

***Trypanosoma brucei brucei* infected rats: Micronucleated polychromatic erythrocytes**

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Abstract. The emergence of bone marrow micronucleated polychromatic erythrocytes (MN-PCE) in rats experimentally infected with *Trypanosoma brucei brucei* was examined in order to understand the bone marrow effects in trypanosomiasis infection. Bone marrow was collected for micronucleus assay while blood samples were collected from infected rat for hematological analysis. The results showed evidence of MN-PCE at 12.75 ± 0.65 micronuclei/1000PCE and 9.60 ± 2.95 micronuclei/1000PCE for rats infected for 21 days and 14 days respectively. The hematology examination revealed changes in packed cell volume, haemoglobin and red blood cells with concomitant increase in parasitemia. This study revealed that the generation of MN-PCE was induced by an acute infection of *T. b. brucei* in rats and this highlights an important phase in the pathogenesis of the disease that may indicate possible damage to genetic information.

INTRODUCTION

Trypanosomiasis or sleeping sickness is a parasitic disease of the genus *Trypanosoma*. The parasite lives and multiply in their mammalian host and are transmitted by the bite of infected Tsetse fly (Steverding, 2008). *Trypanosoma brucei brucei* infects a wide range of mammals and is the causative agent of a chronic wasting condition in cattle which is called Nagana (Shiflett *et al.*, 2007). The parasites can get into the bloodstream by entering lymphatic or blood vessels evading the immune system and latter into the central nervous system (Mulenga *et al.*, 2001; Gibson, 2012). Trypanosomiasis is usually characterized by high levels of parasitemia, severe anemia, cellular infiltrations and marked changes in the lymphoid system (Morrison *et al.*, 1981). The presence of

trypanosome DNA in bone marrow has been reported (Berlin *et al.*, 2009) in infected animals but little is known whether this parasite can cause chromosomal aberrations or DNA double strand breaks.

Micronuclei are functionally small, round to oval bodies within the cytoplasm of a cell, having the same staining properties as the major nucleus, and are assumed to arise from the loss of a whole chromosome or fragment of a chromosome from the nucleus (Peace & Succop 1999) by micronucleus (MN) assay (Krishma *et al.*, 2000; Odunola *et al.*, 2007). The expression of micronuclei is dependent on mitotic cell division and it occurs during the first but also at later cell divisions (Streffer *et al.*, 1998). The presence of *T. b. brucei* in the bone marrow has raised the prospect of other possible effects in genotoxicity.

Several reports (Mabbott & Sternberg, 1995; Bosketal *et al.*, 2011) have studied the bone marrow effects of *T. b. brucei* and in spite of these efforts, studies on the effects of *T. b. brucei* on micronucleated polychromatic erythrocytes has been neglected. The MN test is used to identify substances that can cause cytogenetic damage which can result in the formation of micronuclei that contains lagging chromosome fragments or whole chromosome (USFDA, 2000). Therefore, the purpose of this study is to investigate the damage (if any) induced by *T. b. brucei* to the chromosomes or mitotic apparatus of erythroblasts by analysis of erythrocytes as sampled in the bone marrow to understand the genotoxic effect in trypanosomiasis.

MATERIALS AND METHODS

Chemicals and Reagents

Phusion blood direct kit, Dread Tag polymerase, dNTPs and primer set were obtained from Finnzymes Vantaa Finland, Fermentas St Leon-Rot Germany, Eurofins worldwide Germany respectively. Diminaveto® (contains 1.05 g of Diminazene diacetate and 1.31 g Antipyrine) was obtained from Strides vital Nigeria limited. May Grunwald stain, Bovine serum albumin, Xylene was from Sigma-Aldrich. All other reagents were of analytical grade.

Trypanosome species

Trypanosoma b. brucei (Federe) strain NITR/14 blood stream form was isolated from a cow at Federe in Plateau state Nigeria and preserved at National Institute of Trypanosomiasis Research (NITR) Vom-Nigeria by serial passages in donor rats.

