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The Effect of Dimethylsulphoxide on the Topical Application of Diminazene aceturate (Berenil®) in an Experimental *Trypanosoma brucei* Infection in Albino Rats

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SUMMARY

The difficulty associated with the intramuscular route of administration of diminazene aceturate coupled with tissue reactions often elicited at the injection site was the reason for the use of dimethylysulphoxide (DMSO), a potent non-polar solvent in enhancing the absorption of the drug topically in rats infected with *Trypanosoma brucei*. A total of 35 rats were divided into 7 groups (A – G) of five rats each. All the rats in Groups A - F were infected with x 10^3/µl of *T. brucei* intraperitoneally. Parasitaemia became evident by day 4 post-infection which corresponded with decline in packed cell volume. Groups A and B were treated respectively with graded doses (3.5 and 7.0mg/kg) of diminazene aceturate dissolved in DMSO while Groups D and E were treated with diminazene aceturate (3.5 and 7.0mg/kg) respectively without DMSO. The treatments were topical and commenced from day 8 post-infection. The efficacies of the treatments were compared against the standard intramuscular regime (Group C). Groups F and G served as infected and uninfected controls respectively. Diminazene aceturate dissolved in DMSO (Groups A and B) cleared parasitaemia and the associated haematological and pathological changes as in Group C. Meanwhile, the infection took its full course among rats treated without DMSO (Groups D and E) and the infected control (Group F). It was therefore, concluded that DMSO caused topical or percutaneous absorption of diminazene aceturate and that, the topical route may be adopted as an easier alternative for the administration of the drug in rats.

KEY WORDS: Topical, diminazene aceturate, dimethylsulphoxide, *Trypanosoma brucei*, rats

INTRODUCTION

Trypanosomosis is a debilitating disease of man, domestic and wild animals caused by the haemoflagellate protozoa of the genus *Trypanosoma* (Kahn,2005). Cyclical transmission is carried out by tsetse-fly (*Glossina* species), commonly found in Asia, Africa and South America (Ugochukwu, 1983). The disease is responsible for severe socio-economic losses and it is characterized by fever, apathy, pale mucous membranes, swollen lymph nodes, progressive emaciation and death (Mbaya et al, 2011). Over ten million square kilometers of land in African continent are infested by *Glossina* and thus, rendered unsuitable for livestock production (Murray and Jennings, 1982).

The disease in man is caused by *Trypanosoma brucei gambiense, T. b, rhodesiense* and

Trypanosoma cruzi (Soulsby, 1982). Although, a lot have been done on the chemotherapeutic management of the disease in various animal species (Bida and Aliu, 1981; Onyeyili and Anika, 1989; Mbaya et al., 2009a; Mbaya et al., 2009b; Mbaya et al., 2011). The importation of the drugs into Africa requires foreign currency which is difficult to come by (Onyeyili and Egwu, 1995). As such, plant extracts were explored as alternatives with varied success (Asuzu and Chineme, 1990; Mbaya et al, 2007; Mmbaya et al, 2010). Despite these achievements, diminazene aceturate still remains the drug of choice. However, its conventional intramuscular route of administration often elicits pain and tissue reaction at the site of injection. Other trypanocidal drugs such as melarsoprol dissolved in DMSO have been used topically against *T. brucei* infection in mice (Atougia *et al*., 1995; Jennings *et al*., 1996a). Despite these achievements, there is a paucity of information on the topical application of diminazene aceturate in *T. brucei* infected rats. It is against this backdrop that, this study was designed to evaluate the efficacy of dimethylsulfoxide (DMSO) in facilitating the absorption of graded doses of diminazene aceturate (Berenil®) through the intact skin of *T. brucei* infected rats. This is with a view of finding an alternative route of its application, rather than the conventional intramuscular route.

