



Blood tumour necrosis factor- α and the pathogenesis of anaemia in *Trypanosoma brucei* infected rabbits

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Abstract

Trypanosomosis is a protozoan infection of domestic and wild animals characterized by anaemia, however, the pathogenesis of trypanosomosis-induced anaemia is not fully understood. This study evaluated the possible roles of Tumour Necrosis Factor-alpha (TNF- α) in the pathogenesis of anaemia induced by *Trypanosoma brucei* in rabbits. Twelve adult rabbits of both sexes with mean weight of 2.1 ± 0.1 kg were randomly assigned into two groups of six rabbits each. Group A rabbits were intraperitoneally (i.p) infected with blood containing 2×10^6 /ml of *T. brucei*, while group B (control) rabbits were injected with one ml of normal saline i.p. Blood was collected from the ear vein before infection and weekly thereafter for six weeks post-infection (p.i), in order to determine the packed cell volume (PCV), haemoglobin (Hb) concentration, red blood cell (RBC) count, reticulocyte count (RC) and serum concentrations of TNF- α . The PCV, RBC count and Hb concentration were significantly ($p < 0.05$) lower in group A than group B—rabbits throughout the duration of the study. Serum concentration of TNF- α was significantly ($p < 0.05$) higher in group A (227.5 ± 8.1 ng/ml) than group B (51.3 ± 8.2 ng/ml) at week four post-infection. The serum concentration of TNF- α negatively correlated with PCV ($r = -0.513$) and Hb ($r = -0.769$) in group A. The study concluded that anaemia observed during experimental infection in rabbits with trypanosomosis was associated with increased levels of TNF- α .

Keywords: Anaemia, Rabbits, Pathogenesis, Trypanosomosis, Tumour Necrosis Factor- α

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Introduction

African Animal Trypanosomosis (AAT) also known as Nagana is a protozoan disease caused by flagellated protozoan parasites that inhabit the blood, lymph and various tissues of their hosts (OIE, 2013).

A pathological feature of African trypanosomosis is anaemia which develops very early during infection (Omotainse & Anosa, 1992). Witola & Lovelace (2001) reported that the cause of anaemia in African trypanosomosis is complex and involves a variety of mechanisms which includes: haemolysis (Anosa & Isoun, 1980), haemodilution (Anosa & Isoun, 1976; Anosa, 1988), haemorrhages (Hill & Esuruoso, 1979) and dyshaemopoiesis (Omotainse & Anosa, 1992).

It has been postulated that pro-inflammatory cytokines such as Tumour Necrosis Factor (TNF- α) negatively affects the effectiveness of

erythropoiesis (Stenvinkel, 2001; Macdougall & Cooper, 2002). It has also been observed that cytokines might mediate anaemia associated with inflammation, which accompanies protozoan infections (Naessens *et al.*, 2005). The pro-inflammatory cytokines, interferon (IFN- γ), TNF- α , and interleukin-1 (IL-1) have been shown to suppress erythropoiesis *in vitro* in chronic inflammatory disease (Dai *et al.*, 1998; Allen *et al.*, 1999).

Tumour Necrosis Factor-alpha is a potent proinflammatory cytokine (Isaac *et al.*, 2010) synthesized and secreted by mononuclear phagocytes (monocytes and macrophages), neutrophils, activated lymphocytes, fibroblast, natural killer cells, mast cells, keratinocytes and neurons in response to injury induced by infections, immunological, toxic, traumatic, and

ischemic stimuli (Leibovich *et al.*, 1987; Köck *et al.*, 1990; Montesano *et al.*, 2005). TNF- α has been observed to impair erythropoiesis through its ability to inhibit proliferation and differentiation of Erythroid Colony Forming Unit and Erythroid Burst Forming Unit via the induction of apoptosis, down-regulation of erythropoietin-receptor expression and reduce formation of stem cell factor (Guenther & Lawrence, 2005). Naessens *et al.*, (2005) reported TNF- α to mediate the development of anaemia in murine *T. brucei rhodesiense* infection. The hypothesis of this study is that there shall be no significant changes in the sera concentration of TNF- α in rabbit infected with *T. brucei*. This study aims to evaluate the changes in the haematology and the TNF α concentration in the plasma of *T. brucei* infected rabbits.

