EFFECTS OF MYCOTOXINS ON THE PATHOGENESIS AND EFFICACY OF CHEMOTHERAPY OF *TRYPANOSOMA BRUCEI RHODESIENSE* IN MICE

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DECLARATION

The work described in this thesis is my own unless where acknowledged otherwise

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DEDICATION

This work is dedicated to the members of my family: my wife Margaret, my daughters Carol and Stella, my sons Robert and Charles, my mother Mrs. Keziah Waithera, my father, the late Mr. R. S. I. Karuku and my siblings; Maurice, Lydiah, Victor, Ruth, Silvia and Philip.

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ABSTRACT

Mycotoxins are secondary metabolites produced by toxigenic fungi that on ingestion induce mycotoxicosis in humans and animals. Among others, aflatoxins and ochratoxins are known to alter pathogenesis of diseases including parasitic infections and through their effects may render drugs ineffective. Such effects have not been evaluated in many tropical diseases including trypanosomiasis. The aim of the present work was to assess the effects of mycotoxins on the susceptibility of Swiss White mice and efficacy of chemotherapy to *Trypanosoma brucei rhodesiense* infection. Forty-nine days old male mice were given oral daily doses of 0.50 mg aflatoxin / kg b. wt. (12 mice) or 1.5 mg ochratoxin / kg b. wt. (12 mice) in vegetable oil for 30 days. These were then infected with *T. b. rhodesiense* on day 7 after commencement of mycotoxin feeding and compared to infected placebo-treated (12 mice), uninfected mycotoxin-fed (12 mice), infected non-mycotoxin-fed (6 mice) and uninfected non-mycotoxin-fed controls (6 mice). Parasitaemia development, clinical and pathological changes were determined. Analysis of variance and mean separation were used to determine differences between the test and control mice. Kaplan-Meier method was used to analyse and compare the survival between infected mycotoxin-fed mice and controls. In a separate study, aflatoxin-fed mice (6 groups of 6 mice) and placebo-treated controls (6 groups of 6 mice) were infected with *T. b. rhodesiense* and each treated with 4.0, 4.5, 5.0, 5.5, 6.0 or 6.5 mg suramin / kg b.wt. at the on-set of parasitaemia, and their curative dosage values determined and compared using a logistic linear regression model. Results showed that the two mycotoxins aggravate pathogenesis of *T. b. rhodesiense* infection in mice. Compared to the placebo-treated controls there was pronounced dyspnoea. Host survival was significantly ($p<0.05$) reduced in both mycotoxin-fed groups. There was extreme emaciation in the aflatoxin-fed and significantly ($p<0.05$) shorter pre-patent period in the ochratoxin-fed mice. Macrocytic normochromic anaemia characterized by significant ($p<0.05$) reduction in red cell count, packed cell volume, haemoglobin levels and significant ($p<0.05$) increase in mean corpuscular volume were observed in mycotoxin-fed mice compared to controls. There were also massive hepatic haemorrhages and thrombosis, together with cardiac and hepatic embolism in the aflatoxin-fed mice while the ochratoxin-fed mice had massive hepatic-renal haemorrhages and congestion, and massive hepatic haemosiderosis. Kidneys and liver had massive peri-vascular inflammatory mononuclear cellular infiltration in both mycotoxin-fed groups, and inflammatory eosinophils in the ochratoxin-fed mice. Although both mycotoxins aggravated hepatitis, nephritis and heart inflammation, there was more exacerbated liver damage and pancarditis in aflatoxin-fed mice and more intense nephritis in the ochratoxin-fed mice. The ochratoxin-fed group had aggravated endocarditis and pericarditis with necrosis, fibrosis and hydropericardium. Both mycotoxin-fed groups also presented with exacerbated hydrothorax and ascites. These aggravated pathological lesions could have caused reduced survival in the mice. In addition, aflatoxicosis induced a consistent increase in curative dosages of suramin in mice that indicated reduced drug efficacy. It was concluded that aflatoxicosis and ochratoxicosis aggravated the pathogenesis of *T. b. rhodesiense* infection in mice reducing the host survival, and aflatoxicosis may hinder its chemotherapy. Mycotoxicosis should therefore be taken into consideration during trypanosomiasis control programs.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Background

Trypanosomiasis is a group of diseases, transmitted by tsetse and other biting flies, variously known as Nagana in cattle, Surra in horses and camels, Sleeping Sickness or Human African Trypanosomiasis (HAT) in humans and affects other animal species as well (Apted, 1970; Stephen, 1986). The diseases are caused by the protozoan parasites *Trypanosoma ssp.* which are of different species and are made up of syndromes ranging from virtually asymptomatic, mild to fulminating conditions (Losos, 1986) reflecting differences in phylogeny of the parasite and diversity of the host species. These diseases, which are endemic in sub-Saharan Africa, are an obstacle to agricultural and livestock development and a threat to human health (Stephen, 1986).

HAT, caused by two *Trypanosoma brucei* sub-species namely *T. b. gambiense* and *T. b. rhodesiense*, is a zoonotic disease in tropical Africa where it is a medical major concern. More than 66 million people in sub-Saharan Africa are at risk from HAT (WHO, 2004). The disease by *T. b. rhodesiense* is restricted to eastern and southern Africa while that caused by *T. b. gambiense* is found in central and western Africa (WHO, 2004). The clinical symptoms associated with HAT are lymphadenopathy, skin rash, periodic fever, headache, joint and muscle pains, edema, cardiac problems, endocrinological disorders, emaciation and neurological problems (Apted, 1970). Unlike the *T. b. gambiense* infection that manifests as a chronic disease, *T. b. rhodesiense* infection is acute. HAT has a 3-phase course; first the haemo-stage when the parasites are in blood circulation

with no central nervous system (CNS) invasion, then the second phase which is a transitional, when the parasites are in the CSF with no CNS parenchymal infection and lastly the late stage (meningoencephalitic phase) when CNS parenchyma is invaded by the parasites. In the meningoencephalitic phase, a change in sleeping pattern is the most prominent clinical symptoms, hence the clinical term, Sleeping Sickness. The drugs used for treatment of *T. b. rhodesiense* infections are suramin and Mel B respectively for the early (Murray and Jennings, 1983) and late (Jennings *et al.*, 2002; Burri *et al.*, 2000) stages of the disease. Tsetse control and treatment of reservoir hosts are some of the most effective preventive strategies for control of sleeping sickness in the field (Stephen, 1986).

Immunocompetence is one of the most important host factors in the natural history of trypanosomiasis (Barry and Turner, 1991). Humoral responses involved in defense against the parasites are complement-mediated (Ouma *et al.*, 1997; 1998) and immune globulin production (Greenwood, 1974; Kobayashi and Tizard, 1976). Indeed in the absence of chemotherapy, a significant decrease in parasitaemia is only associated with antibody response to trypanosomes (Seed and Sechelski, 1988). Immunosuppressive conditions such as mycotoxicosis are therefore likely to influence pathogenesis and treatment of trypanosomiasis in the field.

