treated and infected and infected untreated that were significantly lower(P<0.05) till the end of the experiment (Table3).

Infection caused a significant decrease (P<0.05) in the superoxide dismutase by day 7 PI. The decrease was progressive in the infected untreated group. On days 14 and 21, SOD was similar in both the isometamidium chloride-treated and diminazene aceturate- treated groups. It was also similar in the isometamidium chloride-treated group from day days 28 – 42 and subsequently decreased until the end of the study. The diminazene aceturate treated group was significantly lower however, by day 28 post-infection to the end of the study than the normal control group. There was also a progressive

Table 1: The parasitaemia of *Trypanosoma brucei* infected dog groups treated with either 7 mg/kg Diminazene aceturate or 0.5 mg/kg Isometamidium chloride

Time (Days post-infection)	Parasitaemia of the various groups			
	Group 1 (Infected treated with 7.0 mg/kg DA	Group 2 (Uninfected control)	Group 3 (Infected untreated control	Group 4 (Infected treated with 0.5 mg/kg IMC
0	A 5/5	A 5/5	A 5/5	A 5/5
1	A 5/5	A 5/5	A 5/5	A 5/5
2	A 5/5	A 5/5	A 5/5	A 5/5
3	A 5/5	A 5/5	A 5/5	A 5/5
4	A 5/5	A 5/5	A 5/5	A 5/5
5	A 5/5	A 5/5	A 5/5	A 5/5
6	P 3/5	A 5/5	P 4/5	P 4/5
7	P 5/5	A 5/5	P 5/5	P 5/5
14	A 5/5	A 5/5	P 5/5	A 5/5
21	A 5/5	A 5/5	P 5/5	A 5/5
28	A 5/5	A 5/5	M 4/5	A 5/5
35	R2/5	A 5/5	M 4/5	A 5/5
42	R 2/5	A 5/5	M 4/5	A 5/5
49	R 2/5	A 5/5	M 4/5	A 5/5
56	R 2/5	A 5/5	M5/5	R 1/5
63	M 1/5	A 5/5	M 5/5	R 1/5

A = Aparasitemic
M= mortality
P= Parasitaemic
R= Relapse infection
Numerator= No. either aparasitaemic, parasitaemic, relapsed or dead as indicated.
Denominator = No. in a group

Table 2: The weekly mean serum malondialdehyde (nmol/ml) of *Trypanosoma brucei* infected dog groups treated with either diminazene aceturate or isometamidium chloride

Time (days)	Mean serum malondialdehyde (MDA)- nmol with standard error in bracket			
	Group 1 Infected and treated with 7.0 mg/kg DA	Group 2 Uninfected, untreated (negative control)	Group 3 Infected, untreated (positive control)	Group 4 Infected and treated with 0.5 mg/kg IMC
0	0.51 (0.15)	0.62 (0.09)	0.54 (0.2)	0.52 (0.08)
7	0.96^a (0.15	0.53^a (0.15)	0.87^b (0.10)	0.91^a (0.14)
14	0.77^a (0.14)	0.56^a (0.11)	1.16^b (0.27)	0.66^a (0.22)
21	0.82^a (0.16)	0.54^a (0.14)	0.99^b (0.43)	0.75^a (0.50)
28	0.95^b (0.22)	0.67^a (0.05)	0.76	0.57^a (0.26)
35	1.18^b (.23) R	0.64^a(0.09)	0.99	0.65^a (0.41)
42	1.29^b (0.15)	0.56^a (0.11)	1.19	0.93^{ab} (0.46)
49	1.16^b (0.16)	0.44^a (0.07)	1.30	0.66^a (0.38)
56	1.07^a (0.39)	0.47^b (0.06)	-	1.08^a (0.59) R
63	1.12^a (0.36)	0.51^b (0.16)	-	1.41^a (0.42)

^{ab} Different superscripts in a row indicate a significant difference between the group means (P<0.05); R = Relapse

decrease in the diminazene aceturate-treated group from day 28 until the end of the study. However, from day 28 post-infection to the end of the diminazene aceturate treated group. The diminazene aceturate treated group was significantly lower than the normal control group. From days 28 – 42, superoxide dismutase in the isometamidium chloride-treated group did not differ from the control but became significantly lower thereafter (P<0.05) till the end of the study. (Table 4).