Materials and Methods

Twelve Chinchilla rabbits of both sexes (six males and six females) weighing between 1.36-2.68 kg and aged between 5-6 months were used for the study. The rabbits were purchased from a local rabbit farm in Abeokuta, Ogun State and housed singly in standard fly proof rabbit cages in the experimental animal unit of the College of Veterinary Medicine, Federal University of Agriculture Abeokuta. They were acclimatized for four weeks before the commencement of the experiment. During the period of the acclimatization, the rabbits were examined for gastrointestinal and blood parasitic infections. The rabbits were prophylactically treated with Albendazole (A-Zole®) and Ivermectin (Kepromec®, Holland) at the rate of 5mg/kg body weight orally and 400 μ g/kg body weight subcutaneously respectively and 20% Oxytetracycline injection (Tetroxyl®) was administered at 22mg/kg body intra-muscularly against helminths and haemoprotozoan infections. They were fed with rabbit pellets (Vita Feeds®, Grand Cereals, Jos, Nigeria) and water *ad libitum*. The study followed the humane method for handling animals as outlined by CACC, 1993 and was approved by research ethical committee with reference number FUNAAB/ COLVET/ REC. 16/ 02.

Rectal temperature and live weight of the rabbits were measured and recorded daily, with a digital thermometer (Vcare®, China) and table scale (Mettler Toledo®, Columbus, USA) respectively.

A simple control randomized experimental study comprising of two groups (Groups A and B) with six animals per group was conducted. Group A rabbits were infected with *Trypanosoma brucei* 2×10^6 trypanosomes in 1ml of blood and Group B rabbits were given 1ml of normal saline and served as uninfected control.

Trypanosoma brucei used in this study was obtained from the Nigerian Institute of Trypanosomiasis Research (NITR), Vom, Plateau State, Nigeria.

Rectal temperature and blood (2ml) was collected from rabbits through aseptical venipuncture of the ear vein, 0.5ml was put into sodium ethylene diaminetetra acetic acid (EDTA) bottle for parasite determination, complete blood count and 1.5ml into plain bottle for the determination of serum concentration of cytokines. This was repeated weekly over a period of 50 days. Surviving infected rabbits at the end of the experiment were treated with diminazene aceturate (Veriben®) at 3.5mg/kg. Haematological indices such as packed cell volume (PCV), haemoglobin concentration, total red blood cell (RBC) and absolute reticulocyte were determined according to Jain (1986). Capillary tubes were filled with blood until three-quarter full. One end was then sealed with plasticin. The capillary tubes were spun in a microhaematocrit centrifuge (Hawksley and Sons, London) for five minutes at 1200 \times g and the PCV values were determined using the Hawksley haematocrit reader (Jain, 1986). Total RBC count was carried out manually (Jain, 1986) using the improved Hawksley haemocytometer. The blood was diluted 1:200 using Dacies fluid (10ml of 40% formaldehyde and 990ml of 30% aqueous solution of sodium citrate). The haemoglobin concentration was measured spectrophotometrically by the cyanomethaemoglobin method (Jain, 1986) using SP6-500UV spectrophotometer (PYE UNICAM, England). Reticulocyte counts were determined by adding equal volumes of Vital stain (New Methylene blue 0.5g, potassium oxalate 1.6g and distilled water 100ml (Schalm *et al.*, 1975), and blood were mixed on a clean glass slide and allowed to stand for 20 minutes, at room temperature. Thin smears were made and air dried. By counting 500 red cells and noting the number of reticulocytes, the percentage of reticulocytes was calculated. For absolute reticulocyte counts, the percentage reticulocyte count was multiplied by the total red cell count.