Mycotoxins are secondary metabolites produced by a variety of toxigenic fungi (Smith and Moss, 1985) that contaminate food, feeds and food products (Azziz-Baumgartner *et al.*, 2005; FAO/UNEP, 1977). The toxins, which are also found naturally in breast milk

(Jonsyn *et al.*, 1995b) and cord blood (Jonsyn *et al.*, 1995a), induce a condition termed mycotoxicosis in humans and animals (Williams *et al.,* 2004). The most common mycotoxins are aflatoxins, ochratoxins, trichothecenes and zearalenone (Smith and Moss, 1985) and are stable to normal cooking and food processing procedures (Al-Anati and Petzinger, 2006).

Mycotoxins are recognized as carcinogenic, hepatotoxic, nephrotoxic, tremorgenic, neurotoxic, teratogenic, mutagenic, immunomodulants in a variety of animals, unicellular organisms and plants (Williams *et al.*, 2004; Smith and Moss, 1985). Others cause reduced reproductive efficiency (Oswald *et al.*, 2005), dermal toxicity (Pier and McLoughlin, 1985; Smith and Moss, 1985) and haemopoeitic alterations (JECFA 47, 2001; Sandhu *et al.*, 1998). These effects are dependent on dose and route of exposure (Bondy and Pestka, 2000), duration of exposure, sex and age animals (Smith and Moss, 1985).

Immunomodulation (Williams *et al.*, 2004), direct toxicity to disease causing pathogens (Young *et al.*, 1988) or pathological effects on various vital organs (JECFA 47, 2001) probably modify manifestation of various experimental (Young *et al.*, 1988; Joens *et al.*, 1981) and naturally acquired diseases (Hendrickse *et al.*, 1986; Peers and Linsell, 1977). The most frequently encountered mycotoxins in Kenya are aflatoxin and ochratoxin A (Azziz-Baumgartner *et al.*, 2005; Kimathi and Siboe, 1994), which are also the most potent (Bondy and Pestka, 2000). No work has been reported on the influence of these molecules on the pathogenesis of trypanosomiasis, an endemic parasitic disease in

Kenya. Since successful chemotherapy is dependent on a competent immune system (Osman *et al.*, 1992), these natural toxins are likely to influence treatment of trypanosome infections in the field.

1.2 Trypanosomiasis

Trypanosomiasis is a group of human and animal parasitic diseases caused by different species of tsetse-borne protozoan parasites of the order- Kinetoplastida, family- Trypanomastidae and genus-*Trypanosoma* (Boyt, 1986; Stephen, 1986). Trypanosomes are parasites with a two-host life cycle in mammals and arthropods. Life cycle starts when trypanosomes are ingested during a blood meal by tsetse flies from a reservoir host. Trypanosomes multiply over a period of 2-3 weeks in the fly mid-gut and then migrate to salivary glands where they develop into epimastigotes. These then develop into metacyclic trypomastigote forms of the parasite, which are transmissible to humans (WHO, 2004; Boyt, 1986). Hoare (1972) categorized the different morphological forms of trypanosomes as amastigotes, sphaeromastigote, promastigotes, epimastigotes and trypomastigotes. These parasites are transmitted to human hosts through the bites of infected tsetse flies (*Glossina* species), which are found only in tropical Africa. The number of metacyclic forms injected by the insect is an important factor in determining whether or not an infection develops (Ormerod, 1970).

1.2.1 Transmission and epidemiology of trypanosomiasis

Trypanosoma brucei rhodesiense causes the East African form of HAT (Apted, 1970). Unlike the West African form caused by *T. b. gambiense* that manifests as a chronic

disease, the East African type is an acute disease. Both forms lead to death if not treated. Trypanosomiasis is zoonotic with animal reservoirs being important factors in epidemiology of the disease (Njiru *et al.*, 2004; Hide *et al.*, 1996).

1.2.2 Morphological changes in infected host

When tsetse probes subcutaneous tissues, trypanosomes become lodged forming a 'trypanosomal chancre', a hard, painful red nodule with clear fluid containing trypanosomes. In the chancre, metacyclic trypanosomes first develop into long-thin forms which undergo rapid division into short-stumpy forms which do not divide and a complete range of intermediate forms appear (Bird *et al.*, 1966). In humans, the long-thin form invades lymphatic vessels to lymphoid tissues, and a stage of acute generalized trypanosomiasis is initiated (Boyt, 1986; Ormerod, 1970). After formation of the chancre, there is direct lymphatic spread of the disease to surrounding tissues (Ormerod, 1970). The tissues prominently invaded are heart, kidney, spleen, skeletal muscles and serous membranes in pericardium, pleurae and peritoneum, where there is an interstitial infiltration of mononuclear inflammatory cells in experimental studies (Kristensson and Bentivoglio, 1999). There then follows a progressive alteration of immunological apparatus, haemopoietic system, tissue damage and cellular infiltration in all organs especially the heart (Murray *et al.*, 1974). The late stage disease is marked by appearance of parasites or mononuclear inflammatory cells in cerebrospinal fluid and brain (Kristensson and Bentivoglio, 1999; Fink and Schmidt, 1979).

In both lymph glands and spleen, there is development of lymphoid germinal centres (Murray *et al.*, 1974) which increase in size and proliferation of plasma cells and macrophages (Kristensson and Bentivoglio, 1999; Anosa and Kaneko, 1984). As the disease progresses, cellular depletion occurs in lymphoid tissues (Murray *et al.*, 1974). In humans, myocarditis in early stage of *T. b. rhodesiense* infection is evident while sclerotic endocarditis and periarterial myocarditis in *T. b. gambiense* disease occurs (Ormerod, 1970). These lesions are caused by heavy infiltration by trypanosomes and inflammatory cells in the myocardium leading to atrophy of myocardial muscle fibers (Kristensson and Bentivoglio, 1999). There are reports of severe myocarditis and pericarditis in rats and mice infected with *T. brucei* (Murray *et al.*, 1974; Anosa and Kaneko, 1984) and in *T. evansi*-infected camel (Maina *et al.*, 2003).

Hepatomegaly (Chisi *et al.*, 2004) and jaundice (Ormerod, 1970) have been reported in HAT patients. Hepatomegaly is consistent in *T. brucei*-infected rats (Murray *et al.* 1974) and mice (Amole *et al.*, 1982). Also reported, are necrosis of hepatocytes with proliferation and hypertrophy of Kupfer cells exhibiting erythrophagocytosis (Anosa and Kaneko, 1984). In kidneys, trypanosomiasis induces proliferative glomurulonephritis in mice (Anosa and Kaneko, 1984) and HAT patients leading to fibrosis (Manson-Bahr and Bell, 1987). In *T. brucei*-infected mice, there is severe glomerulonephritis characterized with invasion of glomeruli by neutrophils, macrophages and lymphocytes (Anosa and Kaneko, 1984).

