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Effect of aflatoxin B1 on the therapeutic efficacy of suramin in *Trypanosoma brucei rhodesiense*-infected mice

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ABSTRACT

Through immuno-suppression, aflatoxins could affect drug and vaccine efficacy. Such effects have not been evaluated in treatment of many diseases including trypanosomiasis. We assessed the effect of aflatoxin B1 on the efficacy of suramin, the drug used for treatment of early stage sleeping sickness, in a murine model. Mice were fed daily on a diet containing 0.50 mg aflatoxin/kg body weight or a placebo. They were infected with *Trypanosoma brucei rhodesiense* on day 7 post-aflatoxin exposure and then treated with one of 6 different doses of suramin (4.0, 4.5, 5.0, 5.5, 6.0 and 6.5 mg/kg body weight) at the onset of parasitemia. The mice were fed on aflatoxin diet for 30 days and the curative dose values ($CD_{50,\ 75,\ and\ 90}$) computed and compared using a logistic linear regression model. Aflatoxin B1 induced transient protection of the host against *T. b. rhodesiense* infection and a consistent increase in suramin CD values in the mice suggesting reduced drug efficacy. Aflatoxicosis hindered curative treatment of *T. b. rhodesiense* infection in mice, and may contribute to reduced efficacy of suramin during treatment of sleeping sickness in man.

Keywords: Aflatoxin B1; trypanosomiasis; suramin efficacy; mice.

INTRODUCTION

Human African Trypanosomosis (HAT) or sleeping sickness, a zoonotic disease caused by 2 *Trypanosoma* species, is an obstacle to rural development and a threat to human health in sub-Saharan Africa (WHO, 2004; Brun *et al.*, 2010). HAT caused by *Trypanosoma brucei rhodesiense* is restricted to eastern and southern Africa while that caused by *Trypanosoma brucei gambiense* is found in central and western Africa, and more than 66 million people in these regions are currently at risk (WHO, 2004; Brun *et al.*, 2010). The drugs used for curative treatment of *T. b. rhodesiense* infections are suramin and melarsoprol for the early and late stages of the disease respectively (Nok, 2003; Brun *et al.*, 2010).

Reports of trypanocidal drug failure, lack of incentive for development of new trypanocides and unavailability of anti-trypanosomal vaccines (Gutterridge and Coombs, 1977; Geerts and Holmes, 1998) emphasize the need to reduce all possible impediments to effective treatment of Rhodesian sleeping sickness using suramin. The emergence of drug resistance in sleeping sickness has been observed in several countries (Nok, 2003; Kagira and Maina, 2007). Factors which are known to affect the efficacy of trypanocidal drugs include drug concentration in the body (Mdachi *et al*., 2009), host nutritional status (Murilla, 1996), state of host immune system (Berger and Fairlamb, 1992; Osman *et al*., 1992) and drug resistance (Geerts and Holmes, 1998). Further, conditions such as mycotoxicosis which are common in trypanosomosis-endemic areas are also likely to influence its chemotherapy. Aflatoxin B1 which is stable during normal cooking and food processing procedures (Smith and Moss, 1985; Bondy and Pestka, 2000) is a common violative mycotoxin in developing countries including Kenya (Peers and Linsell, 1977; Ngindu *et al.*, 1982; Williams *et al.*, 2004; Azziz-Baumgartner *et al.*, 2005). The high human consumption of legumes, maize and other

cereals in the Eastern and Southern African countries could lead to acute and chronic dietary exposure to aflatoxins. Through its immunosuppressive (Bondy and Pestka, 2000) and potent hepatotoxic effects (Smith and Moss, 1985; Williams *et al.*, 2004), aflatoxin B1 is likely to alter the efficacy of trypanocidal drugs in the field. The purpose of the present study was to establish the influence of aflatoxicosis on the efficacy of suramin therapy against *T. b. rhodesiense* infection.

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

Forty-two days old 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^{o}C. These mice were acclimatized for 7 days before the experiment commenced. Wood-chippings were provided as bedding material. A cryo-preserved *T. b. rhod*esiense clone, KETRI 3741, a derivative of KETRI 2537 which was previously isolated from a human host in Uganda in 1972 (Fink and Schmidt, 1980) was used to infect the mice in this study. Purified extracts of AFB_1, (62.5 µg/ml) and a placebo (obtained from Bora Biotech Ltd, Cooper Centre, Nairobi, Kenya) were separately constituted in vegetable oil as described by Ezz El-Arab *et al*. (2006). Suramin (Germanin®, Bayer 205) was used to treat the *T. b. rhodesiense*-infected mice. Pure aflatoxin B1 standard for *in vitro* anti-trypanosomal activity studies was received from Bora Biotech Ltd.

