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Effect of Aflatoxin B-1 on Course of Infection of *Trypanosoma congolense* in Mice

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ABSTRACT
Mycotoxins as secondary metabolites are known to be common contaminants of both human food and animal feed. If ingested in minute but regular doses, they are known to cause suppression of the immune system and therefore, alter pathogenesis of many infectious diseases. *Trypanosoma congolense* an intravascular parasite is the most important cause of African animal trypanosomosis. The aim of this work was to investigate the effect of aflatoxin B-1, a common mycotoxin on progression and response of *T. congolense* to chemotherapy. Female Swiss white mice were intra-peritoneally injected with 0.05mg/kg body weight aflatoxin B-1 every after 3 days upto 10 times and on the 21st day were infected with *T. congolense*. Parasitological parameters including weight, packed cell volume and parasitaemia levels of aflatoxin B-1-injected-*T. congolense*-infected mice were compared with those of *T. congolense*-infected mice. ANOVA and mean separation were used to determine differences between the test and control mice. It was observed that there was significant difference ($p<0.05$) in body weight and mean death time but no significant difference in packed cell volume. It was concluded that aflatoxin B-1 has an effect on pathogenesis of *T. congolense*.

Key words: *Trypanosoma congolense,* Aflatoxin B-1, progression, chemotherapy

1.0 INTRODUCTION

Trypanosoma congolense causes African animal trypanosomiasis (AAT), which is a severe and fatal disease of cattle and other animals. AAT is a wasting disease in which there is a slow progressive loss of condition accompanied by increasing anaemia and weakness to the point of extreme emaciation, collapse and death. Therefore, it can be said categorically that the tsetse-infested belt of Africa cannot be utilized for animal farming because of AAT. Ideal farm practice has been developed by people in the tsetse belt, which involves crop-livestock interactions. It is deemed necessary to apply manure to fertilize crops and use crop residues to feed livestock (Tittonell *et al*., 2009). In doing this, the farmer is able to supplement one agricultural practice with the other hence improving on returns from the ever decreasing land/farm. The use of spoiled farm produce, particularly crop residues like maize stover, is a likely source of mycotoxins. Investigation of effect of mycotoxins on progression of trypanosomiases is yet to be studied (Kibugu *et al*., 2009).
Trypanosomiases compromise the immune system's haemopoietic system(Williams *et al*., 2004). The disease interferes with functions of essential nutrients like vitamins (Anyanwu *et al*., 2004) and exerts pathological effects on vital organs like spleen, liver, kidney, heart, lymphoid tissue and central nervous system (Kibugu, 2008) and may influence the direction of infections of various diseases. Aflatoxin B-1 (AFB-1) on the other hand is a common mycotoxin of the tropics, which contaminates farm produce, food products and feeds (Azziz-Baumgartner *et al*., 2005). Mycotoxic contamination occurs during pre-harvest, harvest and post-harvest activities and is favoured by plant (or crop) genotype, handling, ecological characteristics and edaphic factors (Williams *et al*., 2004). AFB-1 enters the body through ingestion of contaminated food, inhalation, skin contact or vertical transfer and its symptoms among others depends on state of health of the exposed individual (Bennett and Klich, 2003). AFB-1 is carcinogenic, mutagenic, immunotoxic, and is a growth impairment factor particularly for protein calorie malnutrition (Gong *et al*., 2004). Besides these, AFB-1 causes gastrointestinal dysfunction, reduced food utilization and efficiency, anaemia, jaundice, ascites and high death rate. Just like other mycotoxins, AFB-1 may contribute to pathogenesis of many diseases and conditions (Thrasher, 2007). Endemicity of AAT and AFB-1 to the tropics and their immunosuppressive properties forms the quest for this research work.

2.0 MATERIALS AND METHODS

2.1 Ethical approval

All protocols and procedures that were used in this study were reviewed and approved by KARI-TRC Institutional Animal Care and Use committee (IACUC). The room where the study took place was well labeled and access to it limited to a few members of the technical staff. Use and handling of mycotoxin and its detoxification was as described by Scott (1995) and Karlovsky (1999).

2.2 Mice
Fifty mice (2 donor mice and 48 experimental mice), 30-day old female Swiss white mice weighing between 20-30g from the small animal breeding unit colony at KARI-TRC were used (this was based on availability at the facility). The mice were acclimatized for 14 days before commencement of the experiment during which were dewormed using ivermectin at the rate of 20mg/kg. The mice were housed in groups of 12 per cage and maintained on mice pencils, (Unga Feeds Limited, Kenya) and water *ad libitum* at a temperature of 21-25°C and on wood shavings as bedding material (Seed and Sechelski, 1988).

2.3 Trypanosome
A cryo-preserved *T. congolense* stabilate KETRI 2409, from KARI-TRC trypanosome bank was used to infect the experimental mice. The stabilate was isolated by Dr. George Losos in 1978 from a bovine host at Galana, Malindi, Kenya (Losos, 1986).

