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In vitro and *in vivo* changes observed in *Trypanosoma brucei brucei*-infected rats treated with artesunate and/or diminazene aceturate

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Abstract

This study evaluated *in vitro* and *in vivo* antitrypanosomal effect of artesunate and/or diminazene aceturate in Wistar rats experimentally infected with *Trypanosoma brucei brucei. In vitro* screening was carried out in triplicates using 50 µl of 0.2, 2 and 20 µg/µl of artesunate as test drug; diminazene aceturate, normal saline and trypanosome-infected blood served as controls in a 96-well microtitre plate, incubated at 37˚C for 5 minutes. Efficacy was observed over a period of 60 minutes for reduced or complete trypanosomal immobilization. Results showed concentration-dependent cessation of trypanosomal motility was significantly ($p < 0.001$) induced by artesunate when compared to the controls. Seventy Wistar rats of both sexes weighing between 190 and 210 g were randomly divided into 7 groups (5 males and 5 females) are used for *in vivo* study. Groups I and II served as normal control and model control respectively. Groups III to VII were infected with *Trypanosoma brucei brucei* (10^6 trypanosomes/ml) intraperitoneally. At peak parasitaemia (8 days post-infection), group III was treated with diminazene aceturate (3.5 mg/kg) intramuscularly once while groups IV, V, VI were treated with artesunate (200, 100, 50) mg/kg orally for 5 consecutive days and group VII was treated with combination of artesunate (50 mg/kg) orally and diminazene aceturate (1.75 mg/kg) intramuscularly for 5 days. Results indicated pre-patent period of 4 days and increase in levels of parasitaemia post-inoculation. PCV, Hb concentration, RBC count, MCV, MCHC and total leucocyte count decreased significantly ($p < 0.05$) between days 0 and 8 in groups II to VII. Following treatment, significant increases ($p < 0.05$) were recorded except for groups II, IV, V and VI where the rats died. Thus, combination of artesunate (50 mg/kg) and half the standard dose of diminazene aceturate was able to reduce parasitaemia and ameliorate the anaemia elicited by the trypanosomes.

Keywords: Artesunate, Diminazene Aceturate, Haematology, *Trypanosoma brucei brucei*, Wistar rats

Introduction

Trypanosoma is a genus of veterinary and medical concern in sub-Saharan Africa (Giordani *et al*., 2016, Buscher *et al*., 2017). The salivarian group of trypanosomes, *Trypanosoma brucei*, *T. congolense* and *T. vivax* are transmitted through the infected

saliva of the tsetse fly vector (*Glossina* spp.). The most studied of the salivarian trypanosomes *is T. brucei*, with subspecies *T. b. gambiense* and *T. b. rhodesiense* being the causative agents of human African trypanosomosis (HAT) (Rotureau & Van Den, 2013; Silvester *et al*., 2018). Trypanosomosis is an enfeebling disease characterized majorly by anaemia (Stijlemans *et al*., 2018). Currently, there is no effective vaccine against trypanosomosis because trypanosomes possess variant surface glycoprotein which enables them evade the immune system (Onyilagha & Uzonna, 2019). The available means of control involves tsetse (*Glossina*) control, chemoprophylaxis, chemotherapy and use of trypanotolerant livestock breeds (Giordani *et al*., 2016). Artesunate (ARTS) is a derivative of artemisinin; a sesquiterpene lactone endoperoxide isolated from *Artemisia annua*, a herb long-employed in traditional Chinese medicine to remedy fevers (Heller, 2019). Artesunate, also known as dihydroartemisinin-12-α-succinate, is a potent, semisynthetic antimalarial compound (Chekem & Wierucki, 2006). Artesunate has improved solubility, absorption and pharmacokinetics (Li *et al.*, 1998). These allow it to be recommended as an oral, rectal, intramuscular and intravenous medication for uncomplicated and severe malaria (Barnes *et al*., 2004). Artesunate has a short half-life of between 20 and 45 minutes by oral route (Morris *et al*., 2011) and is metabolized through esterase-catalyzed hydrolysis within this short time to dihydroartemisinin, which is the active metabolite responsible for its antimalarial activity (Teja-Isavadharm *et al*., 2001; Gautam *et al.*, 2009). The genus *Trypanosoma* and *Plasmodium* are both protozoan parasites known to affect man. This study was channeled towards investigating the *in vitro* antitrypanosomal effect and efficacy of artesunate alone and its combination with diminazene aceturate in the treatment of Wistar rats experimentally infected with *T. b. brucei.*

Materials and Methods

Ethical approval

Ethical approval was obtained from the Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC) with the approval number ABUCAUC/2020/047.