In the human brain, appearance of inflammatory cells, protein and immunoglobulins in CSF suggests probable meninges infection early in the disease. Meningoencephalitis develops during late meningeal reaction when brain becomes infected by trypanosomes through blood brain barrier (Ormerod, 1970). Appearance of morular cells in perivascular cuffing is considered pathognomonic feature of sleeping sickness (Ormerod, 1970) and in the CNS (central nervous system), they produce IgM (Manson-Bahr and Bell, 1987). Fink and Schmidt (1979) reported meningoencephalitis in *T. b. rhodesiense*-infected mice with inflammatory reactions that correspond to late HAT but in *T. brucei*-infected rats, infiltration of trypanosomes in CNS did not cause pathological lesions (Murray *et al.*, 1974).

1.2.3 Blood changes

The most important blood change that occurs during trypanosome infections is anaemia in humans (Chisi *et al*, 2004), non-human primates (Kagira *et al.*, 2007) and animals (Stephen, 1986; Jennings *et al.*, 1974). There is also thrombocytopaenia (Kagira *et al.*, 2007), leucocytosis (Njiru *et al.*, 2000), hypergammaglobulinaemia with production of non-specific IgM (Vincendeau *et al.*, 1999), autoagglutination, high erythrocyte sedimentation rate and low blood sugar (Manson-Bahr and Bell, 1987). During *T. brucei* infection, major haemolytic crises leading to haemoglobinuria and haemoglobinaemia in rats (Murray *et al.*, 1974) and splenic haemosiderosis and erythrophagocytosis in mice (Anosa and Kaneko, 1984).

Anaemia is a cardinal feature in trypanosome infections of humans (Chisi *et al.*, 2004), non-human primates (Kagira *et al.*, 2007) and domestic animals (Stephen, 1986). It has been suggested that the mechanisms involved in development of anaemia associated with trypanosomiasis depend on the species of trypanosome involved (Naessens *et al.*, 2005). Haemolysis is the most important mechanism of anaemia during African trypanosomiasis (Manson-Bahr and Bell, 1987). Trypanosome antigens have been demonstrated on the surface of erythrocytes (Stephen, 1986) and this could sensitize erythrocytes and prime them for complement-mediated haemolysis (Kristensson and Bentivoglio, 1999; Stephen, 1986). Pre-formed antigen-antibody complexes (Amole *et al.*, 1982) or IgM (Murray *et al.*, 1974) absorbed onto erythrocytes may fix complement leading to intravascular haemolysis, erythrophagocytosis or both (Murray *et al.*, 1974). It has been suggested that peroxidative injury to erythrocytes, probably due to loss of cell membrane integrity and hypocholesteraemia, are a major cause of haemolysis and anaemia in *T. brucei*-infected sheep (Taiwo *et al.*, 2003).

Dyshaemopoeisis is another important mechanism. During the chronic stage of trypanosomiasis, exotoxins produced by parasite have a degenerative effect on various organs of the host especially the haemopoietic system. This hinders haemopoiesis and is the most important cause of anaemia during the late phase of the disease (Boyt, 1986; Stephen, 1986). Signs of impaired erythropoiesis such as microcytosis (Kagira *et al.*, 2007; Fiennes, 1970), hypoferremia (Tautour and Indris, 1973) and low plasma-iron turnover rates, (Stephen, 1986; Dargie *et al.*, 1979) have been observed in chronic trypanosomiasis. Histological finding of massive haemosiderin deposits in the spleen

(Murray *et al.*, 1974) may be indicative of defective iron utilization. Recently, TNF-α mediated development of anaemia in *T. b. rhodesiense*-infected mice through regulation of erythropoietin production has been reported (Naessens *et al.,* 2005). Haemodilution may result in significant fall in haematocrit, red cell count and haemoglobin levels and other haematological values (Stephen, 1986). Trypanosome infections in mice (Amole *et al.*, 1982) induced a marked hypervolemia with significant increases in plasma volume which was related to widespread microvascular damage. This haemodilution and low level erythrophagocytosis were probably the causes of anaemia in the chronic phase of infection (Amole *et al.*, 1982).

1.2.4 Pyrexia

Pyrexia is known to occur during many infections including trypanosomiasis and is induced by a range of pyrogens. In trypanosomiasis, phagocytes brought into contact with trypanosomal antigens secrete pyrogens such as TNF-α (Naessens *et al.*, 2005). Antibody-antigen complexes and cell mediated immune responses are potent stimuli for pyrogen release (Stephen, 1986). Anaemia becomes progressively more severe during prolonged fever (Stephen, 1986; Murray, 1979).

1.2.5 Immune responses to trypanosome infection

Invasion of trypanosomes into the mammalian hosts triggers a series of events involving first, innate immunity followed by specific immunity. The first line of defense are the trypanolytic factors followed by chancre formation induced by trypanosome proliferation (Stephen, 1986), increase in lymphoblasts and surface immunoglobulin-bearing cells

(Vincendeau *et al.*, 1999). Lymphadenopathy, splenomegaly with destruction of lymphatic tissue architecture and hypergammaglobulaemia are the major modifications (Murray *et al.*, 1974) that limit effectiveness of immune system leading to immunopathological phenomena (Vincendeau *et al.*, 1999). Specific immunity requires efficient presentation of parasitic antigens, activation of T and B cells implying specific antigen receptor recognition and development of effector cells and molecules (Vincendeau *et al.*, 1999). In trypanosomiasis, complement activation by both alternate and classical pathways is detected (Vincendeau *et al.*, 1999). Njiru *et al.* (2000) and Ouma, *et al.* (1997; 1998) observed activation of classical complement pathway in *T. evansi*–infected camels leading to control of parasitaemia. Complement activation in infected animals (Njiru *et al.*, 2000; Kobayashi and Tizard, 1976) led to hypocomplementaemic state (Ouma, *et al.*, 1997; 1998) and increased vascular permeability expressed as perivascular edema, swelling and degeneration of vessels, which is a consistent pathological finding in trypanosomiasis (Musoke and Barbet, 1977).

Elevated levels of IgM and IgG have been reported in animal (Njiru *et al*, 2000; Stephen, 1986; Luckins, 1972) and humans (Lejon *et al.*, 2003) trypanosomiasis. There was enhanced and prolonged production of IgM antibodies (Lejon *et al.*, 2003; Moulton and Sollod, 1976) leading to hypergammaglobulinaemia (Orhue *et al.*, 2005). Hypergammaglobulinaemia is attributed to dysfunction in the control of IgM responses due to dysfunction or loss of T-suppressor cells allowing uncontrolled IgM response, a trypanosome-derived mitogen (Kobayashi and Tizard, 1976) or continuous production of specific antibodies against several variant antigen types (Musoke *et al.*, 1981). In

trypanosome-infected animals (Luckins, 1976; Luckins and Mehlitz, 1976), IgM containing serum fractions were more protective than IgG antibodies (Luckins, 1976). There were two peaks of antibody activity, the second one being higher than the first (Luckins and Mehlitz, 1976). Specific IgM antibodies from the first peak were more protective than specific IgG_1 with the reverse being true of antibodies from the second peak (Musoke *et al.*, 1981). However, although IgM is a better anti-trypanosomal antibody, it does not enter extra-vascular fluids as rapidly as IgG molecules hence the relapse parasitaemia phenomena thus IgG molecule is more active in extra-vascular locations (Sacks *et al.*, 1980; Seed, 1977).