2.4 Drug for treatment
Diminazene aceturate powder 7%, (Veriben®, Sanofi) was used to treat infected mice in groups C and D, (Table 1) at the rate of 20mg/kg body weight at the on-set of parasitaemia (Mdachi personal communication).

2.5 AFB-1
Analytical column purified extracts of AFB-1 from Bora Biotech Limited, Cooper centre, Nairobi, Kenya were used during the experiment.

2.6 Experimental design
Four groups of mice were used in the study, and divided and treated as indicated in Table 1. Group A mice were injected with AFB-1 intraperitoneally (ip) at rate of 0.05mg/kg (Gathumbi, *et al*., 2001) every third day and then infected with *T. congolense* 21 days post exposure to AFB-1. Group B mice were not injected with AFB-1, but only infected with *T. congolense*. Group C mice were injected with AFB-1 ip at rate of 0.05mg/kg every third day and then infected with *T. congolense* 21 days post exposure to AFB-1, then were treated with diminazene aceturate at the on-set of parasitaemia. Group D mice were not injected with AFB-1, but only infected with *T. congolense* and then treated with diminazene aceturate at the on-set of parasitaemia.

Table 1: Summary of mice groups and treatments

Group	Number of mice	Treatment
A	12	Injected with AFB-1 ip at rate of 0.05mg/kg every after 3 days then infected with *T. congolense*
B	12	Injected with *T. congolense*
C	12	Injected with AFB-1 ip at rate of 0.05mg/kg every after 3 days then injected with *T. congolense* and treated with diminazene aceturate at rate of 20mg/kg upon detection of parasites
D	12	Infected with *T. congolense* then treated with diminazene aceturate at rate of 20mg/kg upon detection of parasites

2.7 Determination of body weight
Body weight of experimental mice was determined twice a week using an electronic analytical balance (Mettler PM34, DoltaRange®) (Kibugu, *et al*., 2009).

2.8 AFB-1 injection in mice
AFB-1 was dissolved in distilled de-ionized water at the rate of 0.05mg/ml (El-Arabi, *et al*., 2006) and ip injected in the mice at the rate of 0.05mg/kg body weight every third day upto 10 times.

2.9 Infecting mice with trypanosomes
Two donor mice were immunosuppressed for three consecutive days with cyclophosphamide at rate of 8.3mg/kg body weight (Mdachi personal communication) and then on the third day injected with *T. congolense* KETRI2409 stabilate.

2.10 Preparation and administration of trypanocidal drug
The trypanocidal drug, diminazene aceturate (Veriben®, Sanofi) was prepared by dissolving 44.5mg in 10ml of distilled de-ionized water to give a dosage of 20mg/kg body weight. This was then administered ip in group C and D mice (Table 1) at on-set of parasitaemia, as a single dose (Mdachi personal communication).

2.11 Determination of parasitaemia
Blood of all experimental mice was microscopically examined for parasites daily by a tail clip and a drop of blood was put on a clean slide then coverslip placed and observed at x400 magnification (Gichuki and Brun, 1999) starting second day post-infection for the first 10 days and twice weekly for subsequent days. Parasitemia levels were assessed by matching technique of Herbert and Lumsden (1976).

2.12 Pre-patent period and survival
The time between infection and appearance of trypanosomes in blood, i.e. pre-patent period for each mouse was determined and recorded. The time the mouse took to succumb to disease in absence of treatment i.e. survival time (Seed and Sechelski, 1988) was monitored and recorded.

2.13 Pathological changes
In the course of the experiment, clinical conditions of the mice were observed and recorded as described by Gichuki and Brun (1999) but mainly hair appearance, breathing rate and any other clinical signs.

2.14 Determination of packed cell volume
Taking of packed cell volume (PCV) was done for each experimental mouse twice weekly by a tail snip and about 75µl of blood collected in EDTA capillary tubes and centrifuged at 10,000 revolutions for 5 minutes. The PCV was then measured by haemacytometer (hawksley® micro-haematocrit reader, England) and expressed as a percentage of the total blood volume as described by Naessens, *et al*. (2005).

2.15 Detoxification of mycotoxic wastes
All safety precautions of handling and storage of mycotoxins and their wastes as described by Gathumbi, *et al*. (2001) were adhered to. The housing cages holding AFB-1-injected mice were well labeled. Glassware and equipment used were rinsed in methanol then immersed in 1% NaOCl solution for 2hours and then rinsed in acetone, (Scott, 1995). The beddings and droppings of AFB-1-injected mice were put in a well labeled eco-garb® heavy duty plastic bag and drenched in diesel and incinerated (Kibugu, *et al*., 2009).

2.16 Data analysis
Data was collected in data sheets and entered into spreadsheets (Microsoft excel) and analyzed using Graph pad Prism ver. 5.01 statistical software package (Graph pad Prism Inc. USA). All the tests were two-tailed with critical limit being equal to a p value of 0.05 for significance.