Animals

Male wistar rats weighing between 180-210g were purchased from the animal house of National Research Institute for Chemical Technology Zaria-Nigeria. They were housed under standard laboratory conditions, fed normal rat chow (PLS feeds, Zaria, Nigeria) with given access to clean water ad libitum. They were allowed to acclimatize for a period

of 2 weeks before the commencement of the experiment. The rats were randomly divided into five groups of 5 rats each. Group A was infected with 10^4 trypanosomes *T. b. brucei* and monitored for 21 days, group B was infected with 10^4 *T. b. brucei* and monitored for 14 days, group C was infected with 10^4 *T. b. brucei* and treatment commenced on day 7 when parasitemia was detected for 5 days with 3.5mg/kg/day diminaveto®, group D received 0.2ml of normal saline only and group E received sodium arsenite (SA) at 2.5mg/kg body weight on day 1, 3 and 6. The dosage used is equivalent to one tenth of oral LD₅₀ (Preston *et al.*, 1987). All the infection and treatments were by intraperitoneal injection.

Parasite estimation

The presence and degree of parasitemia was determined daily using the rapid matching method by blood film examination (Herbert & Lumsden, 1976). A drop of blood was collected from the animal tail and a thin smear was prepared and examined under light microscope X 400 magnification.

Trypanosome species identification by polymerase chain reaction

The *T. b. brucei* identification from the donor rat was carried out in a nested PCR as described by Adams *et al.* (2006) using a 25µL reaction volume containing 0.5µL of each primer, 0.5µL of dNTPs, 0.5µL of Dream Taq Polymerase, 2.5 µL of Dream Taq buffer and double distilled water to a final reaction volume of 25 µL. Cycling conditions were as follows: 1 cycle at 95°C for 300s followed by 30 cycles at 94°C for 60s, 56°C for 60s, 72°C for 30s and a final extension of 33s. Two consecutive reactions were carried out using a Phusion blood direct kit and a set of outer primers (OSK 1425 and 1426) following the manufacturer's instructions. This was followed by adding the inner primers (OSK 1423 and 1424) in the second round of reactions in which 1 µL of PCR product from the first round reaction was added to 25 µL of the second round of reaction in a fresh PCR tube. The Sequences of PCR Primers are given as: OSK 1423 Forward Outer (5'TGC AAT TAT TGG TCG CGC 3'); OSK 1424

Reverse Outer (3'CTT TGC TGC GTT CTT 5'); OSK 1425 Forward Inner (5'AAG CCA AGT CAT CCA TCG 3'); OSK 1426 Reverse Inner (3'TAG AGG AAG CAA AAG 5').

Hematological analysis

Blood was collected from the rats by jugular puncture on day 14 for group B and on day 21 for group A, C, D and E prior to micronucleus assay (MA). Packed cell volume (PCV) was determined by sealing capillary tubes before centrifuging in microhaematocrit. The PCV was calculated as percentage of the total blood volume occupied by RBC mass in the haematocrit which depends mostly on the number of the RBCs. Differential white cell count was done by dropping blood from the edge of glass slide then it was air dried before Leishman stain was added on the dry blood smear. Immersion oil was dropped at the edge of the slide before viewing in a light microscope X 100. The haemoglobin (Hb) concentration was evaluated by a spencer haemoglobinometer chamber with a drop of blood and the colour was matched against the standard colour of the haemoglobinometer. The corresponding value on the scale in g/dL was read.

Preparation of bone marrow smears

The rats were weighed and intraperitoneally injected with 0.04% colchicine (1ml/100g body weight) 2 hours prior to when they will be sacrificed. The rats in groups A, C, D and E were sacrificed by cervical dislocation on day 21 while group B rats on day 14. The femur of each rat was removed and stripped clean of muscle then a scissors was used to make an opening in the iliac region of the femur. A small pin was then introduced into the marrow canal at the epiphyseal end. As the pin was pushed inside the canal, the marrow exuded through the hole at the iliac end. The marrow was placed into a slide and a drop of fetal calf serum was added to the smear using a Pasteur pipette. The mixture was made homogeneous and then spread on the slide as smear was air dried.

Micronucleus assay

The slides of bone marrow smears were fixed with methanol for 5 minutes and air-dried then

pretreated with 0.4% May Grunwald stain-1 for 5 minutes and with 0.4% May Grunwald stain 2 for another 5 minutes. The slides were air-dried and stained with 5% Giemsa for at least 30 minutes then rinsed in phosphate buffer (0.1M, pH 6.8) for about 30s and then in distilled water. The slides were air-dried then fixed in xylene for 20 minutes then the presence of micronucleated polychromatic erythrocytes (MN-PCE) was detected under light microscope X 400.