MATERIALS AND METHODS

Source of trypanosomes

Federe strain of *Trypanosoma brucei brucei* used in this study was obtained from the Nigerian Institute for Trypanosomosis Research of the National Veterinary Research Institute Vom, Nigeria. The organism was initially isolated from Muturu and N'dama cattle in Plateau State, Nigeria and maintained by serial passage in donor albino rats in which acute and sub-acute infections were established. Following the development of parasitaemia, the donor rats were bled and each recipient rat was inoculated with 0.5ml of the infected blood containing a uniform dose of x 10^3 /µL trypanosomes in 5% dextrose intraperitoneally. The uniform dose of the inoculums was achieved by serial dilution. The infected rats were examined every four days using wet mount for the presence of the parasites in the blood (Murray and Jennings, 1982).

Experimental animals

Thirty five adult Wister albino rats of both sexes weighing between 111g to 299g were obtained from the Faculty of Pharmacy, University of Maiduguri, Nigeria. The rats were transferred to the Parasitology Laboratory of the Department of Veterinary Microbiology and Parasitology where the research was conducted. The rats were fed with Growers mash (Vital Feed, PLC Nigeria) and water was provided *ad-libitum*. The rats were allowed to acclimatize for 30 days before the commencement of the experiment. The Ethics and Research Committee of the Faculty of Veterinary Medicine, University of Maiduguri, Nigeria approved this experiment in accordance with the international guidelines for the use of animals for biomedical research (EC, 1996).

Experimental drugs

Diminazene aceturate (Berenil® 2.3g, Ferberwarke, Germany) was obtained and dissolved in 15ml of sterile distilled water. Another sachet was dissolved in 15ml of dimethylsulfoxide (DMSO, Mallinckrodt Chemicals, MSDS, Canada).

Experimental protocol

The thirty five rats were randomly separated into seven groups (A, B, C, D, E, F and G) with each group containing five rats. The infected rats in Groups A to E were treated as follows; Group A was treated with 3.5mg/kg of diminazene aceturate dissolved in dimethylsulphoxide (DMSO) and applied topically on the tail (single dose); Group B was treated with 7.0 mg/kg of diminazene aceturate dissolved in DMSO and applied topically on the

tail (single dose); Group C was treated with 3.5mg/kg of diminazene aceturate dissolved in distilled water and administered through its conventional intra-muscular route (single dose); Group D was treated with 3.5mg/kg of diminazene aceturate without DMSO and applied topically on the tail (single dose); Group E was treated with 7.0 mg/kg of diminazene aceturate without DMSO and applied topically on the tail (single dose) and finally Groups F and G served as infected and uninfected controls respectively. All treatments commenced by day 8 post-infection. The experiment was terminated by day 32 post-infection, when the infected controls died of the infection.

Monitoring of infected and control animals

Clinical signs were monitored daily while parasitaemia was determined every 4 days by examining wet blood film from the tails of the rats. Parasitaemia was estimated every four days using the rapid matching technique (Packed Cell Volume (PCV) was determined by the microhaematocrit technique (Herbert and Lumsden, 1976). The carcasses of the dead and euthanized rats were subjected to detailed necropsy and the tissues from heart, lungs, spleen, kidneys and liver were collected in 10% formalin, embedded in paraffin wax, sectioned at 5µ thickness, stained with haematoxylin and eosin stain and examined for lesions (Drury and Wallington, 1976).

Statistical analysis

The Students't' Test was used to analyze the differences between mean values while one way Analysis of Variance (ANOVA) was used to analyze the extent of variation between groups and 'p' values ≤ 0.05 were considered significant using the statistical package (GraphPad, Instat, Version 2000).

RESULTS

Typical signs of trypanosomosis such as anaemia, characterized by pallor of the pinnae, snout and feet pads were observed from day 4 post-infection (p.i.) in all infected groups (A-F). These symptoms were however alleviated in rats treated topically with diminazene aceturate a