Drug acquisition, source and preparation

Analytical grade of artesunate was sourced from Abcam Plc Cambridge, United Kingdom with CAS number - 88495-63-0. Diminazene aceturate (DA) was acquired from Livestock Pharma Arendonk, Belgium with batch number-DG/094/18.

Source of parasite

T. b. brucei was obtained from blood sample collected via the jugular vein of a trypanosome infected cattle in Bukuru, Jos, Plateau State. The parasite was confirmed using microscopic and molecular (polymerase chain reaction) techniques. It was further maintained in the laboratory by continuous passage in rats until required. Each cycle of passage was carried out when parasitaemia was in the range of 35-40 parasites per field, which corresponded to an interval of 6 days post infection.

Determination of parasitaemia

From day 1 post infection (PI) and throughout the course of the experiment, blood was collected directly from the tail pre-sterilized with methylated spirit unto a clean glass slide and covered with cover slip. The wet mount was viewed under the light microscope at × 40 magnification to monitor and score parasitaemia according to the rapid matching method described by Herbert and Lumsden (1976).

Experimental animals

Seventy adult Wistar rats (35 male and 35 female), weighing between 190 and 210 g were used for the experiment. The animals were obtained from the animal house of the Department of Veterinary Pharmacology and Toxicology, Ahmadu Bello University, Zaria, Nigeria. They were kept in metal cages under room temperature and were allowed to acclimatize for two weeks before commencing the study. They were given free access to commercial rat chow (Chukun Feed ®: Crude protein, Fat, Fiber, Calcium, Phosphorous, Metabolized Energy) and water *ad libitum*.

Infection of experimental animals

The infected blood was collected from a donor rat at peak parasitaemia and diluted with physiological saline. About 1.0×10^6 parasites in 0.2 ml solution was injected intraperitoneally into a Wistar rat previously unexposed to trypanosomal infection, as described by Adeyemi *et al*. (2010).

In vitro antitrypanosomal screening

The *in vitro* trypanocidal screening was performed in triplicates. Fifty microlitres of 0.2, 2.0 and 20 µg/µl of artesunate, diminazene aceturate, normal saline (respectively) were pipetted into separate wells of 96-well microtitre plate and 50 µl of blood containing trypanosomes from donor rats sacrificed at 40 parasites per field were added. The mixture was rocked gently and then incubated at 37˚C for 5 minutes. At the end of incubation, 2 µl of individual test mixture were placed on microscopic slides,

covered with coverslips and observed under a light microscope at x 400 magnification. The parasites were observed at 10 minutes interval for death, rate of immobilization and/or any morphological alterations for a duration of 60 minutes. Cessation or reduction in motility of the parasites in treated blood compared to that of parasite-loaded control blood without drug served as antitrypanosomal activity, since motility constitutes a relatively reliable indicator of the viability of most zooflagellate parasites (Atawodi, 2005).

In vivo antitrypanosomal screening

Seventy Wistar rats were randomly divided into seven groups of ten rats each (5 males and 5 females). Group I was the normal control (uninfected control); group II served as model control (infected and untreated). Groups III to VII were infected with *T. b. brucei* (10^6 trypanosomes/0.2 ml of blood) intraperitoneally. Eight days PI Wistar rats in group III were treated with diminazene aceturate (3.5 mg/kg) intramuscularly once, groups IV, V, VI were treated with artesunate 200, 100, 50 mg/kg orally for 5 consecutive days respectively, group VII was treated with artesunate 50 mg/kg orally and diminazene aceturate 1.75 mg/kg intramuscularly for 5 consecutive days respectively.

Determination of PCV, HB, MCV, MCHC, RBC and WBC counts

Blood samples were collected in labelled sample bottles containing Ethylenediaminetetraacetic acid (EDTA) at four days interval for thirty-two days and processed for haematology. Thirty-two days PI, all Wistar rats were sacrificed by jugular venesection. Haematological parameters of packed cell volume (PCV), haemoglobin (Hb) concentration, red blood cell (RBC), differential leucocyte counts were determined using Mindray automated haemotology analyzer (BC 3600, Germany) as described by Dacie & Lewis (1991). Erythrocytic indices of mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated from the values of PCV, Hb concentration and RBC count as described by Schalm *et al*. (1975).