Table 3: The weekly serum catalase (ku/L) of *Trypanosoma brucei* infected dog groups treated with either diminazene aceturate or isometamidium chloride

Time (days)	Mean serum catalase (CAT)- ku/L with standard error in bracket			
	Group 1 (Infected and treated with 7.0 mg/kg DA	Group 2 (Uninfected, untreated (negative control)	Group 3 Infected, untreated (positive control)	Group 4 Infected and treated with 0.5 mg/kg IMC
0	47.04 (10.54)	43.34 (6.71)	48.78 (4.38)	50.84 (10.05)
7	27.44[a](6.90)	45.32[b] (6.50)	29.06[a] (2.81)	32.04[a](2.29)
14	32.66[a] (10.02)	50.12[b] (7.49)	24.28[a] (8.95)	48.40[b] (3.57)
21	34.30[a] (6.85)	48.64[b] (9.12)	32.73[a] (14.36)	41.34[ab] (7.51)
28	30.00[b] (7.47)	47.26[a] (9.75)	43.3	44.98 (5.96)
35	28.50[a] (3.75) R	49.04 (7.43)	30.2	40.78[b](10.14)
42	23.24[a] (6.52)	45.82[b](5.12)	33.7	36.12[b](10.50)
49	20.56[a] (6.68)	46.23[c] (4.51)	30.61	33.73[b] (10.75)
56	18.04[a] (6.84)	46.26[b](5.63)	-	31.04[a] (11.14) R
63	19.73[a] (12.76)	49.40[c] (3.56)	-	28.30[b] (11.04)

[abc] Different superscripts in a row indicate significant difference between the group means (P<0.05); R = Relapse

Table 4: The weekly serum superoxide dismutase (SOD) of *Trypanosoma brucei* infected dog group treated with either diminazene aceturate or isometamidium chloride

Time (days)	Mean serum superoxide dismutase (SOD)- µ/ml with standard error in bracket			
	Group 1 Infected, treated with 7.0 mg/kg DA	Group 2 Uninfected, untreated (negative control)	Group 3 Infected, untreated (positive control)	Group 4 Infected and treated with 0.5 mg/kg IMC
0	5.19 (1.05)	4.64(0.51)	4.96 (0.92)	5.27 (0.97)
7	3.78[a](0.46)	4.82[b] (0.28)	3.43[a] (0.85)	3.36[a] (1.15)
14	4.62[b] (0.83)	5.01[b] (0.31)	2.49[a] (0.94)	4.35[b] (0.55)
21	5.06[b] (028)	4.47[ab] (0.89)	3.34[a] (2.14)	4.58[ab] (0.56)
28	3.66[a] (0.45)	5.03[b] (0.70)	3.22	5.30[b] (1.03)
35	3.96[a] R	5.01[ab] (0.63)	3.31	5.40[b] (1.22)
42	2.62[a]	3.77[b] (0.49)	2.77	4.21[b] (1.59)
49	2.39[a]	4.81[b] (0.63)	1.95	2.85[a] (0.57) R
56	1.60[a]	4.87[b] (0.62)	-	1.47[a] (0.50)
63	2.07[a]	7.60[ab] (0.45)	-	3.41[a] (0.65)

[ab] Different superscripts in a row indicate a significant difference between the group means (P<0.05); R = Relapse

Infection again caused a significant reduction (P<0.05) in the serum reduced glutathione in the infected untreated group when compared with the normal control by day 14 post-infection. From day 28 PI the infected and treated groups became also significantly lower (P< 0.05) than the normal control group and beyond (Table 5).

Discussion

Infection caused a significant increase in the serum level of malondialdehyde, a marker of oxidative stress. This is suggesting that trypanosomosis caused by *Trypanosoma brucei* induces oxidative stress. This agrees with the work of Kobo *et al.* (2014) who got the same result in *T. brucei* infected Wistar rats.

Similarly, Eze *et al.* (2008); Eghiaruwa (2012) also got the same result in rats.

The activities of catalase, reduced glutathione, superoxide dismutase were significantly decreased by the infection. This agrees with the works of Eghianruwa (2012) in *T. brucei* infected rats, and Omobowale *et al.* (2015) in *T. brucei* infected dogs treated with *Azadirachta indica*. These enzymes are antioxidants naturally produced in cells to neutralize the effect of oxidative stress.