3.0 RESULTS
3.1 Pre-patent period
The pre-patent period expressed as mean days of AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected mice was 3.75 ± 0.7538 and 3.583 ± 0.2652 respectively. There was no significant difference ($p>0.05$) in the pre-patent period of the two groups of mice.

3.2 Parasitaemia patterns
The daily mean number of trypanosomes in the blood of AFB-1-injected-*T. congolense*-infected and *T. congolense* infected mice was as shown in figure 1 below. It was observed that AFB-1-injected-*T. congolense*-infected mice showed three parasitaemic waves (on days 12, 14 and between 18-19). While, *T. congolense*-infected mice showed two parasitaemic waves (on days 11 and 14). Although no significant ($p>0.05$) statistical difference in the levels of trypanosome estimates at the peaks, AFB-1-injected-*T. congolense*-infected mice showed a higher initial parasitaemic wave than *T. congolense*-infected mice. The second parasitaemic wave in both groups was almost similar.

3.3 Clinical changes associated with AFB-1
In *T. congolense*-infected mice clinical changes included raised hair coat, facial oedema, higher breathing rate and lethargy. However, these signs became highly visible starting day 12 post-infection. In AFB-1-injected-*T. congolense*-infected mice the changes were highly pronounced particularly facial oedema, emaciation, raised hair coat and hair fall.

3.4 Survival Time of mice
Survival period for AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected mice was 12-20 and 10-17 days respectively. Mean death time was determined on the basis of weight and parasitaemia as indicated in Figure 2 and Figure 3 below. On the basis of weight, mean death time for AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected mice was 13.08 ± 2.234 and 15.0 ± 1.477 days respectively. This showed a significant difference ($p<0.05$) in the mean death time of the two groups of mice. On the other hand on the basis

of parasitaemia the mean death time for AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected mice was 16.0 ± 2.55 and 15.25 ± 2.56 days respectively. This did not show a significant difference ($p>0.05$) in the mean death time of the two groups of mice. On the basis of weight on the mean death time of the two groups of mice the Log-Rank (Mantel-Cox) test p-value for both AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected mice was significant ($p<0.05$) and less than that of Gehan-Breslow-Wilcoxon test. On the basis of parasitaemia on the mean death time of the two groups of mice the Log-Rank test p-value was not significant and less than that of Gehan-Breslow-Wilcoxon test.

3.5 Effect of AFB-1 on weight of mice

The weekly mean weight of untreated and treated mice was as shown in Figure 4 and Figure 5 respectively. The mean weight for AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected mice that were not treated was 27.47 ± 2.076 and 30.31 ± 2.417 grams respectively. This was significantly different ($p<0.05$) for the two groups of mice. The mean weight for AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected mice that were treated was 28.49 ± 2.87 and 28.76 ± 2.53 grams respectively was not significantly different ($p<0.05$) for the two groups of mice.

3.6 Effect of AFB-1 on PCV of mice

The mean PCV of untreated and treated mice was as shown in Figure 6 and Figure 7 respectively. The mean PCV of AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected mice that did not receive chemotherapy was 49.95 ± 3.07 and 51.8 ± 3.6 percent respectively. This was not significantly different ($p>0.05$) for the two groups of mice. On the other hand PCV for AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected mice that received chemotherapy was 49.65 ± 6.223 and 50.6 ± 4.216 percent respectively. This was significantly different ($p<0.05$) for the two groups of mice.

3.7 Effect of AFB-1 on *T. congolense* response to chemotherapy

Upon treatment with diminazene aceturate both AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected mice had the parasites cleared by the second day after administration of the drug. The two groups of mice regained their weights that stabilized at means of 28.49 ± 2.87g for AFB-1-injected-*T. congolense*-infected mice and 28.76 ± 2.53g for *T. congolense*-infected mice as indicated in figure 5**.** However, the PCV of AFB-1-injected-*T. congolense*-infected mice remained lower than that of *T. congolense*-infected mice as shown in figure 7**.** Two (16.67%) of AFB-1-injected-*T. congolense*-infected mice that were treated relapsed on day 20 post-tratment and survived upto day 41 and 45 post-treatment. The relapsed mice had a depressed PCV of upto 24 percent and parasitaemia estimates of upto 1.0×10^9 trypanosomes/ml of blood.

4.0 DISCUSSION

4.1 Effect of AFB-1 on establishment of *T. congolense*, its virulence and progression in mice

As shown in this study AFB-1 has an effect on pathogenesis of *T. congolense* infection in mice. The pathogenesis included alteration of pre-patent period, aggravation of clinical signs and symptoms, lengthened host survival and change in drug efficacy of diminazene aceturate. Though not significant, the prolonged pre-patent period in AFB-1-injected-*T. congolense*-infected mice in this study is in agreement with findings obtained by other researchers working on effect of aflatoxin on protozoan parasites. AFB-1 extended the pre-patent period in *T. b. rhodesiense*-infected mice (Kibugu, *et al*., 2009). The lengthened pre-patent period in this study can be attested on inbred strains of mice which showed that two host responses interact to control parasitaemia; a non-immunological process which regulates the rate of parasite multiplication and an antibody response, specific for exposed epitopes on the VSG of the parasites which determine the rate of parasite destruction. These responses therefore appear to make the host animal have an inherent capacity to control parasite growth and therefore mount superior parasite-specific immune responses. Sacks *et al*. (1980) found a correlation between pre-patent period and virulence of trypanosomes and described further that the virulence correlates with parasite intrinsic immunosuppressive activities particularly depression of IgM responses.