Statistical analysis

The non-parametric data for *in vitro* screening were analyzed using Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks, followed by Dunn's test. For the *in vivo* study, data were presented as mean ± standard error of mean (SEM). Two-way ANOVA followed by Bonferroni post hoc test (using treatment and sex as factors) was used to analyze the data. GraphPad prism version 8.0.2 for windows (GraphPad software San Diego, California) was used with $p < 0.05$ considered statistically significant.

Results

At 20 µg/µl, there was complete cessation of motility ($p < 0.001$) by artesunate from 10 minutes of observation to 60 minutes when compared to diminazene aceturate where complete cessation of motility was observed by 30 minutes and normal saline that had very motile trypanosomes ($P > 0.05$) throughout the observation (Table 1). At 2 µg/µl, artesunate was observed to elicit complete cessation of trypanosome motility by 30 minutes when compared to diminazene aceturate that had complete cessation of motility by 50 minutes and normal saline that had very motile trypanosomes ($p > 0.05$) (Table 1). At 0.2 µg/µl, artesunate exhibited significant difference ($p < 0.01$) with moderately motile trypanosomes for the first 30 minutes of observation followed by slightly motile trypanosomes

Table 1: *In vitro* screening for antitrypanosomal efficacy

Time (minutes)	Normal saline	Diminazene aceturate (µg/µl)			Artesunate (µg/µl)		
		20	2	0.2	20	2	0.2
10	$+++^a$	$++^b$	$+++^a$	$+++^a$	$-^c$	$+^d$	$++^b$
20	$+++^a$	$+^b$	$++^c$	$+++^a$	$-^d$	$+^b$	$++^c$
30	$+++^a$	$-^b$	$++^c$	$+++^a$	$-^b$	$-^b$	$++^c$
40	$+++^a$	$-^b$	$++^c$	$++^c$	$-^b$	$-^b$	$+^d$
50	$+++^a$	$-^b$	$-^b$	$++^c$	$-^b$	$-^b$	$+^d$
60	$+++^a$	$-^b$	$-^b$	$++^c$	$-^b$	$-^b$	$+^d$

$^{a, b, c, d}$ Means with different superscript letters across rows are significantly different ($^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$). +++ = Very motile trypanosome, ++ = Moderately motile trypanosome, + = Slightly motile trypanosome, - = No viable (motile) trypanosome

by 40 to 60 minutes of observation when compared to diminazene aceturate that had no significant difference ($p > 0.05$) with normal saline from 10 to 30 minutes but had moderately motile trypanosomes for the rest of the observation period. Figure 1 shows the effect of treatment on mean survival rate of Wistar rats experimentally infected with *T. b. brucei*. There was no significant difference ($p > 0.05$) in survival rate between groups I, III and VII (normal control, infected and treated with DA stat., infected and treated with combination of ARTS and DA, respectively). Significant differences ($p < 0.05$) were observed in the model control (infected/untreated), ARTS (200 mg/kg, 100 mg/kg and 50 mg/kg) groups when compared to the normal control group.

Figure 2 shows the effect of treatment on the level of parasitaemia in all the treatment groups. There was no significant ($p > 0.05$) difference between the sexes. All the groups that were infected showed presence of trypanosomes 4 days PI There was significant ($p < 0.05$) decline in the level of parasitaemia in groups III and VII when compared to groups II, IV, V and VI where elevated parasitaemia was observed in all rats and death was recorded. Decline in weights were observed in both infected

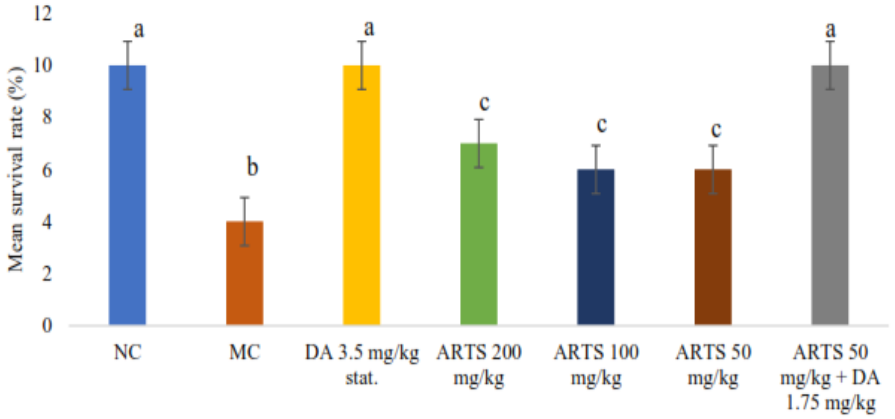

Figure 1: Effect of treatment with artesunate, diminazene aceturate on the mean survival rate of male and female Wistar rats experimentally infected with *T. b. brucei.* [a, b, c] Means with different superscript letters are significantly different $P < 0.05$, Values are expressed as mean ± SEM (n=10)
Key: NC = Normal control, MC = Model control, DA stat. = Diminazene aceturate once, ARTS = Artesunate, ARTS + DA = Artesunate and Diminazene aceturate

