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Aggravation of pathogenesis mediated by aflatoxin B_1 in mice infected with *Trypanosoma brucei rhodesiense*

Kibugu, J.K.[1, 2, *], Makumi, J.N.[2], Ngeranwa, J.J.N.[2], Kagira, J.M.[1] , Gathumbi, J.K.[3] and Mwangi, J.N.[4]
[1]Kenya Agricultural Research Institute, Trypanosomiasis Research Centre, P. O. Box 362, Kikuyu, Kenya, [2]Kenyatta University, Department of Biochemistry and Biotechnology, P. O. Box 43844, Nairobi, Kenya, [3]University of Nairobi, Department of Veterinary Pathology, Microbiology and Parasitology, P. O. Box 29053, Nairobi, Kenya., [4]Kenya Agricultural Research Institute, Social Economics and Biometrics Division, P. O. Box 00200-57811, Nairobi, Kenya.
* Corresponding author: James Karuku Kibugu, Kenya Agricultural Research Institute, Trypanosomiasis Research Centre, P. O. Box 362, Kikuyu, KENYA., *E-mail*: jkkibugu@yahoo.com

ABSTRACT
Aflatoxins are known to alter the pathogenesis of many infectious diseases, but such effects have not been evaluated in trypanosome infections. The aim of the present work was to assess the effects of aflatoxin B1 on the pathogenesis of *Trypanosoma brucei rhodesiense* infection using a murine model. Mice fed on 0.50 mg/kg aflatoxin b. wt. were infected with *T. b. rhodesiense* and compared to trypanosome infected and uninfected aflatoxin-fed controls. The clinical and pathological changes were determined and the quantitative data statistically analysed using standard methods. The results showed that infected aflatoxin-fed mice had pronounced dyspnoea, significantly ($P<0.05$) reduced survival, extreme emaciation, pronounced macrocytic normochromic anaemia characterized by significantly ($P<0.05$) reduced red cell count, packed cell volume, haemoglobin levels and significantly ($P<0.05$) increased mean corpuscular volume compared to controls. Grossly, there were pronounced hydrothorax and ascites while histologically, haemorrhages, thrombosis, embolism, massive peri-vascular inflammatory cell infiltration were observed in the infected aflatoxin-fed mice. Severe anaemia, liver damage, nephritis and pancarditis were the major complicating factors which could have caused reduced host survival. It was concluded that aflatoxicosis aggravated the pathogenesis of *T. b. rhodesiense* infection in mice, and should therefore be taken into consideration during trypanosomosis control programs.

Key words: Aflatoxin B_1, trypanosomiasis, pathogenesis, mice

INTRODUCTION
Sleeping sickness is of great medical concern in sub-Saharan Africa where more than 66 million people are at risk (WHO, 2004). The disease, which is caused by *T. b. gambiense* and *T. b. rhodesiense*, and transmitted by tsetse flies, is endemic in areas where other diseases such as HIV-AIDS, parasitic diseases, food-borne diseases (FBD) occur, and whose interaction could lead to serious implications on animal and human health. Mycotoxicosis is an important FBD caused by ingestion of mycotoxins produced by a variety of toxigenic fungi that contaminate food and animal feeds (Azziz-Baumgartner *et al*., 2005; FAO/UNEP, 1977). The syndromes caused by ingestion of moderate to high levels of mycotoxins range from acute mortality (Azziz-Baumgartner *et al*., 2005), slow growth and reduced reproductive efficiency (Oswald *et al*., 2005) while lesser amounts may result in impaired immunity and decreased resistance to infections (Pier and McLoughlin, 1985). The most common mycotoxins are aflatoxins (difuranocoumarins), ochratoxins, trichothecenes and zearalenone (Smith

and Moss, 1985) and are stable to normal cooking and food processing procedures (Al-Anati and Petzinger, 2006). Through depression of the immune and haemopoeitic systems (Williams *et al*., 2004; Duguala *et al*., 1994; Cukrova *et al*., 1991), interference with functions of essential nutrients like vitamins (Anyanwu *et al.,* 2004) and toxicity effects on vital organs (Pier and McLoughlin, 1985), mycotoxins influence the course of many diseases.