The parasitaemic waves observed in this study though not significant show that AFB-1-injected-*T. congolense*-infected mice had a higher initial wave than *T. congolense*-infected mice. Subsequent wave was similar and hence also not significant. This result is in accord with those observed by Kibugu *et al*. (2009) in which he observed that mycotoxin fed *T. b. rhodesiense*-infected mice had higher parasitaemic waves and that the parasitaemia was characterized by prominent wave. The waves observed are indicative of the mice body's effort to put under control the parasitaemia. Mycotoxin are known to depress antibody response (Al-Anati and Petzinger, 2006) particularly IgM production is reduced by blocking protein synthesis through inhibition of phenylalanyl t-RNA synthetase (Pier and McLoughlin, 1985). AFB-1 inhibits *in vitro* phagocytosis, intracellular killing and the spontaneous production of oxygen radicals of rat, chicken and/or turkey macrophages and

decreases their functional properties. AFB-1 also modifies the synthesis of inflammatory cytokines (Jakab, *et al*., 1994). In piglets AFB-1 decreased pro-inflammatory (IL-1β and TNF-α) and increased anti-inflammatory (IL-10) cytokine m-RNA expression by stimulated blood cells (Oswald *et al*., 2006). This therefore gives a preview of the parasitaemic waves observed for the AFB-1-injected-*T. congolense*-infected mice in this study.

The aggravated clinical signs in AFB-1-injected-*T. congolense*-infected mice such as raised coat, facial oedema, emaciation and hair fall are in tandem with findings by other scientists (Kibugu, 2008). The extensive emaciation could probably be explained by inhibition of protein synthesis by AFB-1 as has been reported in studies by other mycotoxins in animals (Smith and Moss, 1985). The inhibition of protein synthesis gave way to muscle wasting in the AFB-1-injected-*T. congolense*-infected mice consequently enhanced host energy deficit (Pier and McLoughlin, 1985). The observation is in agreement with clinical signs in dogs infected with AAT which included depression, rough coat, corneal opacity, conjunctivitis with muccopurulent discharge, loss of vision and lethargy (Matete, 2003). This therefore explains the sharper fall in average body weight of untreated AFB-1-injected-*T. congolense*-infected mice than *T. congolense*-infected mice.

Pyrexia, a common sign in trypanosomiasis is attributed to antibody-antigen complexes and TNF-α. In these studies up-regulation of TNF-α during *T. b. rhodesiense* infections, aflatoxicosis and ochratoxicosis has been observed (Kagira, *et al*., 2007). The anaemia observed in AFB-1-injected-*T. congolense*-infected mice could be due to additive or synergistic action of the trypanosomiasis and aflatoxicosis leading to TNF-α up-regulation and massive haemolysis. Therefore hypothesizing aggravation of pyrexia in these mice (Thrasher, 2007). Kibugu (2008) also observed dyspnoea in *T. b. rhodesiense*-infected mycotoxin fed mice. He attributed this to mycotoxin induced decline in red blood cells and lung pathology that was characteristic of the mice. This gives an explanation to the depressed PCV in AFB-1-injected-*T. congolense*-infected mice and hence a good measure of anaemia which is characteristic of AAT and aflatoxicosis. Although the PCV difference for untreated mice was not significant in this study could be a contradiction to studies elsewhere that indicated aflatoxin mediated aggravation of anaemia in swine and salmonellosis (Miller *et al*., 1981). Studies done by Kibugu *et al*. (2009) attributed enhanced anaemia in *T. b. rhodesiense*-infected aflatoxin fed mice to be due to severe obstructive coagulopathy leading to heart coagulation. This reinforced findings by Stephen (1986) in which he related mycotoxin aggravated anaemia in trypanosome-infected mice to have been through trapping or emigration of erythrocytes into extra-vascular space due to coagulopathy and vasculopathy. This is best explained by the fact that erythrophagocytosis which is an important mechanism for development of anaemia in trypanosomiasis is blocked by immunodepressants like corticosteroids (Halliwell and Gorman, 1989) leading to attenuation of trypanosome-induced anaemia.