Cellular immune responses constitute antigen presentation (Vincendeau *et al.*, 1999), activation and mitogenic proliferation (Njiru *et al.*, 2000; Lutje *et al.*, 1996) leading to relevant effector mechanisms (Halliwell and Gorman, 1989). Macrophages play a key role in the inflammatory phase as antigen presenting cells, in production of cytokines, prostaglandins (Vincendeau *et al.*, 1999) and receptor-mediated phagocytosis (Stevens and Moulton, 1978). Activated macrophages also produce IL-1 and TNF-α (Vincendeau *et al.*, 1999) and high levels of nitric oxide (Kristensson and Bentivoglio, 1999; Sternberg *et al.*, 1998). However, although nitric oxide is trypanocidal (Stenberg *et al.*, 1998), it may also mediate immunosuppression (Vincendeau *et al.*, 1999). Through secretion of cytokines, specific T cells markedly modify functions of B cells and those of macrophages (Vincendeau *et al.*, 1999). Leucocytosis occurs in trypanosome-infected monkeys (Ndung'u *et al.*, 1994), camels (Njiru *et al.*, 2000) and mice (Vincendeau *et al.*, 1999) and specifically B cell proliferation in *T. congolense*-infected cattle (Lutje *et al.*,

1996). Mast cell activation with production of histamine has also been reported in *T. brucei* infected mice (Ben-Rashed *et al.*, 2003).

1.2.6 Antigenic variation

Variant surface glycoprotein (VSG), the predominant surface antigen that constitutes an important molecular interface between trypanosomes and host immune system (Vincendeau *et al.*, 1999), acts as a receptor for host components and a highly immunogenic protein whose antigenecity is continuously changing (Pays, 1999). The VSG elicits production of trypanocidal antibodies which effectively removes parasites of a corresponding antigenic type (Hudson and Terry, 1979), but parasites change composition of VSGs to a new variant antigenic type (VAT) that cannot be recognized by the earlier antibody and this cycle continue throughout the infection (Stephen, 1986). Two distinct processes drive trypanosomal antigenic variation (Pays, 1999). The first involves alternative transcriptional activation of different VSG gene expression sites and frequent DNA re-arrangement due to spontaneous homologous recombination in the active telomeric expression sites. Inactivation of former expression site and simultaneous activation of a new one occurs. The second mechanism involves changing the VSG gene present in the active expression site. The events involved in VSG gene switching are gene conversion and reciprocal recombination.

1.2.7 Trypanosome-induced immunosuppression

Several mechanisms of trypanosome-induced immunosuppression have been proposed. Trypanosome-induced generation of suppressor macrophages (Grosskinsky and Askonas,

1981) and suppressor T cells (Eardly and Jayawardena, 1977) leading to production of immunosuppressive molecules (Schleifer and Mansfield, 1993; Fierer *et al.*, 1984) has been reported. Hypocomplementaemia leading to immunosuppression (Vincendeau *et al.*, 1999) caused by complement activation by dead (Musoke and Barbet, 1977) and live (Njiru *et al.*, 2000; Ouma *et al.*, 1998) trypanosomes has been observed. Recently, Stiles *et al.* (2004) reported apoptosis-mediated destruction of host immune cells through a trypanosome-derived procyclin molecule.

1.2.8 Chemotherapy to trypanosome infection

Drugs commonly used for treatment of animal trypanosomiasis are diminazene aceturate, homidium salts, isometamedium chloride and quinapyramine (Geerts and Holmes, 1998). Early stage HAT is treated by two drugs; suramin for *T. b. rhodesiense* and pentamidine for *T. b. gambiense* infections (Murray and Jennings, 1983). In late stage, interstitial trypanosome infiltration make chemotherapy difficult (Poltera, 1985) and melarsoprol (Mel B) is the drug of choice for *T. b. rhodesiense* and *T. b. gambiense* infections (Jennings *et al.*, 2002; Burri *et al.*, 2000) and difluoromethylornithine (DFMO) for *T. b. gambiense* infections (Burundi *et al.,* 1995). However use of melarsoprol is marred by reactive arsenical encephalopathy due to hypersensitivity, inflammatory responses or massive death of trypanosomes (Nok, *et al.*, 2003; Ormerod, 1970).

Several factors are known to affect efficacy of trypanocidal drugs. Drug-associated factors include time interval between infection and initiation of therapy (Geerts and Holmes, 1998; Ndung'u and Akol, 1989), quality and appropriateness of drug

formulation (Karanja *et al.*, Unpublished data; Maina *et al.,* 2003), drug administration route and dose (Mdachi, 1999; Geerts and Holmes, 1998) and drug concentration in the body (Mdachi, 1999). Genetic make-up (Landers, 2004), breed, sex of host and disposition of drug (Mdachi, 1999), host nutritional status (Murilla, 1996) and state of host immune system (Berger and Fairlamb, 1992; Osman *et al.*, 1992) are host factors that influence efficacy of chemotherapy. Parasite-associated factors are the infecting trypanosome species (Mdachi, 1999), level of parasitaemia, disease challenge (Geerts and Holmes, 1998; Murilla, 1996) and drug resistance (Geerts and Holmes, 1998).

1.2.9 Interaction of trypanosomiasis with other diseases

Trypanosomiasis is endemic in areas where other diseases occur. HIV-AIDS, malaria, helminthosis, schistosomiasis, food-borne infections and toxicoses are important conditions that are likely to interact with trypanosome infections in the field. These interactions could lead to serious implications on animal and human health. By compromising the immune system (Williams *et al.*, 2004), haemopoeitic system (Dugyala *et al.*, 1994; Cukrova *et al.*, 1991) or interference with functions of essential nutrients like vitamins (Anyanwu *et al.,* 2004) may also exert pathological effects on vital organs like the liver, kidney, heart, lymphoid tissues (Pier and McLoughlin, 1985), thereby influencing the courses of various diseases.

Although both mycotoxicosis and trypanosomiasis are endemic in Kenya, no work has been done on their possible interaction. Given that chronic mycotoxicosis down regulates immune system (Bondy and Pestka, 2000) with a wide spectrum of pathological

effects (Pier and McLoughlin, 1985), it would be interesting to see how this may affect development and pathogenesis of trypanosomiasis. Also since both aflatoxin B1 and ochratoxin A are nephrotoxic agents (Bondy and Pestka, 2000; Smith and Moss, 1985), they may have a possible influence on anaemia, which is a cardinal feature of trypanosomiasis (Chisi *et al.*, 2004) during trypanosome infections. Successful chemotherapy depends on a competent immune system (Osman *et al.*, 1992) and the effect of naturally occurring immunodepressive agents such as aflatoxin B1 on the efficacy of trypanocidal drugs like melarsoprol and suramin require investigation.