The most toxic aflatoxin is aflatoxin B1 (AFB_1) produced as a secondary metabolite by the fungal species *Aspergillus flavus*, *A. parasiticus* and *A. nomius*. Chronic aflatoxicosis is immunomodulatory and causes pathology on many vital organs (Pier and McLoughlin, 1985; Bondy and Pestka, 2000; JECFA 47, 2001; Williams *et al*., 2004). The condition also affects outcome of experimental and natural infections (Peers and Linsell, 1977; Joens *et al*., 1981; Smith and Moss, 1985; Hendrickse *et al*., 1986; Young *et al*., 1988; Bondy and Pestka, 2000). Also, through its potent nephrotoxicity, this mycotoxin could affect the production of renal-produced erythropoietin leading to aggravation of trypanosome-induced anaemia. Despite the endemicity of both trypanosomosis and aflatoxicosis in the sub-Saharan Africa, the interaction of the two diseases has not been investigated. The purpose of the present study was to assess the effects of AFB_1 on the pathogenesis of human infective *T. b. rhodesiense* in the murine model.

MATERIALS AND METHODS

Ethics

All protocols and procedures used in this study were reviewed and approved by the KARI-TRC Institutional Animal Care and Use Committee.

Materials

Male inbred adult Swiss White mice from KARI-TRC colony were maintained on mice pellets (Unga Feeds Ltd, Kenya) and water *ad libitum* at a temperature of 21-25°C. Wood-chippings were provided as bedding material. A cryo-preserved *T. b. rhod*esiense clone, KETRI 3741, prepared from KETRI 2537 previously isolated from a human host in Uganda in 1972 (Fink and Schmint, 1980) was used to infect the mice. Extracts of AFB_1, (62.5 µg/ml) and a placebo which were separately constituted in vegetable oil as described by El-Arab *et al*. (2006) were obtained from Bora Biotech Ltd, Cooper Centre, Nairobi, Kenya.

Experimental design

Five groups of mice were used in this study as shown in Table 1. Group A mice were fed on AFB_1 extract for 7 days and then infected with *T. b. rhodesiense*. After infection the feeding on AFB_1 was continued up to the 30th day. Group B was fed on placebo extract and then infected as in Group A. Group C had the same aflatoxin treatment as in Group A but was not infected with trypanosomes. Groups D and E did not receive the AFB_1 or placebo treatment but the former was infected with *T. b. rhodesiense* while the latter was not. In groups A and C mice, the toxin was administered orally through a gavage needle for 30 days at a daily dosage of 0.50 mg/kg AFB_1 body weight. Group B mice received the equivalent volume of the placebo. To infect mice, cryo-preserved trypanosome stabilates were first expanded in donor mice which were euthanized at peak parasitaemia and blood harvested as described earlier (Kagira *et al.,* 2007). Groups A, B and D mice were injected intraperitoneally (i.p.) with 10^4 trypanosomes per mouse (Kagira *et al.,* 2007; Gichuki and Brun, 1999) on day 7 post-commencement of aflatoxin administration.

Table 1. Groups of mice used in the study

Mice group	Number of mice	Treatment
Group A	12	*T. b. rhodesiense*–infected and fed on AFB_1
Group B	12	*T. b. rhodesiense*–infected and fed on placebo
Group C	12	Uninfected and fed on AFB_1
Group D	6	*T. b. rhodesiense*-infected
Group E	6	Uninfected and not fed on AFB_1 (clean)