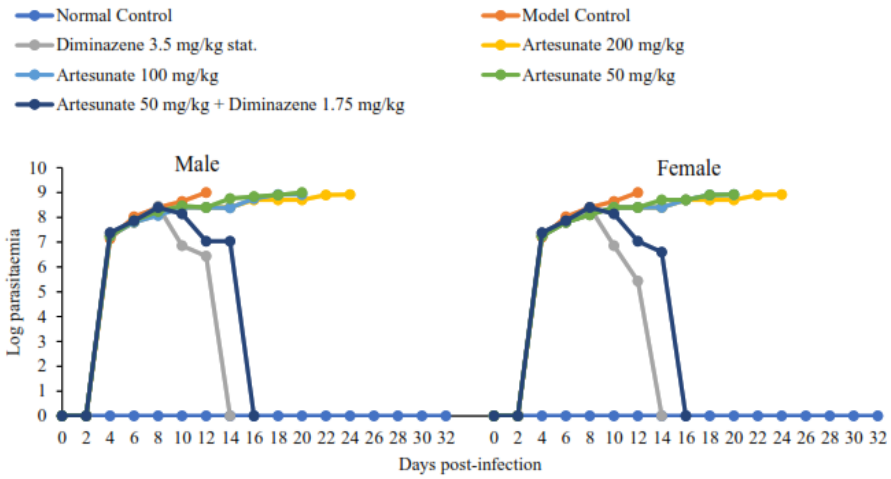

Figure 2: Effect of treatment on the level of parasitaemia in male and female Wistar rats experimentally infected with *T. b. brucei.* Values are expressed as mean ± SEM (n = 5)

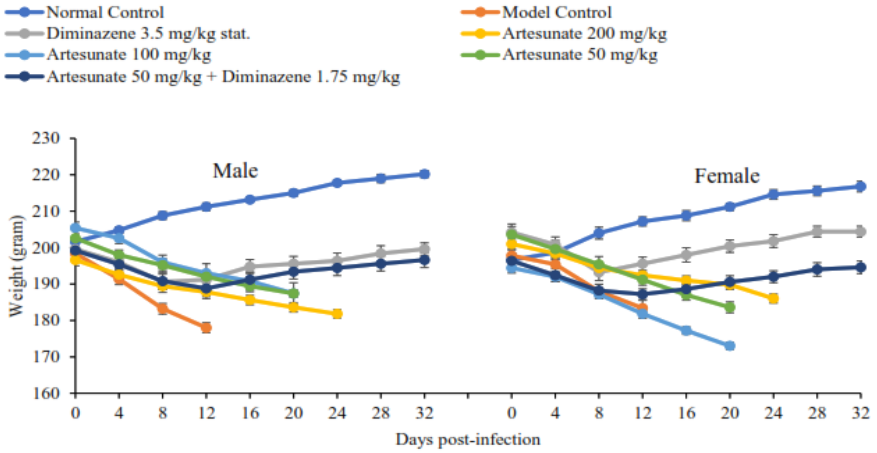

Figure 3: Effect of treatment on weight of male and female Wistar rats experimentally infected with *T. b. brucei.* Values are expressed as mean ± SEM (n=5)

male and female rats between days 0 and 8 PI However, by day 20 PI there was significant weight gain following treatment in groups III and VII when compared to groups II, IV, V and VI where the weights abated continuously. A significant increase in weight was seen throughout the study period in group I for both sexes (Figure 3).