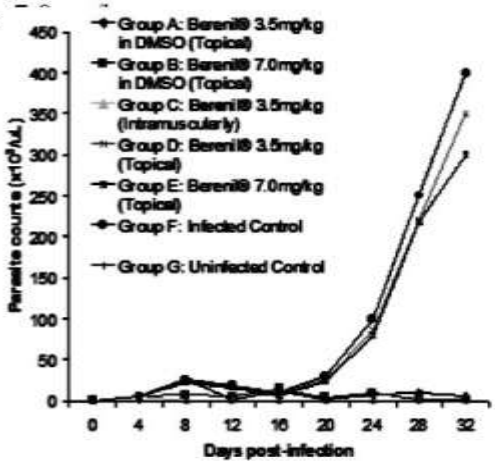

Fig. 1: Parasite counts ($x10^3/\mu L$) of Wister albino rats infected with *T. brucei* and treated either topically with graded doses of diminazene aceturate (Berenil®) or intra muscularly and their controls

dissolved in DMSO (Group B) and in rats treated via the intra muscular route with diminazene aceturate at 3.5mg/kg by day 12 p.i. or day 4 post-treatment (p.t.). In Group A, treated topically with diminazene aceturate at 3.5mg/kg dissolved in DMSO (Group A), these symptoms were alleviated by day 32 p.i. or day 24 p.t. among the 4 surviving rats. All the rats from groups D, E and F showed symptoms of malaise, anorexia, fever, starry hair coat, prostration from day 4 (p.i) prior to death.

The parasitaema of all groups of *T. brucei* infected rats and their controls are presented in Figure 1. Uniform parasite counts of 5.7 ± 0.21 occurred by day 4 p.i. in all infected groups (A-F). In Group A, parasitemia reached a peak count of 25.8 ± 0.45 by day 8 p.i. and kept on fluctuating but declined significantly ($p<0.05$) to 5.6 ± 0.21 by day 32 p.i. or day 24 p.t. For group B, a peak count of 15.0 ± 0.35 occurred by day 16 p.i. or day 8 p.t and declined significantly ($p<0.05$) to 1.0 ± 0.09 by day 32 p.i. or day 24 p.t. In Group C, a parasitaemia of 5.7 ± 0.21 disappeared by day 8 p.i. In Group D,

a parasitaemia of 5.5 ± 0.29 appreciated significantly without abatement ($p<0.05$) and attained a peak count of 350 ± 2.34 by day 32 p.i. or day 24 p.t. In Group E, a parasitaemia of 5.3 ± 0.29 appreciated without abatement to 300 ± 2.17 by day 32 p.i. or day 24 p.t. In Group F, which is the infected control, parasitaemia appreciated slightly ($p>0.05$) to 25.8 ± 0.45 by day 8 p.i. and thereafter declined to 10.0 ± 0.45 by day 16 p.i. and further appreciated significantly ($p<0.05$) to 400.0 ± 1.79 by day 32 p.i.

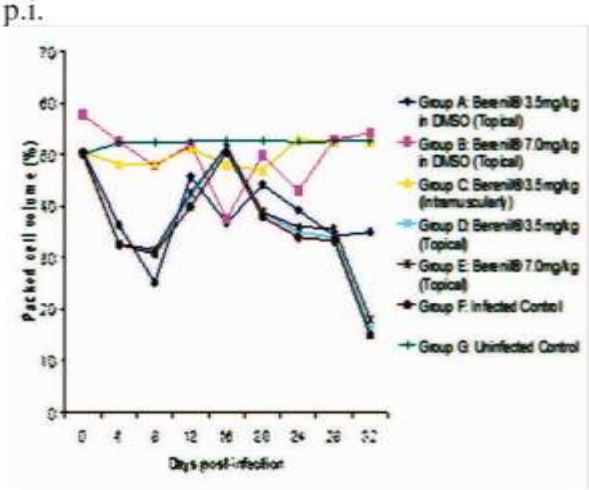

Fig. 2: Packed cell volume (%) of Wister albino rats infected with *T. brucei* and treated either topically with graded doses of diminazene aceturate (Berenil®) or intramuscularly and their controls.