Monitoring of AFB_1 levels, parasitaemia and clinical changes

Body weight of mice was determined weekly as described by El-Arab *et al*. (2006) using an electronic balance (Mettler PM34, DoltaRange®). At 31 day post AFB_1 exposure, 80 µl of blood was collected by tail-snip method from groups A and C mice, transferred to a clean capillary tube, serum prepared as described by Boyt (1986) and stored at -20°C until required. To determine the aflatoxin serum levels in the mice, a competitive AFB_1-ELISA described by Gathumbi *et al*. (2001) was used. Blood (20µl) from mouse tail snip method was examined daily for parasites from the 2nd day post-infection for the first 2 weeks, every second day for the next 4 weeks and twice weekly for further 7 weeks. The matching technique of Herbert and Lumsden (1976) was used to assess parasitaemia levels. Fifty (50) microlitre of tail blood was collected once a week, analysed by automated Coulter Counter (Beckman Coulter® A^C-T diff™) and a full haemogram of each of the experimental mouse obtained. Thin blood smears were prepared, fixed and stained with Giemsa stain for differential cell counts (DCC) (Bain and Bates; Baker *et al*., 2001). The survival times for each animal i.e. time the animal took to succumb to disease in the absence of chemotherapy, were monitored for 67 days post-trypanosome infection. For animals surviving beyond this period, the survival time was recorded as 67 days and categorized as censored data. The clinical picture, gross pathology and histopathology of mice were determined. The clinical status of the mice was determined as described by Gichuki and Brun (1999). From each mice group, 4 animals were randomly selected and sacrificed on the last day of mycotoxin exposure (30 day post-commencement of aflatoxin administration). At autopsy and euthanasia, the major organs were harvested, stored in formalin, and histologically processed and slides stained with Haematoxylin and Eosin. The prepared slides were then examined under the microscope.

Statistical analysis

The means of peripheral AFB_1 levels in mice were determined using the SAS (SAS Institute Inc., Cary NC, USA, 1999-2001) and StatView (SAS Institute, Version 5.0.1) statistical packages and the individual variation determined from the coefficient of variation (CV). Parasitaemia data were subjected to analysis of variance and mean separation using SAS. Skewed haematological data were first subjected to the square root transformation, $\sqrt{(x+1)}$, and then subjected to repeated measures analysis. Due to the unbalanced nature of the data, least square means were calculated. Survival data analysis was carried out using the Kaplan-Meier method for determination of survival distribution function. Rank tests of homogeneity were used to determine the effect of treatment on early (during early phase of infection) and larger (during late phase of infection) survival times respectively (Everitt and Der, 1998).

RESULTS

Levels of AFB_1 in mice

The range of aflatoxin serum levels in mice was 21.36-521.3 pg/ml with coefficient of variation (CV) of 61.2%. Further, 6.8% of the aflatoxin-fed mice had levels below the detection limit (15.06 pg/ml), and all the pre-treatment and negative control samples were detected as negative.

Parasitaemia development

The pattern of parasitemia is shown in Fig.1 and was characterized by two prominent peaks; the first occurring in the first week of infection followed by a smaller wave that persisted until death. Although there was no significant difference (P>0.05) in the level of parasitaemia in the second peak between the groups, the aflatoxin-fed mice showed a higher second peak than the controls.