Statistical analysis

The results were expressed as Mean $\pm$ SEM and the significance of the differences between means were determined by ANOVA and P values <0.05 was considered as significant.

RESULTS

Parasitemia and course of the infection

The parasites were observed in the peripheral blood of the infected animals on day 7 post infection with progressive increase of parasitemia over time for group A and group B (Figure 1). Clinical signs such as apathy and weakness were observed in all animals of group A and B except for group C for which the animals were cured as from day 11 after they received treatment with diminaveto.

The rats in group D showed no *T. b. brucei* trypomastigotes in blood smear and remained clinically healthy during the experimental period with decrease in weight in all the animals that were infected with the parasite.

Micronucleus assay

The bone marrow MA shows the frequency (Figure 2) of MN-PCE induced in the bone marrow cells of group A rats as compared with control groups D and E ($P < 0.05$).

Trypanosoma b. brucei significantly induced MN-PCEs formation in the bone marrow cells to 12.75 ± 0.65 micronuclei/1000 PCE for 21 days and 9.06 ± 2.95 micronuclei/1000 PCE for day 14. Group E animals administered with clastogenic agent SA induced MN-PCE formation at 20.75 ± 6.55 micronucleated/1000 PCE which is higher

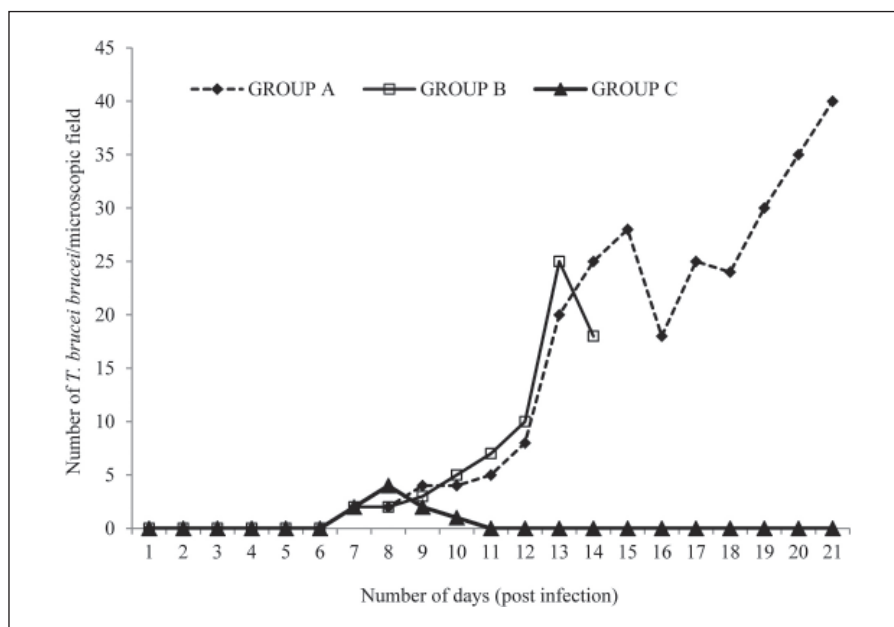


Figure 1. Parasitemia profiles of the *T. brucei brucei* infected rats. Groups A and B were infected with the parasites and monitored for 21 and 14 days respectively, before termination. Group C was infected with the parasites and treated with 3.5 mg/kg bw of diminaveto®