Survival duration of *T. congolense*-infected animal is dependent on a number of factors which are broadly classified as environmental and physiological involving the trypanosome and the hosts. In this study the strain of *T. congolense* used was of a moderate virulence that knocks off its definitive host between 10 and 30 days after infection. This was consistent with findings of other researchers (Masumu *et al*., 2006). From the results though not significant, AFB-1-injected-*T. congolense*-infected mice had a longer survival time than was observed in *T. congolense*-infected mice. The result is in accord with findings elsewhere. On similar studies in *Plasmodium berghei*-infected mice, aflatoxin was observed to increase host survival time (Young, 1988). However, other studies indicated that mycotoxins cause more acute parasitic infections. Kibugu (2008) demonstrated that AFB-1 and ochratoxin A caused a more acute trypanosome infection and shortened the lifespan of *T. b. rhodesiense*-infected mice. The immunosuppressive effect of mycotoxins makes animals more susceptible to infections by reducing host resistance to these infections. Aflatoxin increases severity of coccidiosis and salmonellosis in chicken and Japanese quail (Kubena *et al*., 2001) and ochratoxin A raised susceptibility and severity of salmonellosis in pigs. However, an ambiguous occasion was demonstrated in which on the one hand immunocompetent animal hosts fed on mycotoxins and infected with *Toxoplasma gondii*, the infection progressed to a chronic phase characterized by the presence of encysted parasites mainly within the central nervous system or skeletal muscle and remain latent and reactivation is prevented. On the other hand, in immunosuppressed animals and human subjects, such as HIV infected patients, rupture is associated with the formation of new cysts and disease. Therefore, the AFB-1 acts upon the cellular receptors increasing the ligation points between the parasite and the cell, facilitating the adhesion (Herzog-Soares and Freire, 2004). The differences in the survival periods in all these studies are attributable to species differences, route of entry and quantity of the mycotoxin, age, sex and nutritional status of the animal (Kibugu *et al*., 2009).

5.2 Effect of AFB-1 on *T. congolense* response to chemotherapy

In this study it was observed that at a standard dose of 20mg/kg body weight of diminazene aceturate led to a relapse in 2 of the 12 AFB-1-injected-*T. congolense*-infected mice. Though not significant this would worry

animal farmers on the effect of AFB-1 on management of AAT through chemotherapy. Sones *et al*., (1988) showed that there was relapse upon treatment of cattle by diminazene aceturate of less than 14mg/kg body weight. Immunosuppression has been observed to considerably reduce efficacy of trypanocides. For instance x-irradiation of mice induced rapid development of high levels of stable chemo-resistance in immunocompetent mice (Osman *et al*., 1992). Kibugu *et al*. (2009) demonstrated that 7-day aflatoxin exposure period reduced suramin efficacy and hence need for higher dosages. Research done using other infections has proven that mycotoxins interfere with chemotherapy. T-2 toxin has been shown to cause anticoccidial failure in chicken and AFB-1 has been demonstrated to cause breakdown in vaccine immunity leading to the occurrence of disease in properly vaccinated flocks (Oswald *et al*., 2006). The spleen happens to be the centre-point of AFB-1 infection in these studies and there is down-regulation of specific antibody response towards a specific antigen but serum concentration of IgA, IgG and IgM is not modified (Taranu *et al*., 2005). AFB-1 increases synthesis of IFN-γ a Th-1 cytokine involved in cell mediated immune response and decreases IL-4 synthesis, a Th-2 cytokine involved in humoral response. The alteration of both lymphocyte proliferation and cytokine production might explain failure of chemotherapy and vaccination (Oswald *et al*., 2006).

5.0 CONCLUSION

It can be argued that AFB-1 has an effect on the progression of *T. congolense* in mice. Firstly, the highly pronounced clinical changes which included oedema, emaciation, raised hair coat, and hair fall signified exacerbated pathological conditions of the mice. Secondly, the depressed mean weight of the AFB-1-injected mice was an indication of inhibition of protein synthesis leading to muscle wasting and energy deficit. Therefore, it can be argued that AFB-1 would aggravate weight loss observed in *T. congolense* infections. Additionally, the lengthened survival time of AFB-1-injected mice could be a contributory factor to increased susceptibility of animals to trypanosome infection by enhancing *T. congolense* transmissibility in the field.