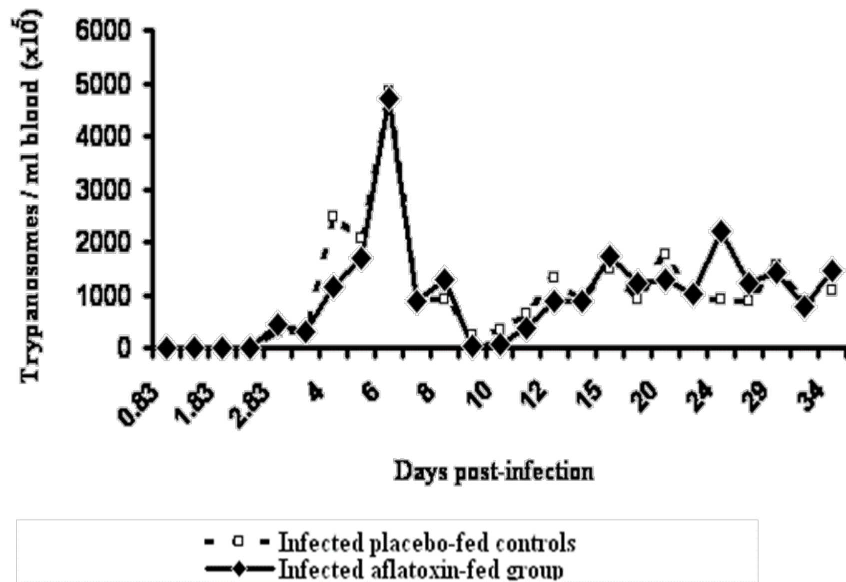

Fig. 1. Mean parasitaemia of control and *T. b. rhodesiense*-infected aflatoxin-fed mice

Clinical changes and survival

The clinical signs of the *T. b. rhodesiense*-infected mice included lethargy, dyspnoea, raised hair coat, facial and scrotal oedema. The signs were more pronounced in the aflatoxin-fed mice than in the infected controls, especially emaciation, raised hair coat and eyelid oedema. The clinical signs became more severe starting 24 days post-infection. The survival for the *T. b. rhodesiense*-infected mice ranged from 30-48 days for aflatoxin-fed group compared to 33-65 days for the infected placebo-fed controls. Figure 2 shows the survival functions for the trypanosome-infected aflatoxin-fed mice and the controls. The death rate was higher in the aflatoxin-fed mice compared to the controls from 45 day post-infection. The rank tests of homogeneity (Log-Rank {p= 0.037}; Wilcoxon {p=0.095}) indicated that the two groups differed primarily at larger survival times with significantly (p<0.05) shorter larger survival time in the aflatoxin-fed group compared to the controls.