Oxidation can only cause cell necrosis when tissue oxidation overwhelms the effect of antioxidation. The depletion of the antioxidation markers is an indication that *T. brucei* infection caused lipid peroxidation which was manifested in elevated levels of malondialdehyde recorded in the study.

Table 5: The serum glutathione (M ± SEM) (nmol/ml) of *Trypanosoma brucei* infected dog groups treated with either diminazene aceturate or isometamidium chloride

Time (days)	Group 1(Infected, treated with 7.0 mg/kg DA	Group 2 (Uninfected, untreated (negative control)	Group 3 (Infected, untreated (positive control)	Group 4 (Infected, treated with 0.5 mg/kg IMC)
0	7.46 (2.18)	6.52 (1.56)	7.26 (2.83)	6.04 (1.21)
7	4.87 (1.51)	6.45 (0.99)	5.73 (1.73)	5.36 (0.72)
14	4.97[ab] (1.37)	6.84[b] (1.78)	3.65[a] (2.06)	5.32[ab] (1.53)
21	4.56 (1.26)	6.50 (2.19)	4.55 (2.69)	6.37 (4.37)
28	5.19[a] (1.30)	7.35[b] (0.89)	8.83	4.64[a] (1.38)
35 R	4.90[a] (1.08)	8.03[b] (1.11)	7.47	4.75[a] (1.23)
42	4.53[a] (1.17)	7.62[b] (0.53)	4.49	5.04[a] (1.31)
49	3.69[a] (0.98)	7.69[b] (0.57)	3.00	4.03[a] (1.02)
56	2.81[a] (1.51)	7.65[b] (0.46)	-	3.54[a] (0.57) R
63	0.80[a] (0.14)	4.81[b] (0.58)	-	1.29[a] (0.55)

ab Different superscripts in a row indicate significant difference between group means (P<0.05); R = Relapse

The trypanocides diminazene aceturate and isometamidium chloride did not satisfactorily reverse this trend. This was similarly observed by Omobowale *et al*. (2015). The results of this study show that experimental infection of dogs with *Trypanosoma brucei* through the intraperitoneal route was established between six to seven days post-infection (PI). The prepatent period of *T. brucei* is known to be variable. However, it agrees with the report of Anene *et al*. (1989), who observed the prepatent period for canine trypanosomosis caused by *T. brucei* to be between four to eight days. It contrasted with the work of Sewell *et al*. (1990) that recorded a prepatent period of 3 – 4 days in dogs. The variations in the prepatent period may be due to strain of the *T. brucei* stock used or due to inherent quality of the infected dogs. In this experiment, one can deduce that canine trypanosomosis caused by *T. brucei* ran an acute course in dogs, as opposed to chronic disease course due to *T. congolense* (Amole *et al.,* 1982; Mario *et al.*, 1997). The death of the dogs in the infected untreated control dogs within 28 days PI can be due to the parasite's immunosuppressive effects, hence not allowing the dogs enough time to produce sufficient antibodies to fight the invading parasite.

The clinical signs of corneal opacity, pale mucosae, staggering movement, ocular discharges, weakness and lethargy were consistent with findings of previous researchers (Ezeh *et al*., 2009; Umeakuana *et al.,* 2019).

The trypanocides cleared the parasitaemia following treatment by day 14 PI indicating that diminazene aceturate (DA) and isometamidium chloride (IMC)

were efficacious in the treatment of *T. brucei* in dogs. However, the trypanocides (diminazene aceturate and isometamidium chloride) did not satisfactorily reverse the antioxidative stress and antioxidant markers to its normal values. This could be due to the relapse infection observed post treatment with the trypanocides. The relapse occurred much earlier in the DA-treated than in IMC treated group hence the seemingly better result recorded among the IMC treated group.

Relapses after treatment have been suggested to be due to drugs resistance and due to invasion of brain tissues by the parasites where the trypanocides' molecule cannot reach (Jennings *et al*; 1980 Barrett, 2001). In the brain, the parasites are protected by the blood-brain barrier which is impervious to the large molecules of the trypanocides. Subsequently, the parasites re-enter the vascular system when the effect of the drugs in the blood stream would have waned. It was thus concluded that the diminazene aceturate and isometamidium chloride though effective in clearing the parasites from the blood after administration could not satisfactorily ameliorate the oxidative stress induced by *T. brucei* infection in dogs.
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Conflicts of Interest
The authors declare no conflict of interest.

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