Experimental design

Twelve completely randomized groups of 6 mice each were used in this study. Six groups (Groups 1-6) were fed on aflatoxin B1, infected with *T. b. rhodesiense* on day 7 post-exposure and treated with either one of 6 daily doses of suramin (i.e. 6.5, 6.0, 5.5, 5.0, 4.5 and 4.0 mg suramin/kg body weight respectively) for 4 days at the onset of parasitemia. Six control groups (Groups 7-12) fed on the placebo, received the same infection and each treated with either one of the above 6 suramin doses. Parasitemia was monitored in all the mice for 90 days post-treatment period. For each group of animals, blood was examined daily for trypanosomes from the 2nd day post-infection for the first 2 weeks, every other day for the next 4 weeks and twice a week during the next 7 weeks. From a drop of blood (20 µl) obtained by tail snip, wet blood films were prepared as described by Boyt (1986) and microscopically examined at x400 magnification (Gichuki and Brun, 1999). Parasitemia was monitored using the matching technique described by Herbert and Lumsden (1976). Buffy coat examination (Boyt, 1986) was used to confirm cure or non-cure at 90 days post-suramin treatment. The pre-patent periods (the time between parasite inoculation in mice and appearance of trypanosomes in peripheral blood) for each mouse were also determined. To collect baseline immunotoxicological data, 3 further groups (Groups 13-15) of non-infected mice were incorporated for determination of total white cell counts. One group of 12 mice was given the above aflatoxin treatment while another, with the same number of individuals, received the placebo as described above. The third group of 6 mice did not receive the AFB_1 or placebo treatment. The safety precaution and detoxification of mycotoxic waste procedures were carried out as described by Scott (1995). The droppings and waste bedding material of the mycotoxin-exposed animals were put in a labeled heavy-duty plastic container, drenched in diesel and incinerated.

Mycotoxin administration and trypanosome inoculation to mice

The appropriate daily aflatoxin dose, based on body weight was determined on a weekly basis

(Ezz El-Arab *et al.*, 2006) on an electronic balance (Mettler PM34, DoltaRange®), and a daily dose of 0.50 mg aflatoxin/kg body weight was administered to a mouse through the oral route using a gavage needle for 30 days while the controls received an equivalent amount of the placebo. Cryo-preserved trypanosome stabilates were first expanded in donor mice (irradiated at 600 rads for 6 minutes). The donor mice were euthanized at peak parasitemia, blood harvested from the heart and diluted in phosphate saline glucose (PSG) buffer before intra-peritoneal inoculation into the experimental mice at 10^4 trypanosomes per mouse (Kibugu *et al.,* 2009a, b; Kagira *et al.,* 2007; Gichuki and Brun, 1999) on day 7 post-commencement of aflatoxin administration.

Administration of Suramin in mice and determination of cure rates and curative doses (CD values)

A stock suramin solution (1 mg/ml) was prepared by dissolving 20 mg of suramin sodium powder (Germanin®, Bayer 205) in 20 ml of injectable water. The suramin treatment solutions of 0.65, 0.60, 0.55, 0.50, 0.45 and 0.40 mg/ml were prepared by appropriately diluting with injectable water mixture. These solutions were administered intra-peritoneally in the control and aflatoxin-fed mice for 4 days, using a daily dose of either, 6.5, 6.0, 5.5, 5.0, 4.5 or 4.0 mg suramin/kg body weight as described above. These doses were based on weekly mice body weights. The response variable was the number of mice cured out of a total of 6. Cure was considered to be achieved if mice remained aparasitemic throughout the observation period of 90 days post-treatment. For each dose of suramin, the proportion of mice cured was recorded as a percentage. Suramin curative dose values ($CD_{50,\ 75,\ and\ 90}$) were computed by substituting the estimates of regression coefficients in a logistic linear regression model.

Hematological analysis

From each mouse in Groups 13-15, 50 µl of tail blood was collected once a week, analyzed by automated Coulter Counter (Beckman Coulter® A^C-T diff™) and total white cell counts obtained.