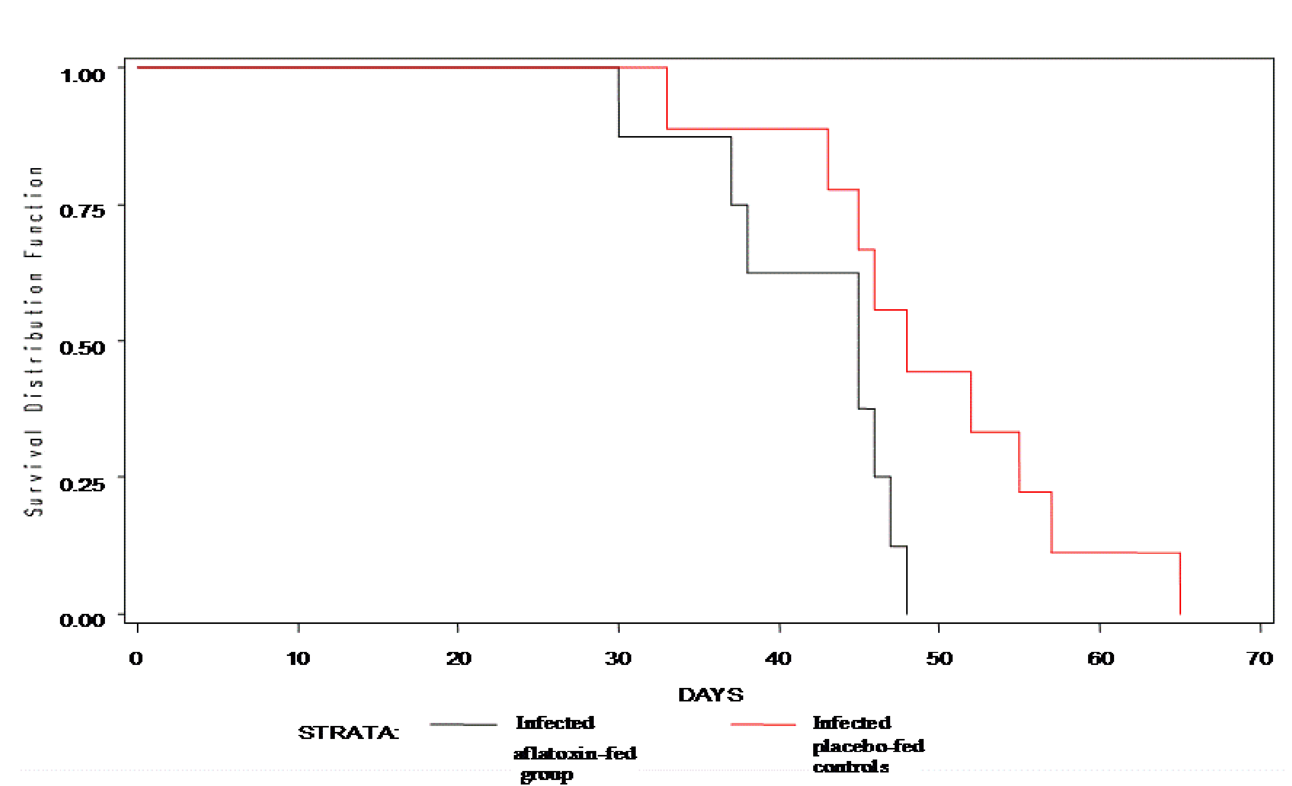

Fig. 2. Survival distribution function for infected aflatoxin-fed and control mice

Clinical pathological changes

Figures 3–6 show the mean haeamatological values of trypanosome-infected aflatoxin-fed and control mice. Red cell counts (RBC) (Fig. 3), packed cell volume (PCV)(Fig. 4) and haemoglobin levels (Hb) (Fig. 5) dropped while mean corpuscular volume (MCV) increased (Fig. 6) in all *T. b. rhodesiense*-infected mice from second week post-infection. These changes were more pronounced in the aflatoxin-fed group. The RBC, PCV and Hb were significantly lower ($P<0.05$) while the mean corpuscular volume (MCV) was significantly ($P<0.05$) higher in the aflatoxin-fed mice than the controls. Grossly, the infected control mice showed emaciation, hepatosplenomegaly, ascites, liver and kidney capsular haemorrhages, heart congestion, cerebral oedema and hydrothorax. Histologically, there was perivascular inflammatory cell infiltration in the liver (Fig. 7), heart and kidneys. The kidneys also showed fatty degeneration and necrosis of tubular cells. The uninfected aflatoxin-fed controls had less severe pathological lesions than trypanosome-infected aflatoxin-fed group (Fig.9). Similar but more severe lesions were observed in the infected aflatoxin-fed group. These were mainly hepatosplenomegaly and hydrothorax. The liver had mononuclear cell infiltration, emboli, fatty degeneration (Fig. 8), necrosis, fibrosis and bile duct hyperplasia. Haemorrhages and thrombosis were evident in mice dying before 21 day post-infection (dpi). The kidneys had focal perivascular infiltration, pinkish exudate within tubules (Fig. 10), dilatation of Bowman's space and degeneration and necrosis of renal tubular epithelium. There was pancarditis characterized by infiltration by lymphocytes, fatty degeneration, embolism and necrosis, and fibrosis of the myocardium. The spleen had macrophage and giant cell hyperplasia, and expanded red pulp.