1.3 Mycotoxicosis

Mycotoxins are secondary metabolites produced by a variety of fungi when they naturally grow on agricultural produce, feeds and food products (Azziz-Baumgartner *et al.* 2005; Smith and Moss, 1985). Aflatoxins are produced by *Aspergillus flavus*, *A. parasiticus* and *A. nomius* (Saad, 2004), ochratoxins by *A. ochraceus*, *Penicilium cyclopium* and *P. viridicatum* while zearalenone and T-2 toxin (a trichothecene) by various *Fusarium spp* (Smith and Moss, 1985). Other toxins produced by *Fusarium species* are nivalenol, deoxynivalenol and fumonisins among others. Ingestion of contaminated food and feeds (Saad, 2004; Pier and McLoughlin, 1985), inhalation (Jakab *et al.*, 1994), skin contact (Smith and Moss, 1985) or vertical transfer (Jonsyn *et al.*, 1995a;b) of these fungal toxins induces mycotoxicosis (Oswald *et al.*, 2005; Bondy and Pestka, 2000; Muller *et al.*, 1999).

1.3.1 Natural occurrence and epidemiology of mycotoxicosis

Mycotoxic contamination occurs during pre-harvest, harvest and post-harvest activities (FAO/UNEP, 1977). The factors that favour mycotoxin production are crop genotypes, drought, soil types and characteristics, insect activity during pre-harvest period (Williams *et al.*, 2004; FAO/UNEP, 1977), untimely harvest and inadequate drying before storage (Azziz-Baumgartner *et al.*, 2005; Williams *et al.*, 2004), warm temperatures, high humidity and water content after harvest (Saad, 2004; FAO/UNEP, 1977) as well as existence of suitable substrate. Hence, mycotoxins occurrence is influenced by factors such as geographical location, agricultural and agronomic practices, susceptibility of produce to fungal invasion during pre-harvest, storage and processing periods (Saad, 2004). Pre- and post-harvest fungal infection leads to mycotoxic contamination in raw commodities like standing crops, stored cereal crops or processed foods (FAO/UNEP, 1977). Host metabolism after consumption of contaminated feeds or food leads to mycotoxic contamination in breast milk, dairy products and human umbilical cord blood (Thrasher, 2007; FAO/UNEP, 1977).

Aspergillus flavus and *A. parasiticus* infect maize, sorghum, peanuts and other oil-seed crops, rice, cassava, nuts, chilies, spices, groundnuts, cottonseeds, coconut, wheat and other foodstuffs. The produce with the highest risk of aflatoxin contamination are maize, peanuts and cottonseed (Saad, 2004). Four major aflatoxins (B1, B2, G1 and G2) occur in fungal-contaminated plant produce, the major producers being *A. flavus* and *A. parasiticus*. These two *Aspergillus spp*, the main producers of aflatoxin B1 (Smith and Moss, 1985), have been isolated in maize meal in which high levels of this toxin were

detected in Kenya (Kimathi and Siboe, 1994). Aflatoxins M1 and M2, the hydroxylated metabolites of aflatoxins B1 and B2 occur predominantly in milk of cows fed on aflatoxin-contaminated rations (FAO/WHO, 1987). In Kenya, ochratoxin A has also been detected in cow milk (Gathumbi, Per. Comm.). Zearalenone produced by *F. graminearum* naturally contaminates maize, hay, barley and feed while T-2 toxin from *F. sporotrichoides* is a contaminant of oats, wheat, and barley (FAO/UNEP, 1977).

Aflatoxins were initially isolated and identified as the causative toxins of Turkey X disease when over 100,000 turkeys died in England (Thrasher, 2007). Variations in aflatoxin exposure exist between countries largely as a function of diet. Aflatoxin exposure varies from 3.5-14.8 ng/kg /day in Kenya, 11.4-158.6 in Swaziland, 38.6-183.7 in Mozambique, 16.5 in South Africa, 4-115 in Gambia, 11.7-2027 in Southern Guangxi province of China, 6.5-53 in Thailand and 2.7 ng/kg /day in USA (Williams *et al.*, 2004). In Kenya, acute aflatoxicosis outbreaks after consumption of aflatoxin-contaminated maize occurred in Eastern Kenya affecting 317 people and led to 125 deaths in the year 2004 (Azziz-Baumgartner *et al.*, 2005).

1.3.2 Health implications associated with mycotoxicosis

Health implications of mycotoxins are categorized as acute, chronic mycotoxicosis and secondary diseases (Smith and Moss, 1985). Symptoms caused by ingestion of moderate to high levels of mycotoxins range from acute mortality (Azziz-Baumgartner *et al.*, 2005), slow growth and reduced reproductive efficacy (Oswald *et al.*, 2005) while lesser amounts may result in impaired immunity and decreased resistance to infections (Pier and

McLoughlin, 1985). Mycotoxins are associated with various diseases in both humans and animals (Pier and McLoughlin, 1985) mainly cancer (Peers and Linsel, 1977), reproductive and gastrointestinal problems (Smith and Moss, 1985), liver abnormalities (Ngindu *et al.*, 1982), nephropathy (Bondy and Pestka, 2000), dermal toxicity (Smith and Moss, 1985) and encephalopathy (Thrasher, 2007). In secondary mycotoxicosis, toxin levels are below those causing a clearly defined toxicosis (Pier and McLoughlin, 1985), with the clinically identifiable disease being the infectious process to which mycotoxin predisposes the host (Bondy and Pestka, 2000), and the true underlying cause may go undetected (Pier and McLoughlin, 1985). Chronic aflatoxin exposure has major effects on nutritional status in animals, but as in immunotoxicities, thresholds for these effects are not defined for any species (Williams *et al.*, 2004).

Aflatoxin, ochratoxin A and T-2 toxin are teratogenic exerting biological effects on the foetus (Pier and McLoughlin, 1985). According to Thrasher (2007) and Pier and McLoughlin (1985) jaundice and decreased birth weight were associated with a higher concentration of aflatoxin B1. In Africa, pre-natal and neonatal mycotoxic exposure is common (Jonsyn *et al.*, 1995a; b). Further, the normal functions of vitamin B12, which plays an important role in haemopoeisis (Baker *et al.*, 2001), are disrupted or interfered with by mycotoxins (Anyanwu *et al.*, 2004).

1.3.4 Mycotoxins and diseases in humans

Intra-uterine and neonate exposure to aflatoxin in contaminated food may contribute to etiology of kwashiorkor, neonatal susceptibility to infection and jaundice, childhood

infections, malignant disease and compromised response to prophylactic immunizations in children (Thrasher, 2007). Acute aflatoxicosis is characterized by high death rate, jaundice, rapidly developing ascites, portal hypertension, massive gastrointestinal bleeding and disorders, pulmonary oedema, convulsions, coma, cerebral oedema and fatty degeneration in liver, kidneys and heart (Saad, 2004; Thrasher, 2007). Aflatoxin B1, a potent liver carcinogen (Saad, 2004), has been implicated in hepatocellular carcinoma in Asia and Africa (Peers and Linsell, 1977), hepatic failure, encephalopathy and Reye's syndrome (Thrasher, 2007).