Evaluation of *in vitro* anti-trypanosomal activity of aflatoxin B1 (AFB_1)

Trypanosomes: Bloodstream trypomastigotes of *T. b. rhodesiense* (KETRI 3741) were used. Cryo-preserved trypanosome stabilates were injected by intra-peritoneal inoculation into donor mice at 10^4 trypanosomes per ml (Kibugu *et al.,* 2009a, b; Kagira *et al.,* 2007). Blood was collected from the trypanosome-infected mice at moderate parasitemia (antilog 8.1) via cardiac puncture using 10% EDTA-rinsed 10 ml syringe and 23-G needle. The parasites were separated from whole mouse blood, counted and diluted employing methods described by Casero *et al.* (1980) with modification. The blood was centrifuged at 180 xg for 10 minutes, with further centrifugation of the supernatant at 1,600 xg for 10 minutes and the pellet re-suspended in 1 ml medium. The trypanosomes were counted using modified Neubauer counting chamber, quantified at 2.4×10^6 and diluted 24-fold to give a seeding concentration of 1×10^5 parasites per ml of culture medium.

Culture conditions: The culture medium consisted of Minimum Essential Medium (MEM) with Earle's salts (Sigma), supplemented with 25mM HEPES, 1 g/L glucose, 2.2 g/L $NaHCO_3$ and 10 ml/L MEM non-essential amino acids and 20 µg/ml gentamicin. The medium was further enriched according to Baltz *et al.* (1985), with 0.2 mM 2-mercaptoethanol, 2 mM pyruvate, 0.1 mM hypoxanthine and 10% (v/v) heat-inactivated fetal bovine serum. The trypanosomes were cultivated axenically at 37°C in a 5% CO_2 atmosphere.

Trypanocidal drug/toxin materials: Pure AFB_1 was quantified employing UV spectroscopy (Shimadzu®, Model 1800, Japan). The toxin was dissolved in 1 ml of methanol (purity 99.9%), its concentration determined by measuring the absorbance at 360 nm and using molar extinction coefficient ε of 21,800 mol^{-1} cm^{-1} (Nguyen *et al.*, 2007) and established to be 120.55 µg AFB_1/ml. The methanol was evaporated. Aflatoxin stock solution of 1,205.5 µg AFB_1/ml in 10% DMSO was prepared and sterilized by

passage through a 0.20 μm Sartorius filter (Minisart®). For use in the assay, the solutions was diluted in culture medium to working solutions of 51.25, 5.125 and 2.5625 ng AFB_1/ml for aflatoxin and 20, 10 and 5 ng Mel B/ml for Mel B (Melasoprol, Arsobal®).

Assay procedure: The assays were carried out as described by Burri and Brun (1992). This was done in a flat-bottom 96-well microtiter plate (Cellstar®). Every test was performed in duplicate in 2 adjacent rows, toxin exposure levels ranging from 0.256-5,125 pg AFB_1 /ml. Three tests were done on one microtiter plate. Border wells were not used since the liquid in them tends to evaporate quickly. One hundred μl of culture medium was placed in wells of rows B-G and columns 2-10. Two hundred μl of toxin stock solution was placed in wells of column 11. Serial dilution was carried out by transferring 100 μl from wells of column 11 to wells of column 10 by a multichannel pipette, then 100 μl from wells of column 10 to 9 and so on. One hundred μl from wells of column 4 was discarded. Columns 2 and 3 wells served as control wells, contained DMSO and complete medium controls respectively. Trypanosomes at density of 1×10^5/ml (Sutherland *et al.,* 1995) in axenic culture medium were seeded into all used wells of 96-well flat bottomed tissue culture plates. The plates incubated at 37°C in a 5% CO_2 atmosphere for 24, 48 and 72 hours.

Pyruvate assay: Pyruvate concentrations in the trypanosome suspensions were measured using a commercial assay system (Sigma Chemical Company) as described by Sutherland *et al.* (1993; 1995). Briefly, proteins were precipitated out of the culture supernatants by incubation with cold perchloric acid (8% v/v). The resulting clear supernatants were diluted in de-ionized and distilled water in spectrophotometer cuvettes, neutralized by addition of 0.5M TRIZMA base and 100 μm NADH added. An initial absorbance reading at 340 nm was taken and 50 units of L-lactic dehydrogenase (Type XV from bovine heart, Sigma) added. A final absorbance at 340 nm was determined after 5-10 minutes incubation at room temperature. The amount of pyruvate in the culture supernatant was calculated from decrease in absorbance using conversion factors provided by Sigma and correcting for the initial dilution factor as described by Burri and Brun (1992).