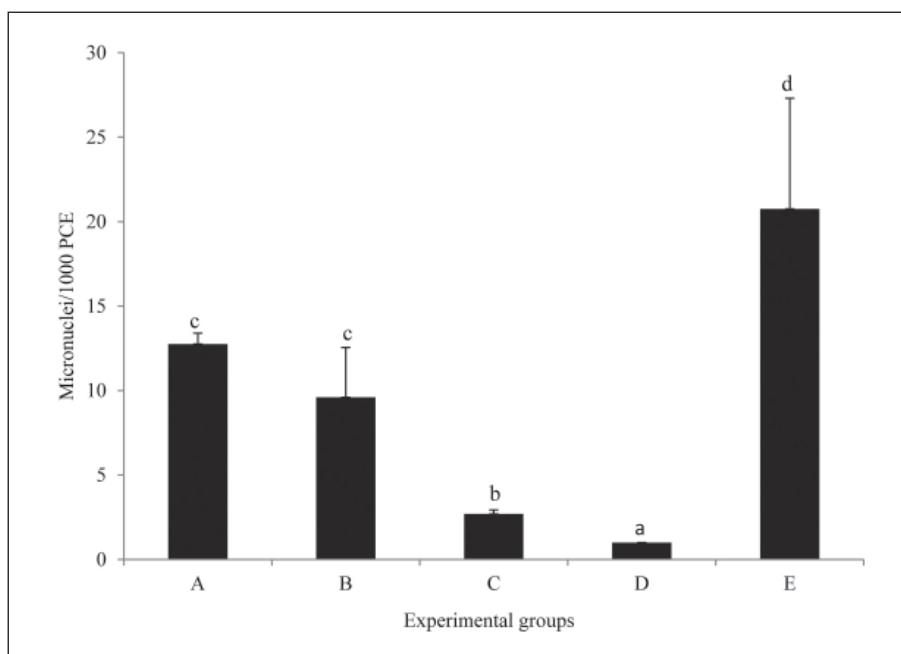


Figure 2. Bone marrow micronucleic polychromatic erythrocytes. Data are presented as mean $\pm$ SEM. ^{a-c}Values with different alphabets over the bars are statistically significant ($p < 0.05$). Groups A and B were infected with the parasites and monitored for 21 and 14 days respectively, before termination. Group C was infected with the parasites and treated with 3.5 mg/kg bw of diminaveto®. Group D is an uninfected (normal) control group while Group E was uninfected but administered 2.5 mg/kg bw of sodium arsenite

than *T. b. brucei* infected groups B and A. The group C animals were infected with *T. b. brucei* and treated with 3.5mg/kg/day diminaveto® did not demonstrate neurological signs, clinical signs such as apathy and weakness were not observed in all the animals as in the case of groups A and B animals but on assessment of MN-PCEs formation group C was 2.71 ± 0.24 micronuclei/1000 PCE which are significantly different from the MN-PCEs formation in group A. The recovery of the rats in group C after induction of MN-PCE was observed to be significant.

Polymerase chain reaction

The blood of an infected rat was used for PCR 3 hours before injecting 0.04% colchicines. The band in Figure 3 runs at 400bp. This was done to verify the presence of the *T. b. brucei* in vivo which is implicated with the pathogenesis of the disease.

Hematological analysis

The mean values for the hematological examination are presented in Table 1. The examination was carried out before intraperitoneal injection of 0.04% Colchicine. Blood was collected to assess the progression of the disease.

Statistical analysis showed a significant decrease between groups A and B as compared to group D in RBC, PCV and Hb

values. Group E animals which received SA had PCV values significantly different from that of groups A and D. The analysis of RBC indices indicates marked anemia in all the infected rats of groups A and B.

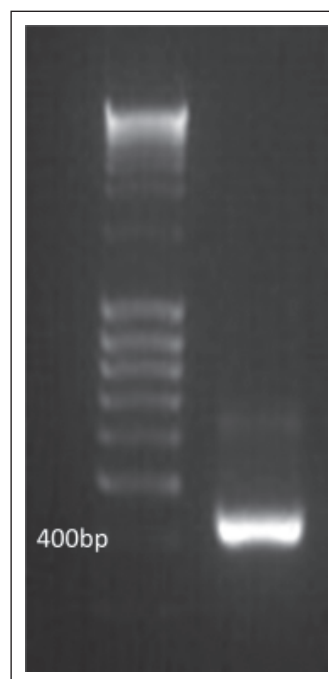


Figure 3. Ethidium bromide-stained nested PCR products after Agarose gel electrophoresis for *Trypanosoma brucei brucei* strain. The band runs at 400bp a DNA fragment which confirms the presence of the parasite

Table 1. Hematological values before intraperitoneal injection of colchicine

GROUPS	PCV (%)	Hb (g/dL)	WBC (X 10 ⁹ /L)	RBC (X10 ⁻¹² /L)
A	28.33±0.58 ^a	10.10±0.55 ^a	1.28±0.21 ^a	3.00±0.22 ^a
B	35.00±1.50 ^b	12.16±0.59 ^b	1.15±0.13 ^a	4.50±0.53 ^b
C	40.00±2.89 ^{bc}	11.70±1.10 ^{ab}	1.25±0.22 ^a	5.75±0.26 ^c
D	42.00±0.71 ^c	13.40±0.81 ^b	1.33±0.57 ^a	6.37±0.15 ^d
E	34.33±0.82 ^b	10.45±0.42 ^a	2.30±0.10 ^b	4.13±0.15 ^b