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FIGURES

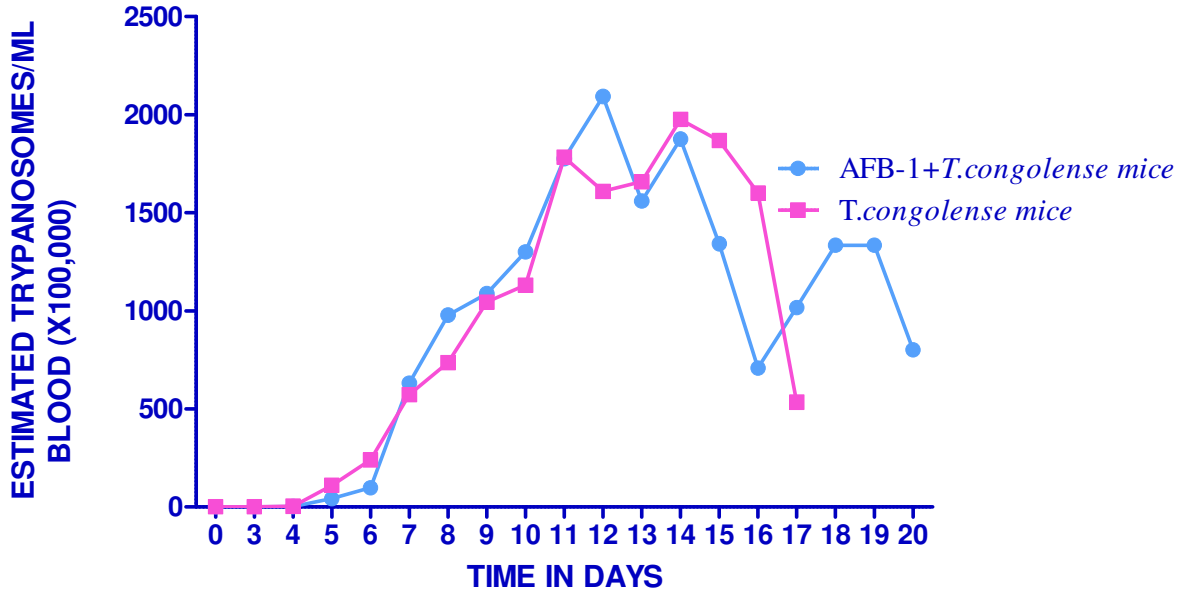

Figure 1: The parasitaemia patterns of AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected untreated mice

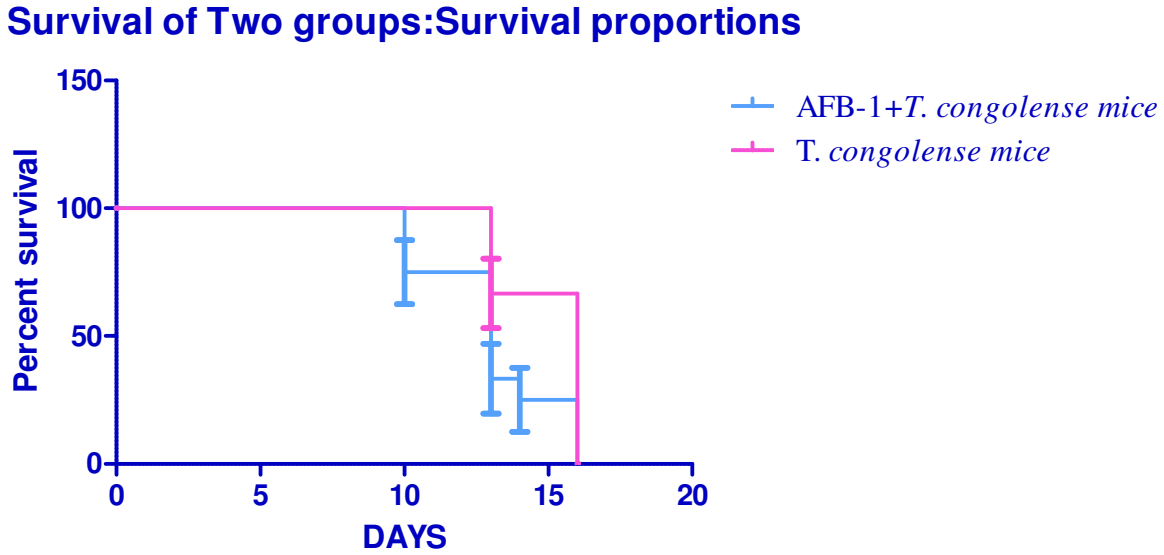

Figure 2: Survival proportions on basis of weight of AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected untreated mice.