Statistical analysis

The response variable was the number of mice cured (cure rates). Since these had a binomial distribution, B (n,p), where, n=number of mice and p=probability of cure, a logistic linear regression model, $\log \{p/(1-p\} = ß_0 + ß_1 \text{ rate} + ß_2 \text{ drug}_j + ß3(\text{rateXdrug interaction}) + \varepsilon_i$, where $ß_i$ are the coefficients of regression, $\varepsilon_{i=}$ random error, was fitted to the data on Genstat computer program (Genstat 5 Release 3.2 Lawes Agricultural Trust, IACR-Rothamsted). Using this model, the suramin dose by treatment interaction was determined. Pre-patent period data were subjected to analysis of variance and Fisher's F-test used to test for significance on StatView statistical package (SAS Institute, Version 5.0.1). The white cell counts 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.

RESULTS

Effect of aflatoxin on the pre-patent period of *T. b. rhodesiense*-infected mice

The pre-patent period (expressed as mean days) for *T. b. rhodesiense*-infected aflatoxin-fed mice was 3.60 ± 0.1 days, and this was significantly longer than that of the controls (3.26 ± 0.1 days) at $p < 0.05$.

Effect of aflatoxin on the therapeutic efficacy of suramin

Table 1 shows the curative doses (CD) in the experimental mice. The CD values of both control and aflatoxin-fed groups were computed from their respective logistic linear regression models for suramin efficacy. These models were as follows:

1. For the placebo-fed controls, $\text{Logit } (p) = \text{Log } \{p/(1-p\} = -5.03 + 1.1178\text{Dose}$. Hence, suramin dose= [Log

{p/(1-p} + 5.03]/ 1.178.

2. For the aflatoxin-fed mice, Logit (p)= Log {p/(1-p}=-5.03 + 1.1178Dose- 0.58 + 0.238 Hence, suramin dose= [Log {p/(1-p} + 5.372]/ 1.178.

The effect of suramin dose was significant at $p < 0.01$ indicating that the higher the dose the higher the cure rate. There was no significant ($p > 0.05$) difference in the proportion of mice cured between the two groups, and also the trypanocidal drug dose by treatment groups interaction was not statistically significant ($p > 0.05$). However the curative doses of suramin were consistently higher in the aflatoxin-fed group compared to the controls (Table 1).

Table 1: Curative doses (CD) of suramin in control and *T. b. rhodesiense*-infected aflatoxin-fed mice

	Suramin curative dosage (CD)		
Mice groups	***CD_{50}***	***CD_{75}***	***CD_{90}***
Controls	4.27	4.67	5.08
Aflatoxin B1	4.56	4.97	5.37

Effect of aflatoxin on the total white cell counts of non-infected mice

Figure 1 shows the total white cell counts of the non-infected mice. From week 4-6 post aflatoxin-exposure, the total white cell count of aflatoxin-fed group significantly declined compared to the placebo-fed controls ($P < 0.05$).