^{a-c} Values with different superscripts letters along a column are significantly different from each other (P<0.05). Groups A and B were infected with the parasites and monitored for 21 and 14 days respectively, before termination. Group C was infected with the parasites and treated with 3.5 mg/kg bw of diminaveto®. Group D was an uninfected (normal) control group while Group E was uninfected but treated with 2.5 mg/kg bw of sodium arsenite. PCV: packed cell volume, Hb: hemoglobin, WBC: white blood count, RBC: red blood cells

DISCUSSION

Trypanosoma b. brucei is among the protozoan diseases that affect animals and causes trypanosomosis which can be fatal if untreated thus presenting an excellent model for the study of trypanosome infection (San-Qiang *et al.*, 2011). It therefore provides an excellent system for trypanosome studies of many aspects of cell biology including cell structure, morphology, organelle positioning and cell division (Mathews, 2005). This study, reports the effect of *T. b. brucei* ability to induce MN in rat bone marrow. The rats infected with *T. b. brucei* induced statistically significant responses observed in the bone marrow in vivo on day 14 and on day 21. The band (400bp) of the PCR products of *T. b. brucei* infected blood of the rats after Agarose gel electrophoresis confirms that the rats in groups A, B, C were positive for the trypanosome species responsible for MN-PCE in the bone marrow. This identification is required because parasite infections frequently contain multiple strains of the same parasites species (Balmer & Caccone, 2008) and high level of multiple genotypes (Gustave *et al.*, 2011) by DNA sequencing (McCulloch & David, 2009) have been reported.

The differences between the MN-PCE production which was high in group A when compared to group B is attributed to the level of parasitemia and the time required for *T. b. brucei* to proliferate. The detection of parasitemia occurs on day 7 post infection and characterized by exponential parasite growth along with clinical signs such as apathy, weakness and anemia. In this study, infected rats showed a significant decrease in RBC, Hb and PCV while there was no significant difference in WBC. *T. b. brucei* infection in rats is known to affect blood levels, tissue leukocyte apoptosis (Happi *et al.*, 2012) and erythropoiesis in the bone marrow (Nishimura *et al.*, 2011). This event is associated with the pathogenesis of *T. b. brucei* (Sandesh *et al.*, 2010; Boreham & Wright, 2012) and alteration of B cell development in bone marrow (Bockstal *et al.*, 2011).

The use of SA as a standard clastogenic agent have been emphasized (Odunola *et al.*, 2007). Therefore, to enable comparison with the *T. b. brucei* infected rats, SA was employed. Micronuclei are formed as a result of chromosomal breakage or spindle damage (Morales-Ramirez *et al.*, 1997; 1999) and this was the significant observation in this study. Fragments of whole chromosomes may not be included in the nuclei of the daughter cells following cell division and single or multiple micronuclei in the cytoplasm of these cells on day 21 during acute *T. b. brucei* infection. However, induce bone marrow damage is implicated with the simultaneous frequency and duration of clastogens (Dertinger *et al.*, 2011). The MN-PCE observed could be associated with the degree of infection and pathogenesis

of the disease from the time the parasite was intraperitoneally injected into the experimental rats. The likely events are: the proliferation of parasites which depends on the dose administered and the nature or species of parasite, distribution of parasite towards the target tissue, re-distribution and transportation of active metabolites, reaction with DNA resulting to strand break production, erythroblast division and the micronuclei formation, normoblast enucleation and the formation of MN-PCE. Bone marrow develops into a polychromatic erythrocyte (PCE) in which the main nucleus is extruded therefore, any MN that have been formed may remain behind in the otherwise anucleated cytoplasm (USFDA, 2000). The production of MN-PCE by *T. b. brucei* was confirmed in this study when treatment was administered to infected rats. The absence of the parasite in the rats after treatment revealed a decrease in MN-PCE production to 2.71 ± 0.24 micronuclei/1000PCE which is compared to that in the control group (1.03 ± 0.50 micronuclei/1000PCE) that was uninfected with the parasite. This superimposes that the parasites had multiplied and have dominated the host dynamics during chronic infections with corresponding parasite-driven pathogenesis.

In conclusion, the absence of main nucleus facilitated the visualization of the micronuclei. The increase in the MN-PCE in this study is an indication of induced chromosomal damage which may further complicate the disease.

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