The packed cell volume (PCV) changes of the *T. brucei* infected Wister albino rats and their treatments are presented in Figure 2. In Group A, PCV of 50.8 ± 0.89% declined significantly ($p<0.05$), to 25.2 ± 0.48%, fluctuated and appreciated significantly ($p<0.05$) to 35.0 ± 0.53% by day 32 p.i. or day 24 p.t. In group B, PCV of 57.8 ± 0.88% declined significantly ($p<0.05$) to 37.6 ± 0.55% by day 16 p.i. and appreciated significantly ($p<0.05$) thereafter almost attaining its pre-infection value by day 32 p.i. or day 24 p.t. Similarly, in group C a slight decline ($p>0.05$) occurred between days 4 and 8 p.i. and appreciated significantly ($p<0.05$) without attaining its p.i. value by day 32 p.i. or day 24 p.t. For Groups D, E and F, PCV of 50.4 ± 0.63%, 50.4 ± 0.63% and 50.2 ± 0.63% declined significantly ($p<0.05$) to 31.2 ± 0.50%, 31.6 ± 0.70% and 30.8 ± 0.50% respectively by day 8 p.i. but appreciated again to 51.2 ± 0.64%, 51.6 ± 0.89% and 50.5 ± 0.64% by day 16 p.i. or day 8 p.i. and declined significantly ($p<0.05$) again to 16.0 ± 0.35%, 18.0 ± 0.53% and 15.0 ± 0.35% respectively by day 32 p.i. or day 24 p.t. while the PCV in Group F remained fairly constant

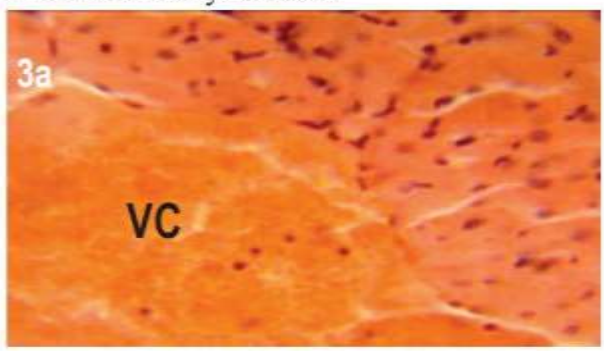

Fig. 3a: Photomicrograph of the heart muscle of a *T. brucei* infected rat treated topically with diminazene aceturate (3.5mg/kg) without DMSO (Group D) with focal area of vascular congestion (V.C.); H&E x 400

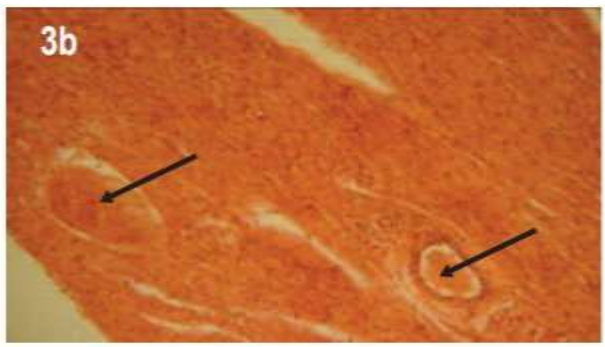

Fig. 3b: Photomicrograph of the heart muscle of an infected control rat (Group F) with multi-focal areas of vascular congestions (arrows);
H & E x 200

Table I: Mortality pattern of Wister albino rats infected with T. brucei and treated either topically with graded doses of diminazene aceturate (Berenil®) or intramuscularly and their controls

Groups	Treatment Protocol	Route of Drug Administration	Number of Dose	Mortality Pattern (%)	No. Dead on specific days
A (n=5)	Berenil®: 3.5mg/kg in DMSO	Topically	Single dose	1(20%)[a]	28[1]
B (n=5)	Berenil®: 7.0mg/kg in DMSO	Topically	Single dose	0(0%)[b]	NIL
C (n=5)	Berenil®: 3.5mg/kg	Intra-muscularly	Single dose	0(0%)[b]	Nil
D (n=5)	Berenil®: 3.5mg/kg without DMSO	Topically	Single dose	5(100%)[c]	8[1], 24[1], 28[1], 32[2]
E (n=5)	Berenil®: 7.0mg/kg Without DMSO	Topically	Single dose	5(100%)[c]	9[2], 25[1], 29[2]
F (n=5)	Infected Control	Nil	Nil	5(100%)[c]	8[1], 24[1], 28[1], 32[2]
G (n=5)	Uninfected Control	Nil	Nil	0(0%)[b]	Nil

[1, 2] Superscripts in 6th column indicate number of rats that died on those days, [a, b, c] Superscripts in 5th column differed significantly ($p<0.05$), n = number of rats in each group.