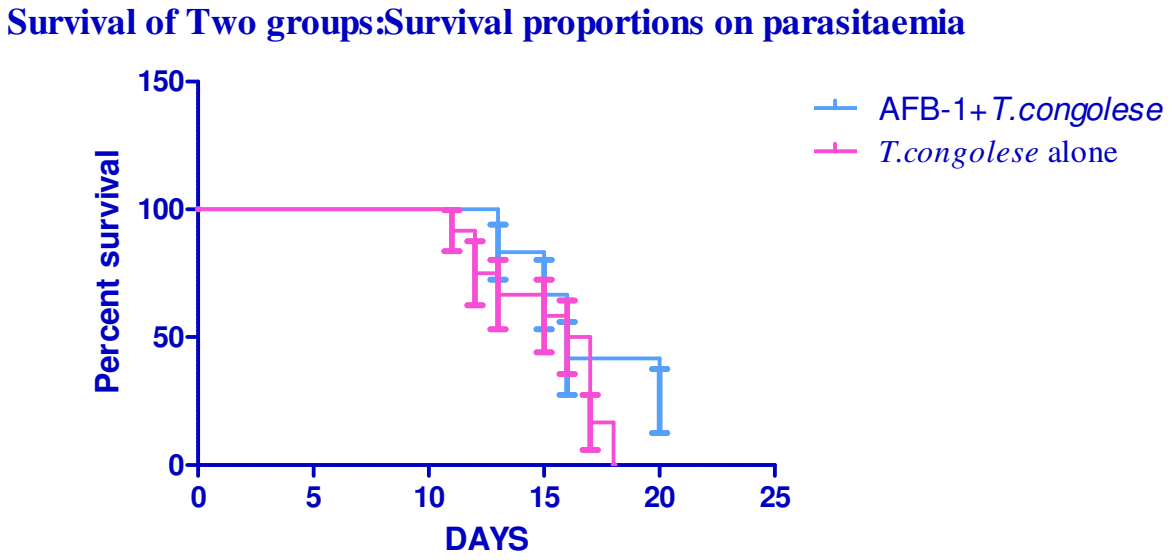

Figure 3: Survival proportions on basis of parasitaemia of AFB-1-injected- *T.congolense*-infected and *T. congolense*-infected untreated mice.

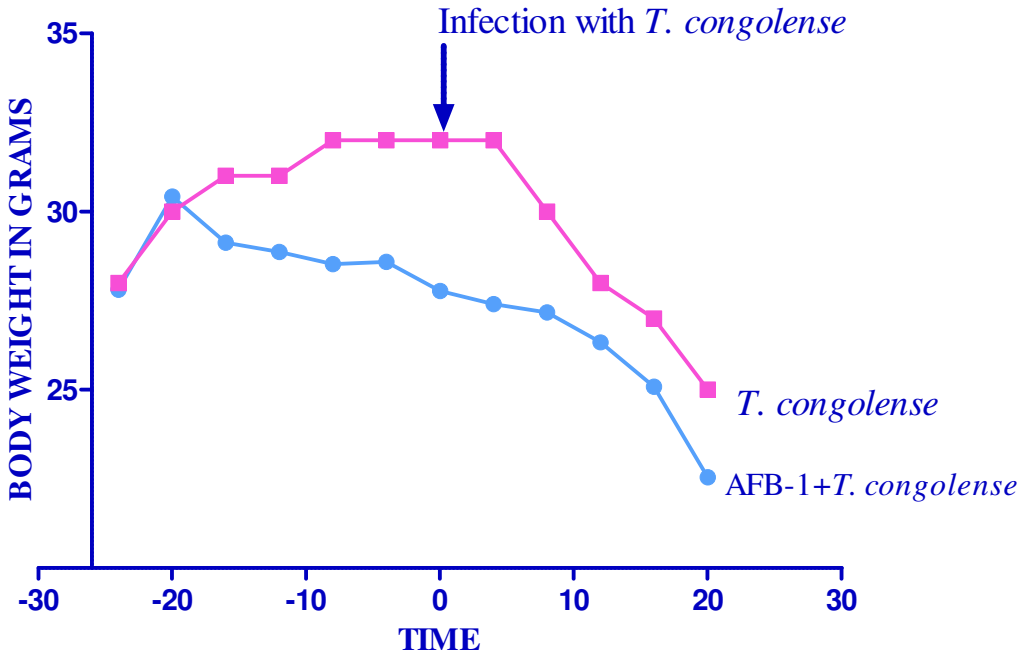

Figure 4: Changes in body weight over time in days of AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected untreated mice.

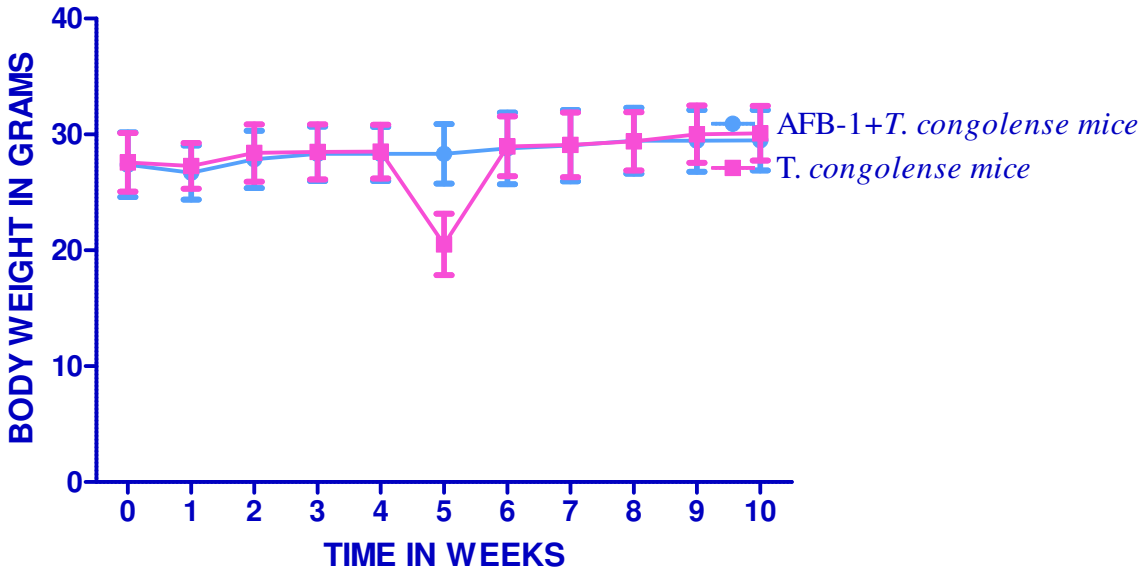

Figure 5: Changes in body weight over time in weeks of AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected and treated mice.