Aflatoxin B1 also causes abnormal mitochondrial structure and function and has been associated with a Reye-like syndrome in Thailand, New Zealand, Czechoslovakia, U.S.A., Malaysia, Venezuela and Europe (Thrasher, 2007). Damage may be to mitochondrial DNA through adducts and mutations, mitochondrial membranes, increased apoptosis or disruption of energy production. This disruption may lead to dysfunction of various organs and concomitant symptoms that escape standard medical diagnostics (Thrasher, 2007).

Ochratoxin A is a potent nephroxin (Pier and McLoughlin, 1985) that has been detected in breast milk (Jonsyn *et al.*, 1995b) and cord blood (Jonsyn *et al.*, 1995a) and is associated with a fatal human kidney disease endemic to the Balkan region (Bondy and Pestka, 2000). T-2 toxin, a natural contaminant of wheat causes alimentary toxic aleukia (Smith and Moss, 1985).

1.3.5 Mycotoxicosis in animals

Acute toxic effects of aflatoxins in all animals studied are characterized by haemorrhagic necrosis of the liver. Aflatoxin B1 is a potent hepatocarcinogen in all animals studied (FAO/WHO, 1987). In laboratory rodents, it induces adducts and mutations, cancer, immunosuppression, liver injury and birth defects. Pathological lesions induced by OTA in animals are increases in PCV, haemoglobin levels, proteinaemia, proteinuria, dehydration, enteritis, gastric and intestinal hyperemia and pale and swollen kidneys in acute ochratoxicosis (JECFA 47, 2001). Proximal tubular necrosis and dilation, focal areas of intestinal mucosal damage in lower intestine, nephrosis, enteritis, lymphoid necrosis and hepatic necrosis are microscopic lesions associated with acute ochratoxicosis.

1.3.6 Immunotoxicity of mycotoxins

Three groups of mycotoxins associated with immunosuppression are aflatoxins, ochratoxin A and some trichothecenes such as T-2 toxin. Much of their immunotoxicity is mediated through inhibition of protein synthesis, but each acts at a different site of protein formation (Pier and McLoughlin, 1985). According to Oswald *et al.* (2005), sensitivity of immune system to mycotoxin-induced immunosuppression arises from vulnerability of proliferating and differentiating immune cells. This may manifest as depressed T or B-lymphocyte activity, suppressed antibody production and impaired macrophage/neutrophil effector functions. Suppressed immune function may decrease resistance to infectious diseases, reactivate chronic infections or reduce vaccine and

therapeutic efficacy (Oswald *et al.*, 2005; Osman *et al*, 1992). Immunotoxicity of ochratoxin A and aflatoxin B1 is dose- and route-dependent (Bondy and Pestka, 2000).

Ochratoxin A (OTA) affects protein synthesis through inhibition of phenylalanyl t-RNA synthetase (Pier and McLoughlin, 1985). Major biochemical effects include inhibition of macromolecular synthesis especially immune globulin synthesis (Pier and McLoughlin, 1985), increased lipid peroxidation and inhibition of mitochondrial respiration (Bondy and Pestka, 2000). Immunosuppressive activity of OTA is characterized by depression of antibody responses (Lea *et al.*, 1989; Dwivedi and Burns, 1984), size reduction of immune organs, alteration in number and functions of immune cells and modulation of cytokine production (Al-Anati and Petzinger, 2006). This results from degenerative changes and cell death following necrosis and apoptosis in combination with slow replacement of immune cells due to inhibition of protein synthesis.

OTA suppresses antibody responses (Lea *et al.*, 1989; Dwivedi and Burns, 1984), and its administration in mice intra-peritoneally (Bondy and Pestka, 2000; Haubeck *et al.,* 1981) and orally at 0.25 or 2.6 mg OTA/kg diet for 28 days (Thuvander *et al.,* 1995) decreased serum antibody titres to sheep red blood cells (SRBC) whose effect was prevented by phenylalanine (Haubeck *et al.,* 1981). Intra-peritoneal administration of OTA at 3 or 6 mg/kg b.wt./day i.p. for 17 days also decreased serum antibody titres to *Pasteurella multocida* (Bondy and Pestka, 2000) and it was postulated that even lower concentrations than 0.005µg OTA /kg b.wt would have affected the immune system (Haubeck *et al.,* 1981).

OTA is also known to affect some aspects of CMI responses (Lea *et al.*, 1989). It inhibits the late stages of T cell activation such as IL-2-induced proliferation (Lea *et al.*, 1989) probably by inhibiting both DNA and protein synthesis (Bondy and Pestka, 2000). Daily oral daily exposure to OTA at 1.5 mg/kg b.wt. in mice for 17 weeks led to suppression of peritoneal macrophage function such as chemotactic activity, IL-1α and TNF-α production (Bondy and Pestka, 2000).

OTA induces dose-dependent immunomodulatory effects in animals (Muller *et al.*, 1999) which include lymphocytosis (Bondy and Pestka, 2000; Pier and McLoughlin, 1985), neutrophilic and monocytic leucopenia, and macrophage migration inhibition in mice (Pier and McLoughlin, 1985). In mice fed on 0.25 or 2.6 mg ochratoxin /kg diet for 28 days, lower thymic CD4+ and CD8+ cells and decreased thymus and spleen lymphocyte proliferative responses to Con A at 0.006, 0.25 or 2.6 mg ochratoxin /kg diets were observed (Thuvander *et al.,* 1995).

OTA also induces apoptosis of lymphocytes (Bondy and Pestka, 2000; Muller *et al.*, 1999) through a mitochondrial pathway leading to caspase activation (Assaf *et al.*, 2004). Assaf *et al.* (2004) also observed that OTA provokes a decrease of Bcl-x_L expression that may be a trigger for OTA-induced apoptosis. There is potential involvement of apoptosis in OTA-induced immunotoxicity (Bondy and Pestka, 2000).

Aflatoxins bind to DNA, suppress DNA-dependent RNA production and thus interfere with transcription (Reddy and Sharma, 1989; Clifford and Ress, 1966). Five key points on aflatoxin immunotoxicity have been identified. These are impairment of immunogenesis without suppressing antibody formation, suppression of formation of non-specific humoral substances namely complement (C4) and interferon, suppression of phagocytosis by macrophages, thymic aplasia and suppression of cell-mediated immunity, notably delayed cutaneous hypersentivity; lymphoblastogenesis and leucocyte migration (Pier and McLoughlin, 1985). The latter authors speculated that the effects on complement, interferon and serum proteins could be a result of liver injury and inhibition of protein synthesis. Aflatoxin-induced immunosuppression increases infectivity and hence the risk of secondary infection (Bondy and Pestka, 2000) of humans and animals. Daily oral administration of aflatoxin at 0.1 mg/kg b.wt. for 50 days activated infection in mice pre-infected with *Toxoplasma gondii* (Oswald *et al.*, 2005), resulted in ruptured cysts and brain lesions indicative of compromised immune function.