Tumour Necrosis Factor-alpha was assayed using rabbit TNF- α ELISA kit (MyBiosource®, USA) which was based on a double antibody sandwich enzyme-linked immune-sorbent assay technology. Fifty micro litres each of the standards solution were added into designated wells in the precoated microtitre plate in an order of highest concentration to lowest concentration. The concentrations of standard solutions were 800 pg/ml, 400 pg/ml, 200 pg/ml, 100 pg/ml, 50 pg/ml, 25 pg/ml, 12.5 pg/ml. The 7th well (G1) was set as the blank well. Thereafter, 40 μ l of the sample diluent and 10 μ l of rabbit test samples were then

added into the rest of the wells in the microtitre plate. Thereafter 50 μ l of horseradish (HRP) was added into each well, except the blank well. Then the plate was sealed and incubated for 60 minutes at 37°C after which the plate was unsealed and washed with the wash solution. Fifty micro litres of chromogen solution A was then added into each well, thereafter, 50 μ l of chromogen solution B was also added into each well. Then the microtitre plate was gently shaken and then incubated for 10 minutes at 37°C in the dark. Thereafter 50 μ l of stop solution was then added into each well. The microtitre plate was then taken to an ELISA plate reader (BioTek®) and the optical density (O.D.) was read at a wavelength of 450nm. Reading was done within 15 minutes of adding the stop solution.

Statistical analysis

Data collation was done in Microsoft Excel while Statistical Package for Social Sciences (SPSS. 17) was used for statistical analysis. Data were presented as means \pm standard error of mean (SEM) and analyzed using 2-way analysis of variance (ANOVA). Relationships between parameters were determined using Pearson correlation. Values of $p < 0.05$ was considered significant.

Results

Parasitaemia was first observed with thick smear 5-8 days post infection (*p.i*) in the *T. brucei*

infected group and there was a progressive significant ($p < 0.05$) increase in rectal temperature of the *T. brucei* infected group while that of the control remained within normal values throughout the study (Figure I).

There was a progressive significant ($p < 0.05$) decrease in the weight of *T. brucei* infected rabbits from week 2 *p.i.* to week 6 *p.i.* At the end of the experiment *T. brucei* infected rabbits had an average weight loss of 0.18kg (Figure II). Pearson's correlation of the *T. brucei* group showed a negative correlation ($r = -0.221$) which was not significant ($p > 0.05$) between live weight and TNF- α .

The pre-infection PCV values were $44.33 \pm 1.48\%$ for the control group and $43.17 \pm 0.65\%$ for *T. brucei* group respectively. This value in the *T. brucei* group decreased progressively but gradually till the termination of the experiment. Throughout the six-week period of the experiment, PCV values of the *T. brucei* group remained significantly ($p < 0.05$) lower when compared with the non-infected control group (Figure III).

Seven days *p.i.*, there was a significant ($p < 0.05$) decrease in the RBC counts of *T. brucei* infected rabbit group ($5.77 \pm 0.38 \times 10^{12}/l$). The RBC counts for *T. brucei* group significantly ($p < 0.05$) remained lower than the control group except at week 6 *p.i.* (Figure IV).

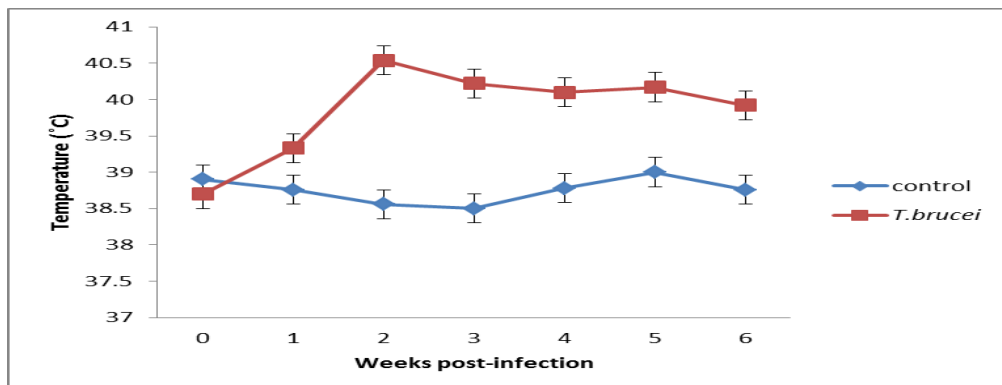


Figure 1: Mean temperature profile of *Trypanosoma brucei* infected and control rabbits