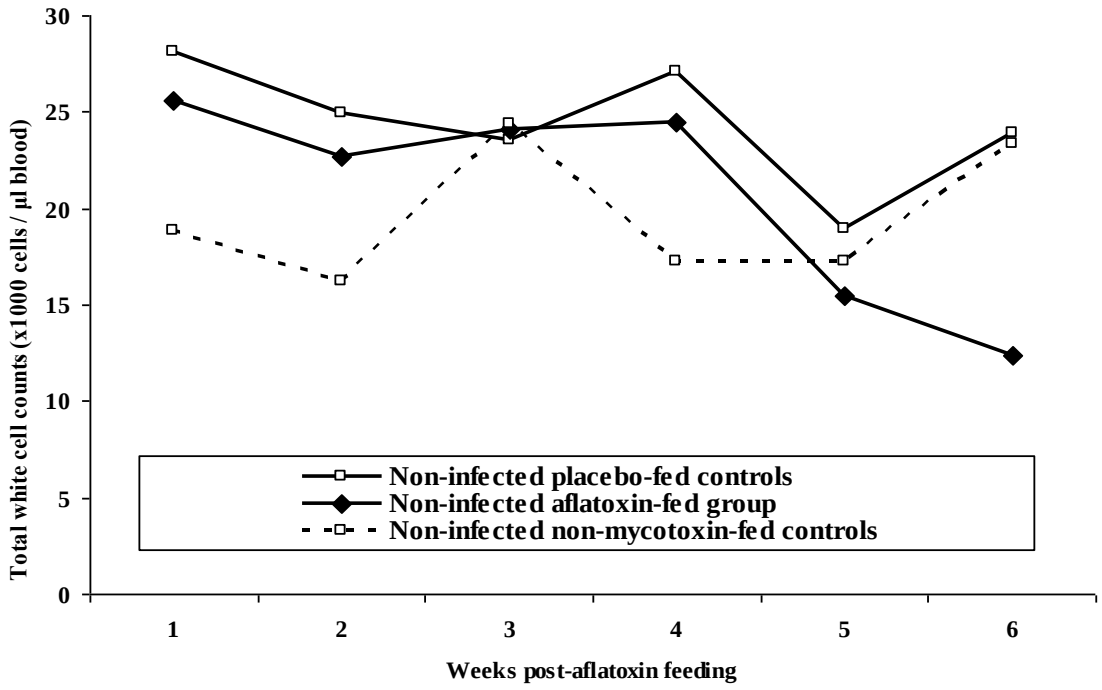

Figure 1. Mean total white cell count of non-infected mice at different time intervals

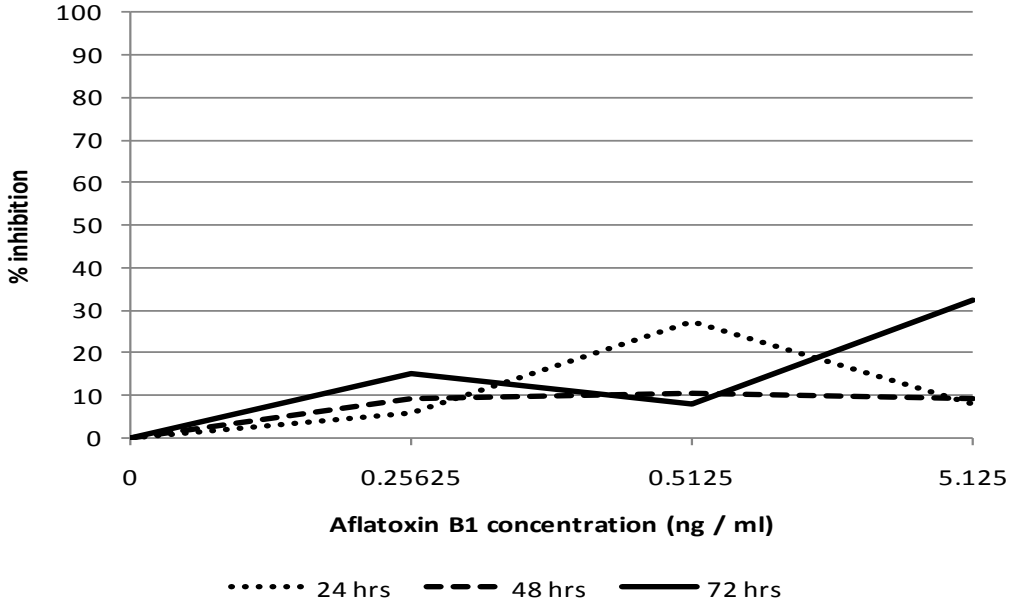

Figure 2. Inhibition curves of aflatoxin B_1 against *T. b. rhodesiense* KETRI 3741

Effects of aflatoxin on trypanosomes in culture

Figure 2 and 3 show inhibition of trypanosomal pyruvate production by aflatoxin B_1 and melarsoprol respectively. There was haphazard and minimal displacement in inhibition at 72, 48 and 24 hrs post-incubation with increasing concentrations of aflatoxin B_1. The highest inhibition recorded was 33% by the highest concentration of 5.125 ng/ml. Inhibition by AFB_1 concentrations below 0.5125 ng AFB_1/ml was negligible (Fig. 2). There was drastic displacement in inhibition of trypanosomal pyruvate production with increasing concentrations of Mel B (Fig. 3). A maximum inhibition of 95% was followed by a plateau phase between 0.5-2.0 ng/ml.