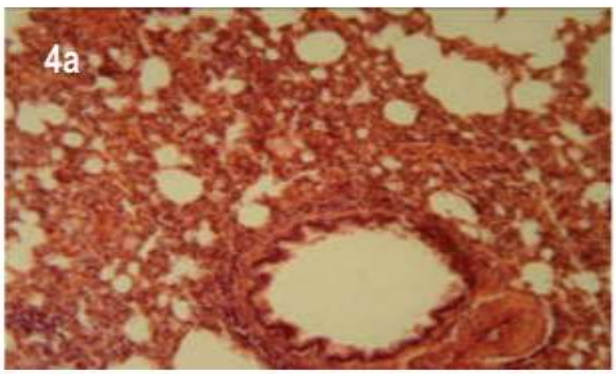

Fig. 4a: Photomicrograph of the lungs of a *T. brucei* infected rat treated topically with diminazene aceturate (7.0mg/kg) without DMSO (Group E) with narrowing of alveolar spaces; H&E x 200

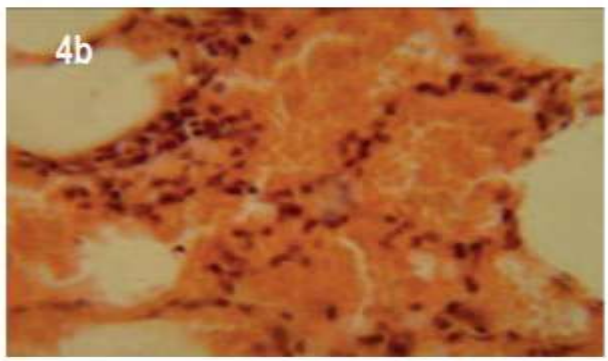

Fig. 4b: Photomicrograph of the lungs of an infected control rat (Group F) with severe inter-alveolar haemorrhages; H&E x 400

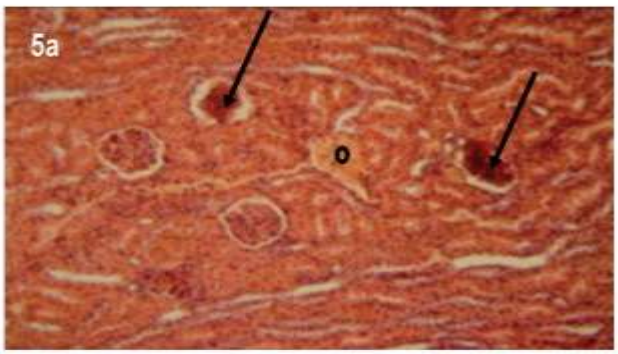

Fig. 5a: *Photomicrograph of the kidney of a T. brucei infected rat treated with diminazene aceturate (3.5mg/kg) without DMSO (Group D) with glomerular degeneration (arrows) and congestion; H&E x 200*

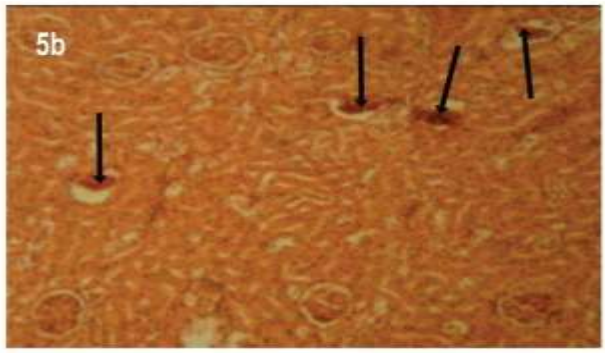

Fig. 5b: *Photomicrograph of the kidney of a T. brucei infected control rat (Group F) with multi-focal glomerular degenerations in the cortex (arrows). (H&E x 200).*