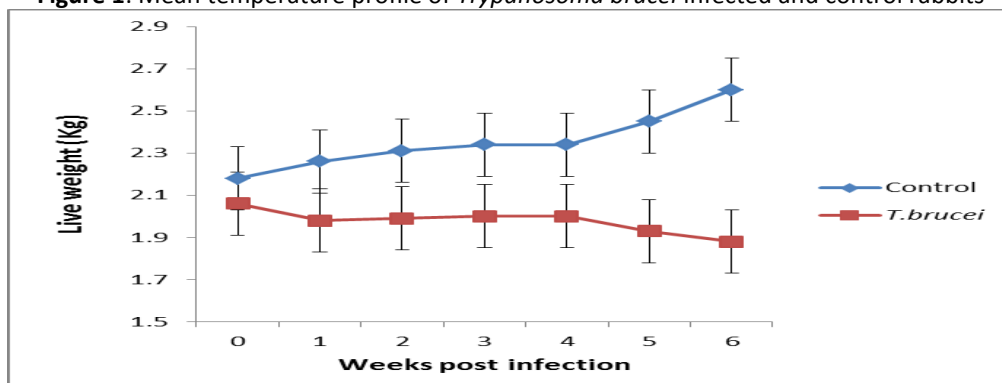


Figure 2: Mean live weight of *Trypanosoma brucei* infected and control rabbits

There was a gradual significant ($p<0.05$) increase in the RET count in rabbits infected with *T. brucei*. This increase in RET post-infection was sustained throughout the six weeks of infection as shown in Figure III.

The pre-infection haemoglobin (Hb) concentration values were 15.52 ± 0.81 g/dl for the control and 15.37 ± 0.86 g/dl for the *T. brucei* infected group. There was a significant ($p<0.05$) decrease of Hb in the infected rabbits as compared with the relatively stable values in the control group (Figure IV).

Following infection, the mean MCV values of infected rabbits significantly ($p<0.05$) increased at week 2 when compared with the control (Figure V) and there was a significant ($p<0.05$) decrease in mean MCHC of *T. brucei* infected rabbit group

when compare to the control at weeks 4 and 5 *p.i.* (Figure V).

The mean pre-infection serum TNF- α concentration in the control group and *T. brucei* group were 51.0 ± 5.83 ng/ml and 50.6 ± 6.4 ng/ml respectively. The Serum TNF- α levels increased significantly ($p<0.05$) in the *T. brucei* infected group at weeks 3 and 4 *p.i.* (165 ± 8.24 ng/ml and 227.50 ± 8.24 ng/ml) when compared to the control group (Figure VI). Pearson's correlation of the *T. brucei* group showed a positive ($r= 0.797$) but not significant ($p>0.05$) correlation between RET and TNF- α . There was a negative ($r= -0.769$) correlation between Hb concentration and TNF- α and also a negative ($r= -0.513$) correlation between TNF- α and PCV in the *T. brucei* group. TNF- α also had a negative ($r= -0.730$ and $r=-0.656$) correlation with MCV and MCHC.

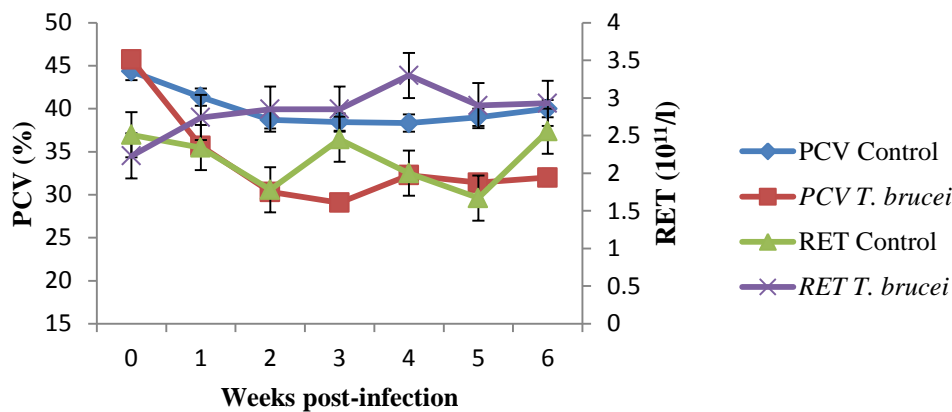


Figure 3: Mean the Packed Cell Volume and Mean Absolute Reticulocyte Count of *Trypanosoma brucei* infected and control rabbits