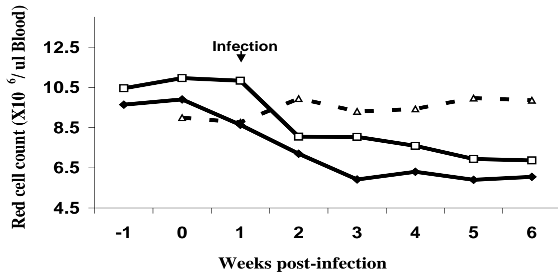

Fig. 3. RCC

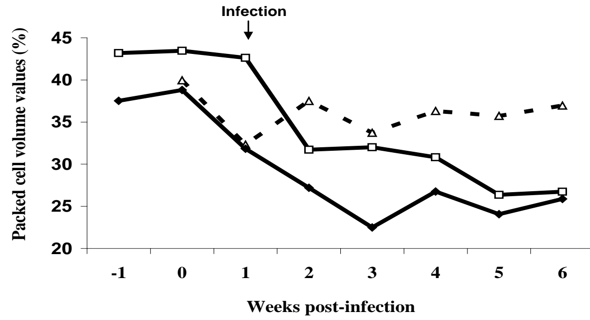

Fig.4. PCV

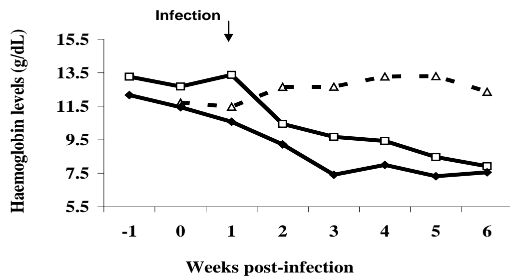

Fig. 5. Hb

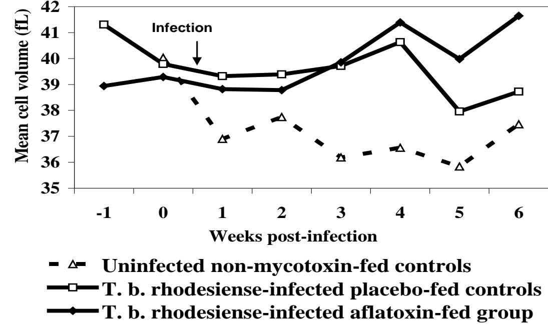

Fig. 6. MCV

Figures 3-6. Haematological values of *T. b. rhodesiense*-infected mice at different time intervals

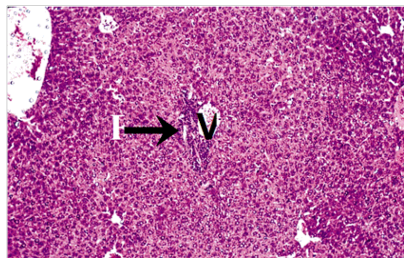

Fig.7

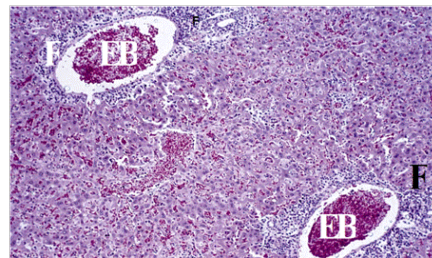

Fig.8

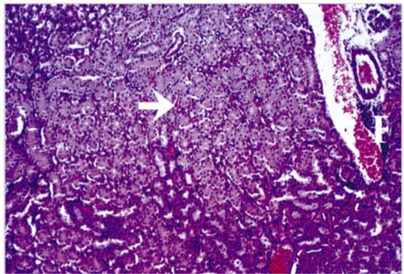

Fig.9

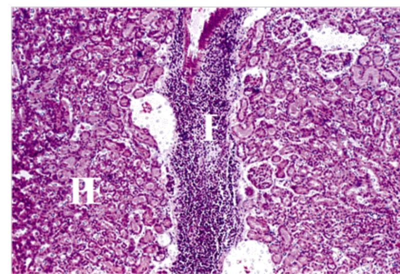

Fig.10

Figures 7-10. The liver of *T. b. rhodesiense*-infected non-mycotoxin-fed control mice at 23 dpi (Fig. 7) showing fatty degeneration of hepatocytes, central vein (V) and perivascular infiltration by inflammatory cells (F, arrow). Fig. 8 shows a more severely affected liver of trypanosome-infected aflatoxin-fed mice at 35 dpi with perivascular infiltration by inflammatory cells (F), embolism (EB) and depopulation of hepatocytes. Fig. 9 shows the kidney of uninfected aflatoxin-fed control mice at day 30 post-AFB_1 exposure showing congestion (arrow) and diffuse infiltration by inflammatory cells (F). The kidney of infected aflatoxin-fed mice at 48 dpi (Fig. 10) showed severe pinkish exudate (PE) in a renal tubules, focal massive perivascular cellular infiltration (F), degeneration and necrosis of tubular epithelium (Haematoxylin and Eosin x131).