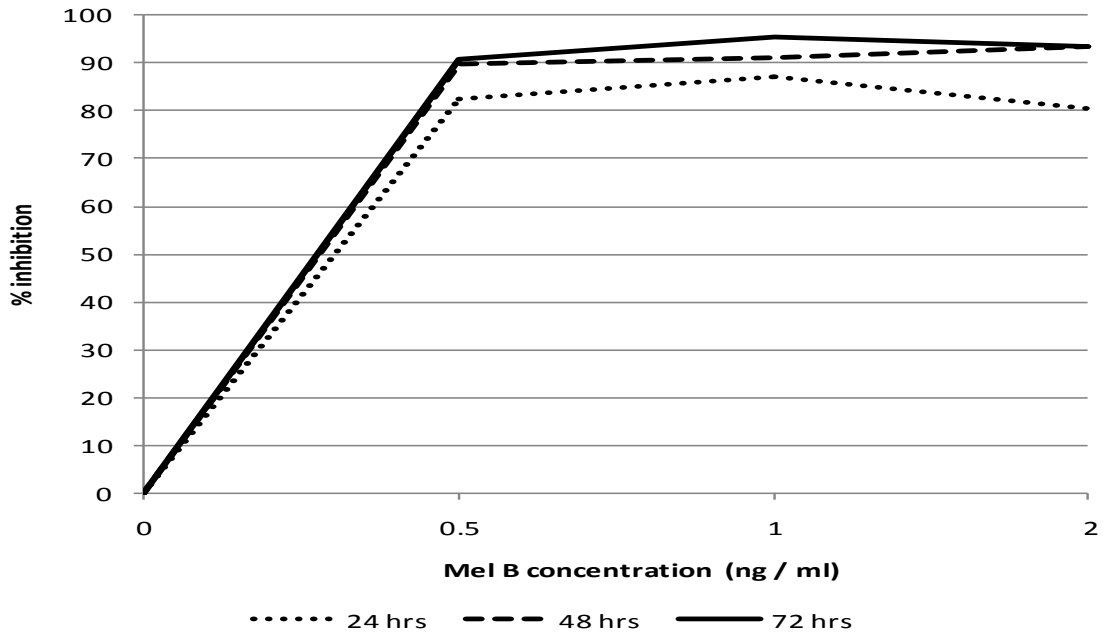

Figure 3. Inhibition curves of melarsoprol against *T. b. rhodesiense* KETRI 3741

DISCUSSION

This study suggests that aflatoxin B1 could affect the curative dose of suramin, whereby higher doses of the trypanocide were required to clear *T. b. rhodesiense*-infection in the aflatoxin-fed mice. Similar findings using immunosuppressive protocols have been reported in other studies, suggesting that intact efficient immune system is necessary during treatment of African trypanosomosis and leishmaniasis (Berger and Fairlamb, 1992; Osman *et al*., 1992). It was earlier demonstrated that x-irradiation in mice considerably reduced the efficacy of trypanocides and induced rapid development of high levels of stable chemo-resistance (Osman *et al*., 1992). Immunosuppression in *Trypanosoma evansi*-infected mice induced a higher level of suramin-resistance (Mutugi *et al.*, 1994) and also elevated suramin doses were required to clear the infections (Fang *et al.*, 1994). In visceral leishmaniasis, use of macrophage activators such as bacterial cell components or γ-interferon to lower the pentavalent antimonial dose required to cure the infection (Berger and Fairlamb, 1992) emphasizes that a strong and effective immune system enhances efficacy of chemotherapy. Aflatoxin B1 binds to DNA interfering with transcription (Clifford and Rees, 1966; Reddy and Sharma, 1989) inducing immunosuppressive effects (Bondy and Pestka, 2000), characterized by decreased antibody (Azzam and Gabal, 1998; Gabal and Azzam, 1998; Turner *et al*., 2003) and lymphoproliferative responses (Reddy and Sharma, 1989; Raisuddin *et al*., 1993). The higher doses of suramin required to cure mice in the present study could therefore have been due to immunosuppressive effects of aflatoxin B1 on the host.

Since suramin inhibits a number of enzymes, its mode of action is not well understood (Pépin and Milord, 1994). However, in the present study, aflatoxin administration to trypanosome-infected mice may have interfered with the anti-trypanosomal action of suramin. At the extra-cellular level, suramin exerts trypanocidal activity by hampering trypanosomal receptor-mediated uptake of cholesterol and phospholipids by forming a complex with low density lipoproteins (LDL) (Gutterridge and Coombs, 1977; Vansterkenburg *et al.*, 1993; Nok, 2003). It is possible that aflatoxin B1 inhibited this trypanocidal action by shifting this competition in favor of cholesterol, reducing trypanosomal LDL receptor-mediated endocytosis of suramin. Consequently, intra-cellular inhibition of the parasite glycolytic enzymes by the drug (Nok, 2003) notably L-α-glycerophosphate oxidase (Gutterridge and Coombs, 1977) could have been reduced.