The mortality pattern of the infected and treated rats is presented in Table I. In Group A, 1/5(20%) mortality was observed by day 28 p.i. In Groups B, C and G 0(0%) mortality was encountered. In Groups D 5/5(100%) mortality was encountered with 1 each occurring on days 8, 24, 28 and 2 on day 32 (p.i.). For Group E, 5/5(100%) mortality was also encountered with 2 each occurring days 9 and 29 and 1 on day 8 p.i. For Group F, 5/5(100%) mortality was encountered with 1 each occurring on days 8, 24, 28 and 1 on day 32 p.i.

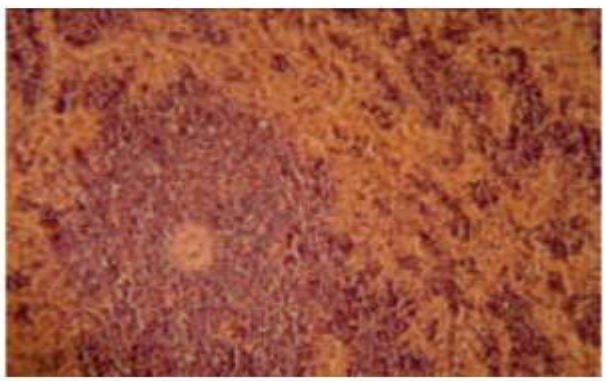

Fig. 6a: *Photomicrograph of the spleen of T. brucei infected rat treated with diminazene aceturate (7.0mg/kg) without DMSO (Group E) with diffuse aggregation of lymphoid cells; H&E x 200*

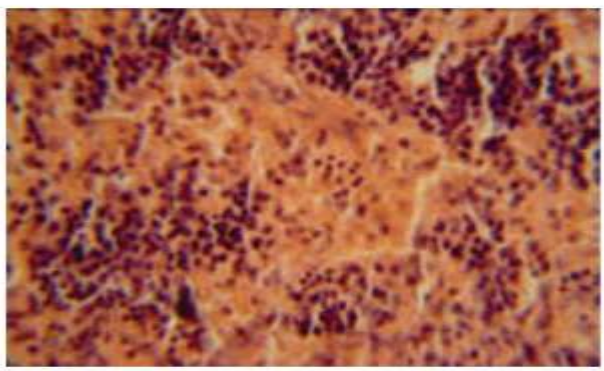

Fig. 6b: *Photomicrograph of the spleen of an infected control rat (Group F) with diffuse aggregation of lymphoid cells; (H&E x 200)*

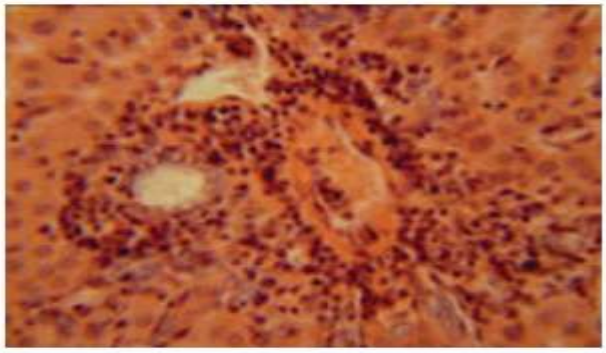

Fig. 7a: *Photomicrograph of the liver of a T. brucei infected rat treated with diminazene aceturate (3.5mg/kg) without DMSO (Group D) with moderate bile duct hyperplasia; H&E x 400*