Irregular patterns of rectal temperature were observed in all the infected groups of both sexes with significant difference ($p < 0.05$) observed in all the groups when compared to uninfected control. Following drug intervention, groups III and VII had their temperature returned to pre-infection value. The increase in temperature was seen to correlate with increased parasitaemia (Figure 4). Significant decrease was recorded in the PCV of Wistar rats in all the infected groups between days 0 and 8 PI in both sexes when compared with values obtained in group I (uninfected control). Significant ($p < 0.05$) increases to pre-infection values were observed in groups III and VII when compared to groups II, IV, V, VI where a continuous decline in the PCV was observed till all rats in the groups died of the infection (Figure 5). Decrease in the HB concentration was observed in all groups between days 0 and 8 PI in both sexes when compared with group I (uninfected control), Following treatment, HB concentration declined significantly ($p < 0.05$) till all rats in the groups II, IV,V, VI died while significant increase ($p < 0.05$) to pre-infection values was recorded in groups III and VII when compared to group I (Figure 6).

Figure 7 shows the effect of treatment on RBC count. Significant decrease ($p < 0.05$) was recorded in RBC count of the infected groups between days 0 and 8 PI in both sexes when compared to group I

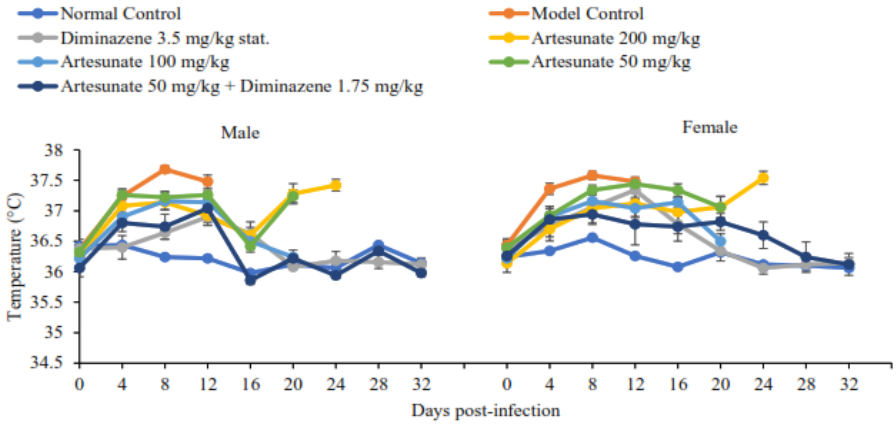

Figure 4: Effect of treatment on rectal temperature of male and female Wistar rats experimentally infected with *T. b. brucei.* Values are expressed as mean ± SEM (n=5)

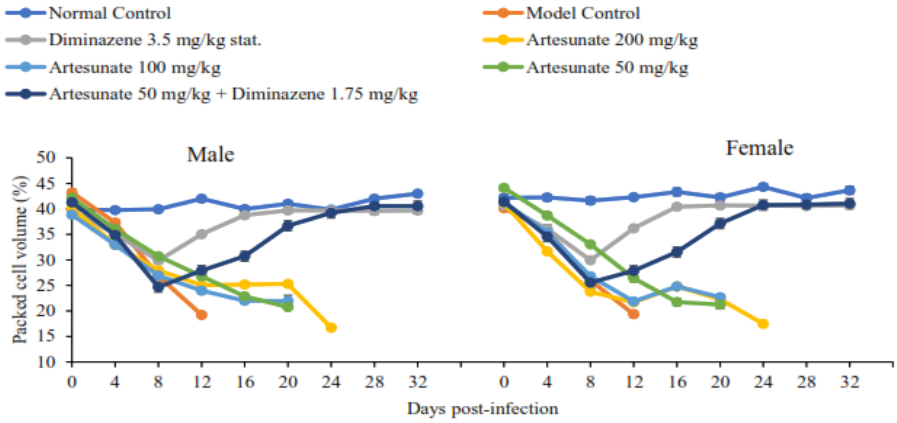

Figure 5: Effect of treatment on packed cell volume of male and female Wistar rats experimentally infected with *T. b. brucei.* Values are expressed as mean ± SEM (n=5)