Aflatoxins inhibit complement formation, primarily C4; diminish serum proteins, primarily albumin, α and β globulins and at very high levels affect IgA and IgG but not IgM (Pier and McLoughlin, 1985). However, Turner *et al.* (2003) observed markedly lower secretory IgA and a weak antibody response to a pneumococcal challenge in children with detectable aflatoxin-albumin concentrations than in those with undetectable concentrations. Oral aflatoxin exposure caused reduced antibody titers to vaccines in poultry (Azzam and Gabal, 1998; Gabal and Azzam, 1998), cattle and pig (Williams *et al.*, 2004), and to bacterial diseases in rabbits (Williams *et al.*, 2004).

Cell-mediated immunity (CMI) and phagocytic cell functions (innate immunity) are more affected by aflatoxins than humoral immunity in most species (Bondy and Pestka, 2000). Impairment of cellular function by aflatoxin could be due to its effects on production of lymphokines and antigen processing by macrophages and decrease in or lack of heat-stable factors involved in phagocytosis (Williams *et al.*, 2004). Chronic exposure to aflatoxin B1 (AFB1) induces thymic aplasia, reduce T-lymphocyte function and number (Williams *et al.*, 2004) and a delayed cutaneous hypersensitivity reaction (Pier and McLoughlin, 1985). Raisuddin *et al.*(1993) observed that chronic aflatoxin B1 exposure mostly significantly inhibited delayed-type hypersensitivity and lymphoproliferative responses. Oral exposure at 60, 300 and 600 μg/kg b.wt AFB1 on alternate days in rats (Raisuddin *et al.*, 1993) and at 0.03-0.07 mg/kg b.wt. in BALB/c mice for 4 weeks (Reddy and Sharma, 1989) inhibited lymphoproliferative responses in rats and *in vitro* proliferation of suppressor cells in mice. In AFB1-treated mice, there have been reports of decrease in peripheral leucocyte counts (Reddy and Sharma, 1989) and splenic CD4 cell numbers after parenteral administration (Williams *et al.*, 2004). Aflatoxins inhibit phagocytosis in experimental animals (Pier and McLoughlin, 1985) with demonstrated suppression of alveolar macrophage phagocytosis at 16.8 μg/kg AFB1 b.wt. aerosol inhalation exposure in rats and suppression of alveolar and peritoneal macrophage phagocytosis in rats and mice (Jakab *et al.*,1994).

1.4 Problem statement and justification

The human population is continuously exposed to dietary mycotoxic contamination (Azziz-Baumgartner *et al.*, 2005; Smith and Moss, 1985). Kimathi and Siboe (1994) isolated various toxigenic mycoflora and a varying range of levels of aflatoxins, zearalenone and ochratoxin A residues in sifted maize meal packaged and sold countrywide in Kenya. The toxins detected by these authors are hazardous to animal and human health (Smith and Moss, 1985). Of special importance is that these mycotoxins are immunosuppressive (Haubeck *et al.*, 1981; Friend, *et al.*, 1985; Pier and McLoughlin, 1985) and their levels in maize flour are far much higher than the acceptable limit in food (FAO/WHO, 1987). They are also teratogenic and capable of blocking acquired immunity in the unborn (Pier and McLoughlin, 1985; Smith and Moss, 1985). Incidence of clinical and fatal aflatoxicosis has been reported in Eastern Kenya (Azziz-Baumgartner *et al.*, 2005), Murang'a (Peers and Linsell, 1977), Machakos (Ngindu *et al.*, 1982), Meru (Daily Nation, 2001) and Makueni (Daily Nation, 2005) districts of Kenya. The high human consumption of maize and other cereals in Kenya could lead to acute and chronic dietary exposure to mycotoxins. The purpose of the present study was to investigate the effect of mycotoxicosis on pathogenesis of human infective *T. b. rhodesiense* infection and response to chemotherapy.

1.5 Research questions

(i) What is the effect of mycotoxins on pathogenesis of *T. b. rhodesiense* infection in mice?

(ii) What is the effect of mycotoxins on *T. b. rhodesiense* response to chemotherapy in mice?

1.6 Hypothesis

Mycotoxins alter the pathogenesis and efficacy of chemotherapy of *T. b. rhodesiense* infection to chemotherapy.

1.7 Overall objective

To assess the effects of mycotoxins on susceptibility, disease progression and efficacy of suramin in mice infected with *T. b. rhodesiense*.

1.7.1 Specific objectives

To determine the effect of mycotoxins on:-

(i) Pathogenesis of *T. b. rhodesiense* infection in mice.

(ii) *T. b. rhodesiense* response to chemotherapy.

1.8 Significance and anticipated output

As an underlying condition that could enhance susceptibility to HAT, the concept of mycotoxicosis would be incorporated in disease control programs and could explain the endemicity and seasonality of the disease in Africa, a situation that has puzzled researchers for long. Enhancement of susceptibility to trypanosome infections by mycotoxins may possibly lead to emergence and re-emergence of other diseases. On the

other hand, effect of dietary aflatoxin B1 exposure may explain the rapid development of drug failure associated with trypanosomiasis in the tropics.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Experimental materials

2.1.1 Animals

Forty-two days old male inbred adult Swiss White mice obtained from the TRC-KARI colony were acclimatized for seven days before the experiment commenced. These mice were maintained on mice pellets (Unga Feeds) and water *ad libitum* at a temperature of 21°C, 45-55% relative humidity and on wood chipping as the bedding material (Gasbarre *et al.*, 1981; Seed and Sechelski, 1988).

2.1.2 Trypanosomes

A cryo-preserved *T. b. rhod*esiense clone, KETRI 3741, from KARI-TRC trypanosome bank was used to infect mice. This clone was prepared from KETRI 2537 stabilate isolated from a human host in Uganda in 1972 (Fink and Schmint, 1980).

2.1.3 Trypanocidal drug

Suramin sodium powder (Germanin®, Bayer 205) was used to treat *T. b. rhodesiense*-infected mice.

2.1.4 Mycotoxins

Analytical column purified extracts of aflatoxin B1, ochratoxin A separately constituted in vegetable oil (El-Arab *et al.*, 2006) at 62.5 µg/ml and 187.5µg/ml respectively and

62.5 µg/ml purified substrate extract in vegetable oil (placebo), were obtained from Bora Biotech Ltd, Cooper Centre, Nairobi, Kenya.

2.2 Experimental design

Four groups of mice were used in this study (Table 2.1). In group A, 36 mice were divided into three sub-groups of 12 individuals each fed on either aflatoxin B1, ochratoxin A or the placebo and then infected with *T. b. rhodesiense*. In group B had the same number of mice, sub-groups and mycotoxin or placebo treatment but was not infected with *T. b. rhodesiense*. Group C had two subgroups each of 6 mice that did not receive mycotoxin or placebo treatment; one was uninfected (clean mice) while the other was infected with *T. b. rhodesiense* (Table 2.1). Group D comprising of twelve sub-groups each of 6 mice were used to determine the effects of aflatoxin B1 on *T. b. rhodesiense*-infected mice response to chemotherapy. Six sub-groups were fed on aflatoxin B1, infected with *T. b. rhodesiense* on 7 day post-exposure and then received either one of six different dosages of suramin at the on-set of parasitaemia. Six control sub-groups on placebo treatment, received the same infection and suramin treatment. Parasitaemia was monitored in all these mice for 90 days after administration of the last dose of suramin (see section 2.3.4). In addition, the group D mice were also used for determination of pre-patent period.