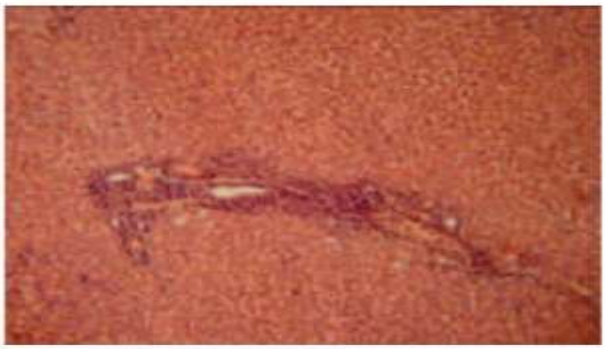

Fig. 7b: *Photomicrograph of the liver of a T. brucei infected control (Group F) with moderate bile duct hyperplasia; H&E x 200*

Following necropsy, splenomegally, hepatomegally, petechial haemorrhages on the serosal surfaces of spleen, liver, kidneys and heart with atrophy of body fat were the gross changes observed in the rats that died of the infection. Histopathologically, varying degrees of degenerative changes were observed in some organs (heart, lungs, kidneys, spleen, liver) of the rats infected with *T. brucei* and treated topically with diminazene aceturate (3.5 and 7.0mg/kg) without DMSO and the infected control (Figures 3–7). The heart muscle of a *T. brucei* infected rat treated topically with diminazene aceturate (3.5mg/kg) without DMSO (Group D) showed focal area of vascular congestion (Fig. a). While the heart muscle of infected control (Group F) showed multi-focal areas of vascular congestions (Fig. 3b).

Narrowing of the alveolar spaces of the lungs of an infected rat, Group F was observed (Fig 4a). While the lungs of an infected control had severe inter-alveolar haemorrhages (Fig. 4b). Fig. 5a shows kidney of an infected rat treated with diminazene aceturate (3.5mg/kg) without DMSO (Group D) with glomerular degeneration and congestion while Fig. 5b shows kidney of an infected untreated rat (Group F) with multi-focal glomerular degenerations in the cortex. Fig. 6a shows the spleen of an infected rat treated with diminazene aceturate (7.0mg/kg) without DMSO (Group E) with diffuse aggregation of lymphoid cells. Fig. 6b shows the spleen of an infected untreated control (Group F) with diffuse aggregation of lymphoid cells. Fig. 7a shows the liver of an infected rat treated with diminazene aceturate (3.5mg/kg) without DMSO (Group D) with moderate bile duct hyperplasia while, Fig. 7b shows the liver of an infected control (Group F) with moderate bile duct hyperplasia. Lesions were however absent in Groups A and B treated topically with 3.5 and 7.0mg/kg of diminazene aceturate dissolved in DMSO, and in *T. brucei* infected rats treated via the intra- muscular route with diminazene aceturate (Group C).

DISCUSSION

In this study where anaemia was characterized by pallor of the pinnae, snout and feet pads with associated low PCV during bouts of parasitaemia is in agreement with several reports that the anaemia in trypanosomosis often started during the 1st wave of parasitaemia and is haemolytic in nature (Anosa (1983, 1988; Nwosu and Ikeme, 1992; Igbokwe, 1994; Mbaya *et al*., 2012). However, the haemolytic nature of the anaemia in most cases would depend on the species of trypanosomes involved (Anosa, 1983). The expanded and active mononuclear phagocytic system (MPS) has been a major player in haemolytic anaemia in trypanosomosis through erythrophagocytosis which develop soon after infection and continued thereafter, in the

various phases of the disease (Anosa, 1983; Igbokwe, 1994; Mbaya *et al*., 2009b, 2012). The presence of the MPS might have been associated with increased demand on the system to remove dead red blood cells, tissue cells, trypanosomes, antigen-antibody complexes and to participate in immune responses (Nwosu and Ikeme, 1992). Similarly, the infected rats showed symptoms of malaise, anorexia, fever, starry hair coat and prostration prior to death. The starry hair coat was due to the fever caused by the parasitaemia, which led to the anorexia. This has been reported extensively in *T. brucei* infection in animals (Anosa, 1983; Igbokwe, 1994; Mbaya *et al*., 2009b, 2012).