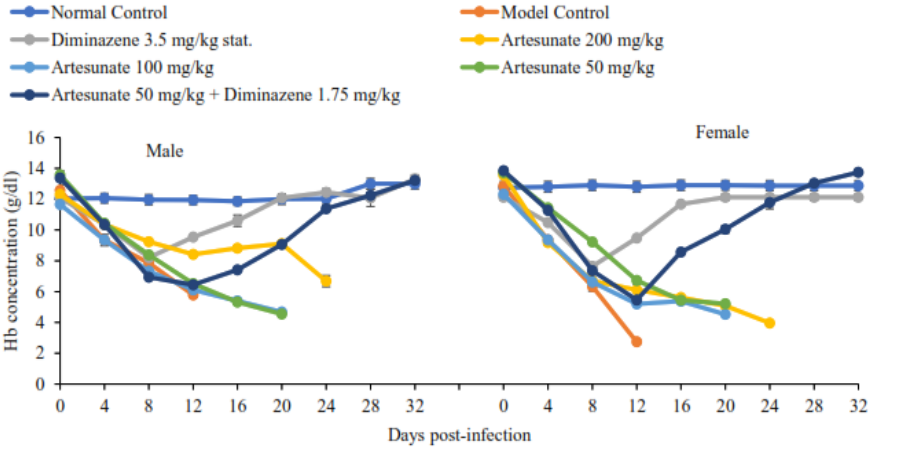

Figure 6: Effect of treatment on haemoglobin concentration of male and female Wistar rats experimentally infected with *T. b. brucei.* Values are expressed as mean ± SEM (n=5)

(uninfected control). Significant ($p < 0.05$) increase to pre-infection values following treatment was seen in groups III and VII when compared to groups II, IV, V, VI where a continuous decline in the RBC count was observed. The effect of treatment on mean corpuscular volume is represented in Figure 8. The MCV of the Wistar rats in group II decreased significantly ($p < 0.05$), when compared to the values obtained in groups I, III and VII respectively. There was a significant ($p < 0.05$) increase in the MCV of Wistar rats following treatment in groups III and VI when compared with group II. The MCHC of group II was significantly ($p < 0.05$) lower than that of groups I, III and VII. There was significant elevation observed in MCHC following decline between days 0 and 8 in the treatment groups that recovered from the infection when compared to groups II, IV, V, and VI where reduced MCHC values were recorded and the rats died (Figure 9).

Between days 0 and 8 PI, significant decline in WBC count of rats was recorded in all the infected groups when compared with group I (uninfected control), However, continuous decline in the WBC count was observed till all rats in groups II, IV, V and VI died on days 12, 24 and 20 respectively. WBC count in Groups III and VII increased significantly ($p < 0.05$) when

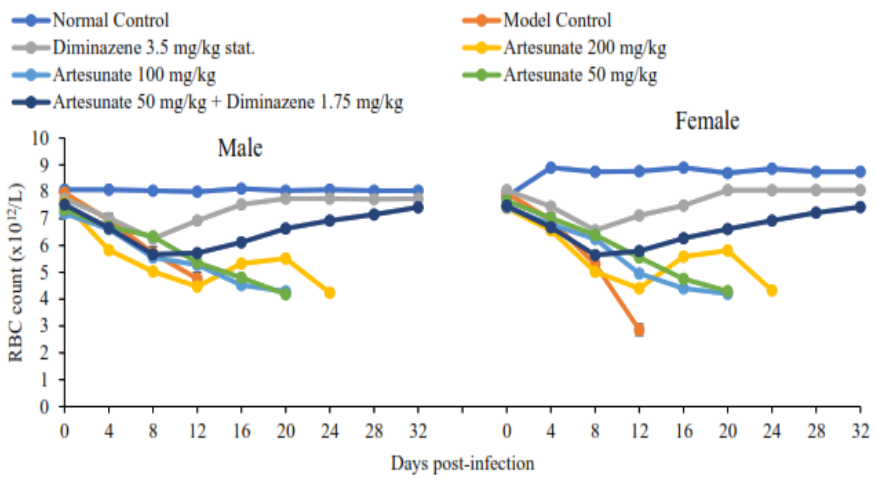

Figure 7: Effect of treatment on red blood cell count of male and female Wistar rats experimentally infected with *T. b. brucei.* Values are expressed as mean ± SEM (n=5)