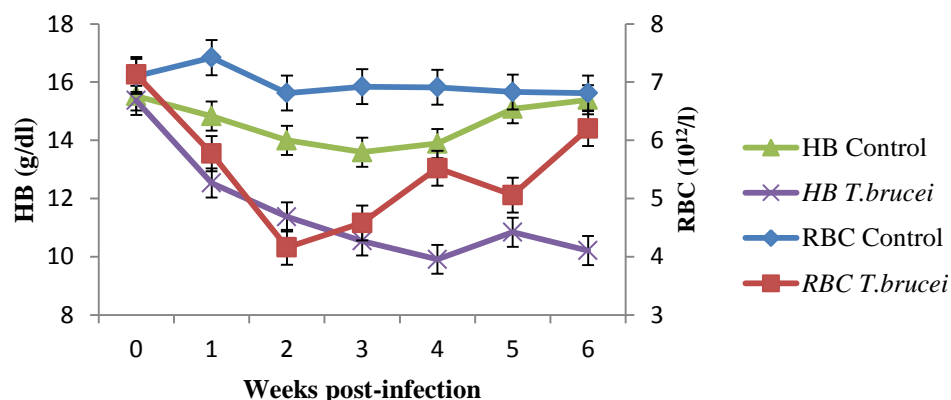


Figure 4: Mean Haemoglobin concentration and Mean Red Blood Cell count in *Trypanosoma brucei* infected and control rabbits

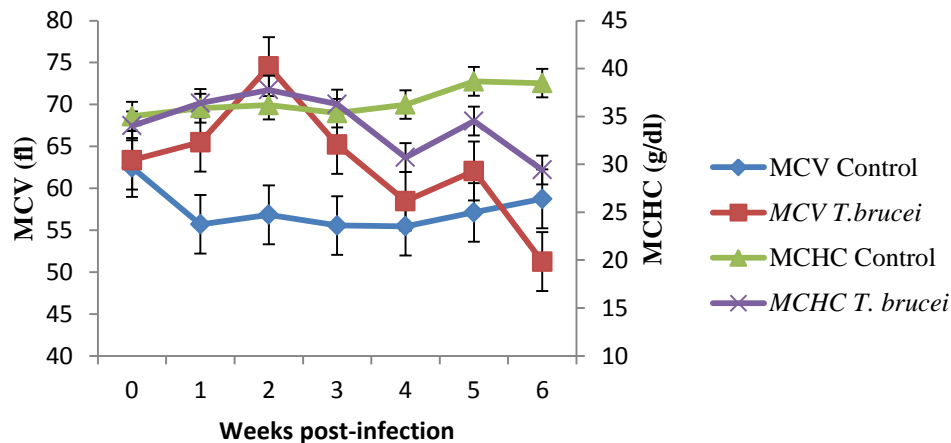


Figure 5: Mean Corpuscular Volume and Mean Corpuscular Haemoglobin Concentration in *Trypanosoma brucei* infected and control rabbits