Alternatively, it is also possible that aflatoxin B1 may have directly interfered with pharmacokinetic profiles of suramin. The trypanocide has a high protein binding capacity (Suramin-Systemic, 2008) especially to plasma proteins leading to extraordinary long half-lives (Collins *et al*., 1986; Pépin and Milord, 1994), high peak plasma levels and slow elimination by renal excretion (Suramin-Systemic, 2008). Since both suramin and aflatoxin B1 are strongly anionic (Gutterridge and Coombs, 1977, Smith and Moss, 1985; Nok, 2003), competition for binding sites on the plasma proteins is likely to modify the pharmacokinetic profiles of suramin. The presence of 2,3-epoxy aflatoxin B1, a highly reactive intermediate generated from detoxification and metabolism of aflatoxin B1 which covalently binds with proteins and other macromolecules (Smith and Moss, 1985) and peripheral aflatoxin B1 observed in our laboratory (Kibugu *et al*., 2009a) may suggest competition between the mycotoxin and suramin for binding sites on blood plasma proteins. This could reduce peak plasma levels and half-life, and increase elimination by renal excretion of suramin leading to the observed reduced efficacy of suramin observed in the present study. Elsewhere, the persistent illness observed in patients resulting from treatment failure and drug resistance, was attributed to interaction of mycotoxins and antifungal drugs due to structural and functional mimicry (Anyanwu *et al*., 2004).

The reduced suramin efficacy resulting from the 7-day aflatoxin exposure period in the present study may lead to treatment failure when used at the recommended dose. Since immunosuppressive effects of mycotoxins depend on a critical exposure window of time and dosage (Pier and McLoughlin, 1985; Müller *et al*., 1999; Hinton *et al*., 2003), probably a more significant effect would have been observed if the aflatoxin exposure period was extended as is the case in the field. In the present study, this hypothesis was

corroborated by the hematological data of non-trypanosome infected mice fed on 0.5 mg aflatoxin/ kg body weight daily for 30 days which suggested significant depression of leucocyte counts indicating immunosuppression.

Significant extension of pre-patent period in the *T. b. rhodesiense*-infected mice mediated by aflatoxin B1 was observed in this study. This suggested protection of the host by aflatoxicosis from *T. b. rhodesiense* infection and which is corroborated by studies involving malarial parasites. Aflatoxicosis decreased morbidity and prolonged survival time in mouse-*Plasmodium berghei* model (Hendrickse *et al.*, 1986; Young *et al*., 1988), and reduced *P. falciparum* parasitemia in children (Hendrickse *et al*., 1986). However data from our laboratory show that this protection is transient since aflatoxicosis aggravates pathogenesis in *T. b. rhodesiense*-infected mice (Kibugu *et al*., 2009a). Our *in vitro* studies did not demonstrate any trypanocidal effects of aflatoxin B_1. Indeed the peripheral AFB_1 levels of 0.021-0.5213 ng AFB_1 /ml previously reported in mice (Kibugu *et al*., 2009a) had no effect on trypanosomal growth in the present study. Therefore the observed transient host protection is mediated through another mechanism other than direct toxin effect on the parasite. It is known that immunomodulatory effects of aflatoxins could be stimulatory or suppressive depending on a critical exposure window of dose and time (Hinton *et al*., 2003; Williams *et al*., 2004). In fact Marin *et al*. (2002) observed biphasic effect of aflatoxin B1 on leucocytes, and cytokine m-RNA expression down regulating and up regulating pro-(TNF-α) and anti-inflammatory (IL-10) responses respectively. It is concluded that aflatoxin B1 induced reduction in suramin efficacy for treatment of *T. b. rhodesiense* infection in mice which could lead to treatment failure. Therefore, it is important to consider the effects of aflatoxicosis during clinical trials and control programs of trypanosomiasis and other tropical parasitic diseases in the field. The role of chronic aflatoxicosis in trypanocidal drug failure in the field needs to be evaluated. Further laboratory investigation of the effect of long exposure duration to aflatoxin B1 on efficacy of suramin against *T. b. rhodesiense* KETRI 3741 infection in mice needs to be carried out.

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