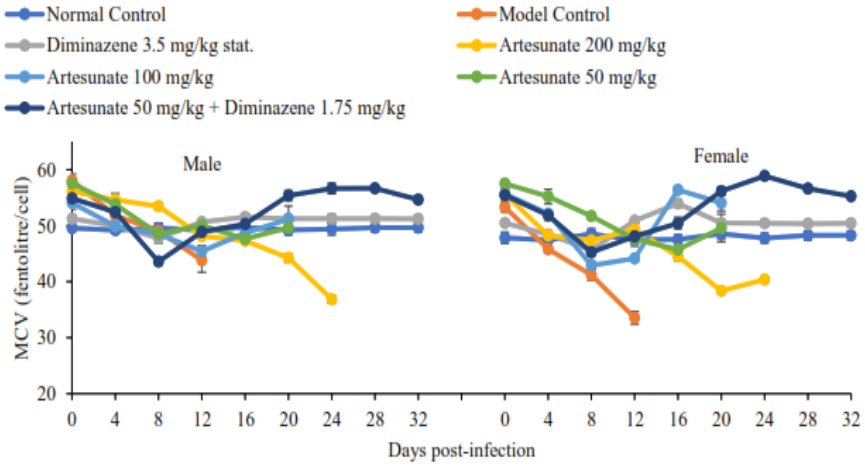

Figure 8: Effect of treatment with on mean corpuscular volume of male and female Wistar rats experimentally infected with *T. b. brucei.* Values are expressed as mean ± SEM (n=5)

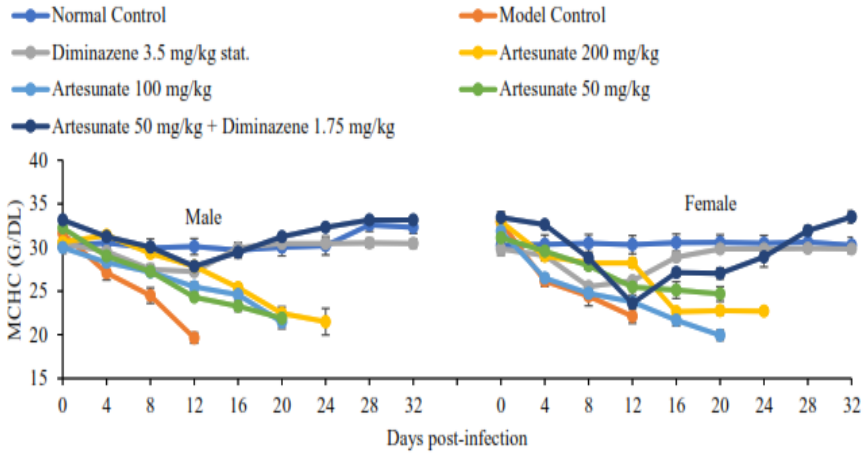

Figure 9: Effect of treatment on mean corpuscular haemoglobin concentration of male and female Wistar rats experimentally infected with *T. b. brucei.* Values are expressed as mean ± SEM (n=5)

compared to other groups (Figure 10).

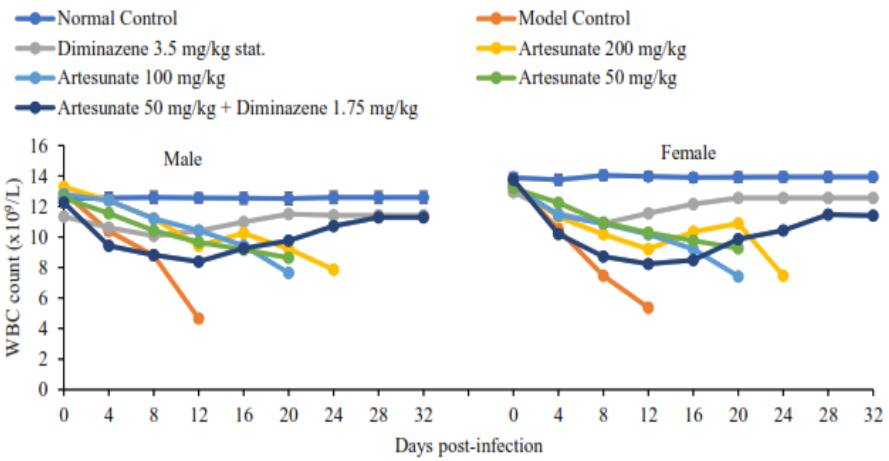

Figure 10: Effect of treatment on white blood cell count of male and female Wistar rats experimentally infected with *T. b. brucei.* Values are expressed as mean ± SEM (n=5)

Discussion

Trypanosomosis is a constraint to livestock development in sub-Saharan Africa with estimated direct annual losses to producers and consumers exceeding US$4.5 billion (Simukoko *et al.*, 2007, Yaro *et al*., 2016). This study investigated *in vitro* and *in vivo* antitrypanosomal effect of artesunate alone and in combination with diminazene aceturate in Wistar rats experimentally infected with *T. b. brucei*.