DISCUSSION

The results in this study showed an aggravation of clinical and pathological lesions in *T. b. rhodesiense*-infected aflatotoxin fed mice indicating that aflatoxicosis influences the pathogenesis of the murine trypanosomosis. Aggravated anaemia observed in the trypanosome-infected aflatoxin-fed animals was expected since anaemia in trypanosomosis and aflatoxicosis is well documented in various animal species. However, anemia is attenuated by immunosuppressive corticosteroids in *T. brucei*-infected mice (Balber, 1974; Halliwell and Gorman, 1989). This shows that while corticosteroids influence anaemia through immunosuppression (Balber, 1974), a different pathway could be at play for the aflatoxin-mediated exacerbation of anaemia in the present study. The mechanism(s) by which AFB_1 aggravated pathogenesis of anaemia in the present study could involve down-regulation of erythropoietin activity (Naessens *et al.*, 2005) by aflatoxin B1. Indeed severe nephritis was observed in the infected aflatoxin-fed mice.

Histopathological evidence suggested exacerbation of inflammation in the trypanosome-infected AFB_1-fed mice in this study. Aflatoxicosis aggravated the inflammation observed in the kidney, liver and heart. The reported lesions have been reported before in trypanosomosis (Maina *et al*., 2003; Stephen, 1986) and aflatoxicosis (El-Arab *et al*., 2006; Azziz-Baumgartner *et al*., 2005). AFB_1 is potently hepatotoxic and nephrotoxic (Smith and Moss, 1985) and could have promoted the degeneration of parenchymal cells in this study as has been observed by other workers (El-Arab *et al*., 2006). Aggravation of facial, eyelid and generalized oedemas was probably secondary to the observed acute renal tubular cell injury.

The severe pathological lesions could have reduced the host survival. The survival time of *T. b. rhodesiense*-infected mice has been estimated between 6-9 weeks (Fink and Schmidt, 1979) and 3-12 weeks for KETRI 3741 (Kagira *et al*., 2007) which was within the range of 3-7 weeks in the control mice in the present study. However, aflatoxicosis significantly reduced the host survival with higher death rates in the late phase of the trypanosome infection therefore causing a more acute disease. This differed with results of similar studies on malarial parasites where aflatoxicosis was shown to increase the survival time of *Plasmodium berghei*-infected mice, an effect that was attributed to direct toxicity of AFB_1 on the parasite (Young *et al.,* 1988; Hendrickse *et al.,* 1986). The cause of this difference is not clear but may be due to difference in species of the parasites. Budovsky *et al.* (2006) reported similar cyclophosphamide-mediated aggravation of pathological lesions in rats infected with *T. lewesi*.

In conclusion, the study has shown that continuous dosing of mice with aggravated the clinical and pathological aspects of *T. b. rhodesiense* (KETRI 3741) infection in mice. This aggravation of pathogenesis observed in the present study may have some implications on the clinical progression and outcome of sleeping sickness and animal trypanosomosis cases in Africa where the two conditions occur concurrently. Indeed, global climatic changes which could result in favourable conditions for mycotoxin production, and the significance of interaction of mycotoxicoses with various infectious agents that may lead to emergence of new disease patterns has already been recognized (Minakshi, 2005). It is therefore important to consider the effects of aflatoxicosis during control programs of trypanosomosis and other tropical parasitic diseases in the field. Further, future studies should evaluate the biological mechanisms involved in the exacerbation of pathogenesis of trypanosomosis by aflatoxins.

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