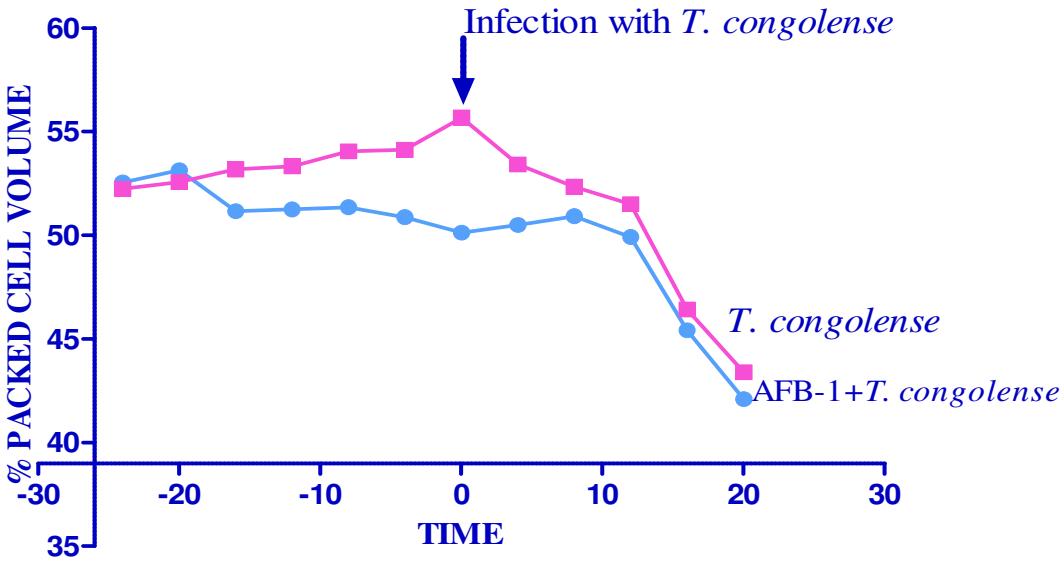

Figure 6: Variations in percentage packed cell volume against time in days of AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected untreated mice.

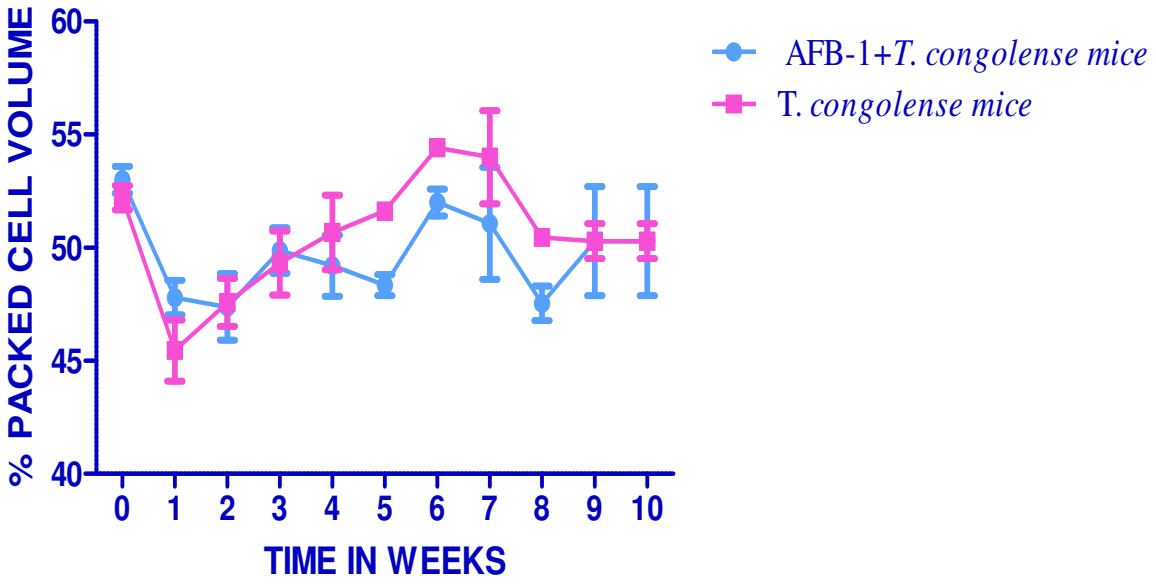

Figure 7: Variations in percentage packed cell volume plotted against time in weeks of AFB-1-injected-*T. congolense*-infected and *T. congolense*-infected treated mice.