In vitro, immobilization of trypanosomes was observed to be dependent on the concentration of the test drugs when compared to controls. This is in agreement with the findings of Mishina *et al*. (2007) who reported *in vitro* inhibition of *Trypanosoma cruzi* and *Trypanosoma brucei rhodesiense* using artemisinin. Nibret & Wink (2010) also reported *in vitro* antitrypanosomal activity against *T. b. brucei* using four Ethiopian *Artesmia spp* extracts. The cessation of trypanosome motility seen in this study may be attributed to the inhibition of sarco-endoplasmic reticulum calcium ATPase (SERCA) by inhibiting H^+/K^+ ATPase (Krishna *et al*., 2004).

Four days PI, the Wistar rats were observed to be parasitaemic. Clinical signs such as weakness, huddling, pale ocular membrane, anorexia, weight loss and death were observed in the untreated group. The signs were milder in the treated groups. The prepatent period of four days seen in rats in this study agrees with the works of Kobo *et al*. (2014) and Erin *et al*. (2020) in rats. Administration of artesunate alone slightly suppressed the level of parasitaemia resulting from *T. b. brucei* in the rats studied but failed to clear the trypanosomes completely from peripheral circulation. However, it succeeded in prolonging the survival rate of the rats in groups IV, V and VI when compared with the duration in the infected and untreated rats (group II). Artesunate when used alone, was not efficacious because of the oral route of administration used in the study and the short half-life of the drug (Morris *et al*., 2011). The combination of artesunate with half the standard dose of diminazene aceturate appeared to be effective by clearing trypanosomes from the systemic circulation and decreased clinical signs. We postulated synergism between the two drugs in achieving therapy. In trypanosomosis, anaemia, pyrexia and decreased appetite are factors that result in body weight decline (Desquesnes *et al*., 2013). In this study, the infected and untreated Wistar rats experienced severe muscle protein degeneration, pyrexia, resulting in loss of body weight (Chaparro & Suchdev, 2019). The pyrexia development in trypanosomosis is due to pyrogens as a result of trypanosomes, migrating from the systemic circulation to the brain where they affect the body's thermoregulation (Dinarello & Gelfand, 2005). Elevated temperature in this study was seen to correlate with increased parasitaemia. The PCV in the *T. b. brucei*-infected Wistar rats declined significantly, as observed in this study. The severity of anaemia is often characterized by a decline in the PCV, which is considered a major indicator of the nature of the infection (Viana, 2011; Chaparro & Suchdev, 2019). Reactive oxygen species generated by trypanosomes destroy membranes of red blood cells, inducing oxidation which results in haemolysis (Stijlemans *et al*., 2018).

The administration of artesunate and diminazene aceturate in combination ameliorated the anaemia seen in this study by inhibiting the parasites from causing further damage. Hence, the return to pre-infection values seen in the treated groups. This could occur by reducing the proliferating parasite load, neutralizing the toxic metabolites produced by trypanosomes or scavenging the trypanosome

associated free radicals (Karori *et al*., 2008; Feyera *et al*., 2014).

Erythrocytic index is a major technique used when categorizing anaemia. In this study, there was a significant decrease in MCV and MCHC in the infected and untreated Wistar rat group which agrees with the previous findings of Kagira *et al.* (2006) and Kobo *et al*. (2014). However, the result of this study disagrees with the findings of Omotainse *et al*. (2016) in rabbits infected with *Trypanosoma brucei*. Microcytic hypochromic anaemia was observed in this study and it has been reported to be associated with deficiency of iron. It is possible that during infection, failure of iron fusing into RBC progenitors, even in the presence of sufficient iron storage, may precipitate the development of microcytic hypochromic anaemia. Also, inefficient recovery of iron from phagocytized RBC may also lead to iron deficiency in the body. The significant decrease in WBC counts observed in the infected and untreated group by day 12 was below the normal range when compared to the treated groups and uninfected control. This is not in agreement with the work of Sulaiman & Adeyemi (2010) who reported an increase in WBC, lymphocytes and neutrophils in rats infected with *T. b. brucei* but agrees with that of Kobo *et al*. (2014). Leucopenia observed in the infected rats was attributed to factors such as variable surface antigen of the trypanosomes, bone marrow depression of their production and immunosuppressive actions of trypanosomes in the body (Happi *et al*., 2012). The relative increase by day 24 in WBC count in the group treated with the combination of artesunate and diminazene aceturate is apparent of defense against leucopenia caused by T. *b. brucei*. This study demonstrated the *in vitro* and *in vivo* antitrypanosomal effect of artesunate alone and in combination with diminazene aceturate, thus, suggesting potential as alternative in the treatment of trypanosomosis.

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Conflict of interest

The authors declare no conflict of interest.

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