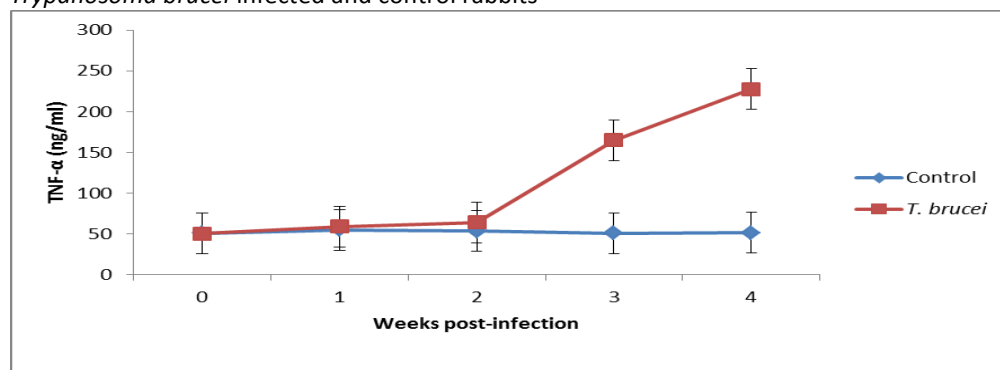


Figure 6: Mean tumour necrosis factor- α concentration in *trypanosoma brucei* infected and control rabbits

Discussion

The *Trypanosoma brucei* isolate used for inoculation showed marked pathogenicity in rabbits. This observation is similar to previous studies by Erah *et al.* (2003) who report that *T. brucei* species are highly pathogenic in rabbits. Intermittent pyrexia has been documented in rabbits infected with *T. brucei* Takeet & Fagbemi (2009) contrary to the observation in the present study in which rectal temperatures of *T. brucei* infected rabbits were persistently elevated. This increase in temperature had been attributed partly to the effect of toxic metabolites produced by trypanosomes, increased parasitaemia and presence of elevated TNF- α (Okomo-Assoumou *et al.*, 1995; Mbaya *et al.*, 2012).

There was a negative but insignificant correlation between body weight and TNF- α in *T. brucei*-infected rabbits. It has been reported that TNF- α is responsible for cachexia in infectious diseases (Larry & John, 2003), that TNF- α is a cachectin that promotes cachexia (Okomo-Assoumou *et al.*, 1995). TNF- α level in infected group had negative correlation with weight in this study, indicating progressive weight loss with increasing levels of TNF- α in *T. brucei* rabbits. The major

haematological changes observed in this study were significant decreases in PCV and Hb in the infected rabbits when compared to the control ($p < 0.05$). This is in consonance with the works of Biryomumaisho & Katunguka-Rwakishaya (2007), and Takeet & Fagbemi (2009), who reported anaemia and leucopenia in *T. brucei* infected rabbits. The *T. brucei* group for most of the period of the experiment had normocytic normochromic anaemia except at weeks 2, 4 and 5 *p.i* where the anaemia was macrocytic hypochromic. There was also high reticulocyte count at week 2 *p.i* which coincided with elevated MCV. The resulting macrocytic anaemia in the infected group was due to the high reticulocyte count. The increase in MCV in the acute phase reaching peak at week 2 *p.i* and a drop to normal and below normal, later in these investigations suggests that erythropoiesis moderately increased during the acute phase but wanes and progresses into chronic phase. This observation is also in agreement with the report of Igbokwe (1989). The MCHC in the *T. brucei* group slightly increased *p.i* in weeks 1, 2, and 3 but later decreased below the control group. This slight increase might have been due to the

presence of circulating free haemoglobin liberated from haemolysed red cells (Igbokwe, 1989). The decrease MCHC might also have been due to the hyperactivity of the mononuclear phagocytic system which is a prominent feature in African Trypanosomosis (Anosa & Kaneko, 1984). In this study, *T. brucei*-infected group had a negative correlation between TNF- α and haemoglobin concentration and TNF- α and PCV. This may likely be a contributory factor to the inadequate erythropoiesis.

Trypanosoma brucei has been reported to be tissue invasive and extravasation of the parasite into body tissues leads to extensive tissue damage (Abenga, 2014). The increase in TNF- α in *T. brucei* infection in this study might be due to the tissue invasiveness of the parasite. This observation agrees with Naessens *et al.* (2005) who reported that higher levels of TNF- α in *T. brucei* infected mice, which have been reported to inhibit erythroid cell development by inhibiting the formation of erythroid colony forming units (Roodman *et al.*, 1987; Davis *et al.*, 1997) and this might have been the reason for the negative correlation between serum TNF- α concentration, PCV and RBC count in *T. brucei* infected rabbits and thus mediate positively the development of anaemia.

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