This study showed that dimethylsulphoxide (DMSO) greatly enhanced the percutaneous absorption of diminazene aceturate (Berenil®) in Wister albino rats infected with *T. brucei*. This is a welcomed development due to the low toxicity associated with the use of DMSO (Jennings et al., 1993; Atongia et al., 1995; Jenning et al; 1996). The ability of DMSO to penetrate intact skin and membranes without damaging them and to carry other chemicals and compounds into biological systems is associated with its high osmotic pressure measuring up to tens of atmospheres (Kabu et al., 1991; Blood et al; 2007). In this study, DMSO successfully caused the topical absorption of diminazene aceturate (Berenil®) in a graded dose manner (3.5mg/kg, 7.0mg/kg), with 7.0mg/kg being the most effective dose. However, rats treated topically with diminazene aceturate (Berenil®) (3.5mg/kg, 7.0mg/kg), without DMSO were not effective. This therefore shows that the efficacy of the treatment topically was associated with the activity of DMSO since diminazene aceturate (Berenil®) does not posses the ability to be absorbed topically on its own. Although DMSO is being used for the first time in facilitating the topical absorption of graded doses of diminazene aceturate in *T. brucei* infected rats, it agrees with the findings, where topical application of diminazene aceturate using propylene glycol (Mbaya et al; 2012) and melarsoprol either singly or in combinations with nitroimidazoles, nitrofurazones and nifurtimox (Jennings and Gray, 1983; Ginoux et al; 1984; Jennings et al.; 1993; Atougia et al, 1995; Jennings et al, 1996a,b) cured mice experimentally infected with *T. brucei*. The topical application of diminazene aceturate at 7.0mg/kg produced similar results as the standard application of the drug via its conventional intra-muscularly route. The topical application of diminazene aceturate (Berenil®) in DMSO was successful in alleviating the symptoms of the attendant disease by eliminating parasitaemia, modulating declined PCV and preventing the development of tissue lesions in the (heart, lungs, liver, kidneys and spleen as compared to what was obtained in the infected controls and those treated with diminazene aceturate topically but without DMSO. This is in consonance with several reports where other drugs such as nitrofurazone and nifurtimox dissolved in DMSO (Atougia et al; 1995) and melarsoprol (Jennings et al, 1993) applied topically cured *T. brucei* infection in mice.

In this study, successive waves of parasitaemia were recorded among the infected rats. Successive waves of parasitaemia are known features of trypanosomosis commonly caused by antigenic variation (Nwosu and Ikeme, 1992; Mbaya, 2007; Mbaya *et al*., 2007). The ability of the host to limit the peak and number of each wave of parasitaemia is however, dependant on whether the infection is acute, sub-acute or chronic (Soulsby, 1982; Katunguka-Rwakishaya *et al.,* 1992). The fact that the PCV decreased sharply during bouts of parasitaemia but maintained a gradual increase during the periods of low parasitaemia showed an inverse relationship with parasitaemia (Nwosu and Ikeme, 1992).

The severe histopathological changes encountered in these organs are probably

associated with the tissue invasiveness of *T. brucei* which allows a period of its complete absence in peripheral circulation. This period is called the "aparasitaemic phase" when the parasites establish in extra vascular sites, connective tissues and tissue fluids thereby, causing severe tissue damage before it re-emerges into peripheral circulation (Mbaya et al., 2009b; Mbaya et al., 2009c; Mbaya et al., 2009d). This probably accounted for the various degrees of degenerative changes and mononuclear cellular infiltrations observed in some of the organs of the infected control, and those treated with diminazene aceturate without DMSO.

CONCLUSION

Based on the findings described above, we report that diminazene aceturate (Berenil®) dissolved in DMSO and applied topically cured *T. brucei* infection in Wister albino rats in a graded dose manner. And that the topical application of diminazene aceturate dissolved in DMSO may be an easier alternative of administering the drug. It is however, recommended that this experiment be conducted in large animal models to further ascertain these findings.

diminazene aceturate (Berenil®)

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