Table 2.1: Groups of mice used in the study

Mice groups	Treatment of sub-groups of mice
Group A (three sub-groups each of 12 mice)	*T. b. rhodesiense*–infected and fed on aflatoxin B1
	T. b. rhodesiense–infected and fed on ochratoxin A
	T. b. rhodesiense–infected on placebo treatment
Group B (three sub-groups each of 12 mice)	Uninfected and fed on aflatoxin B1
	Uninfected and fed on ochratoxin A
	Uninfected placebo-treated mice
Group C (two sub-groups each of 6 mice)	*T. b. rhodesiense*-infected
	Uninfected mycotoxin-free control mice
Group D (Twelve sub-groups each of 6 mice)	*T. b. rhodesiense*–infected, fed on aflatoxin B1 and treated mice with suramin
	T. b. rhodesiense–infected placebo-treated mice on suramin treatment

2.3 Laboratory procedures

2.3.1 Determination of body weight

All mice were weighed once a week using an electronic balance (Mettler PM34, DoltaRange®) for the entire duration of experiment.

2.3.2 Mycotoxin administration in mice

For basic immunotoxicological endpoints, a "Repeated dose 28-day oral toxicity study in rodents", including weights of immune organs, blood cell counts, and histopathology of immune organs such as spleen and thymus as recommended by Bondy and Pestka (2000) was carried out. The appropriate daily mycotoxin dosages, based on body weight determined on weekly basis (El-Arab *et al.*, 2006), were administered orally through a gavage needle for 30 days. These daily dosages were 0.50 mg aflatoxin /kg body weight

and 1.5 mg ochratoxin / kg body weight. Control mice received equivalent volume of vegetable oil containing purified substrate extract, as placebo treatment.

2.3.3 Infecting mice with trypanosomes

The cryo-preserved trypanosome stabilates were first expanded in donor mice (irradiated at 600 rads for 6 minutes) before inoculation at 10^4 parasites per mouse (Gichuki and Brun, 1999) on day 7 post-commencement of mycotoxin administration. Both donor and experimental mice were infected by intraperitoneal (i.p.) administration with parasites suspended in phosphate saline glucose (PSG) buffer.

2.3.4 Preparation and administration of trypanocidal drug

Trypanocidal drug solutions were prepared daily. A stock suramin solution (1mg/ml) was prepared by dissolving 20mg 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. Each of these solutions was administered intra-peritoneally daily in mice at the on-set of parasitaemia on 4 consecutive days at 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5 mg suramin / kg b.wt.

2.3.5 Detection of aflatoxin B1 in biological samples

80 µl of blood was collected from experimental mice by tail snipping using a pair of sterile pair of scissors at 31 day post aflatoxin exposure into plain capillary tubes (Hirschmann®, 75mm/75 µl, D.A. 1,5-1,6 mm), and serum prepared as described by Boyt (1986). Serum was transferred to a clean capillary tube and stored at -20 °C until use.

Pre-treatment samples were appropriately collected. Serum samples were first diluted 6 times in 10% methanol in PBS for analysis by competitive aflatoxin B1-ELISA as described by Gathumbi *et al.* (2001). Optical densities (OD's) were measured at 450 nm using a multi-channel microplate reader (BDSL Immunoskan Plus, Finland) inter-faced to a personal computer (Compaq 5500, Korea). Average absorbance values for each standard and sample extract dilution (B) and that of reagent blank (B0) were used to calculate percentage inhibition (B/B0%) for each standard and sample dilution, and for construction of calibration curve (Standard concentration versus B/B0%) using GraphPad Inplot ™ (GraphPad Software Inc. Version 4.04). Calibration curve was then used to calculate concentrations of unknown samples. The limit of detection of the ELISA was set at 90 % inhibition corresponding to 15.6 parts per trillion (ppt) below which the aflatoxin B1 levels were recorded as 0 ppt.

2.3.6 Determination of parasitaemia

For each group of animals, blood was examined daily for circulating parasites from the 2nd day post-infection for the first 2 weeks, every other second day for the next 4 weeks and twice a week during the next 7 weeks. From a drop of blood (20µl) obtained by tail snip, wet blood films were prepared as described by Boyt (1986) and microscopically examined at X 400 magnification (Gichuki and Brun, 1999). The level of the parasitaemia was assessed by the matching technique of Herbert and Lumsden (1976) after modification as indicated in Table 2.2 (Mdachi, Pers. Comm.) below.

Table 2.2: Trypanosome scoring of wet smear examination

Number of trypanosomes	Score	Antilog	Estimated trypanosomes / ml blood
1 in 20 fields	(+)	5.4	2.5×10^5
Average of 1 per field	+	6.6	4.0×10^6
2-5 per field	(++)	7.05	1.1×10^7
6-20 per field	++	7.7	5.0×10^7
21-30 per field	(+++)	7.9	7.9×10^7
31-40 per field	+++	8.1	1.3×10^8
>40 per field	(++++)	8.5	3.2×10^8
Swarming (More than RBC)	++++	9.0	1.0×10^9

Levels of initial and second parasitaemic waves for each mouse were recorded as average number of trypanosomes per group of mice. Buffy coat examination (Boyt, 1986), was employed as a confirmatory procedure on day 90 after initiation of treatment for mice treated with trypanocides.

2.3.7 Determination of haematological values

Fifty (50) microlitre of blood was collected by tail snipping once a week in heparinised capillary tube and transferred to Eppendorf tubes using a 200-µl adjustable pipette (Gilson® pipetman, France). Sample were analysed using automated Beckman Coulter Counter(Coulter® A^C-T diff™) and a complete blood haemogram of each experimental mouse, giving haemoglobin levels (Hb), haematocrit, red cell count (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and total white cell count (WCC) generated. For differential cell counts (DCC), thin smears were prepared, fixed with absolute methanol and stained with Giemsa stain. The DCC were determined microscopically employing the battlement method described by Baker *et al.* (2001) and Bain and Bates (2001). Different leucocytes were microscopically identified and quantified and results expressed as %

(relative number) of the total for each type of cell. Absolute numbers of each cell type was calculated by multiplying relative number of each cell type by the WCC.

2.3.8 Pre-patent period and survival

Pre-patent periods, i.e. the time between infection and appearance of trypanosomes in blood, for each mouse was determined and recorded. Survival times for each animal i.e. time the animal took to succumb to disease in the absence of chemotherapy (Seed and Sechelski, 1988), were monitored for 67 days post-trypanosome infection. For animals surviving beyond this period, survival time was recorded as 67 days and categorized as censored data.

2.3.9 Pathological changes

In the course of experiment, clinical picture, gross pathology and histopathology of mice were determined. Clinical status of mice was determined as described by Gichuki and Brun (1999). The parameters monitored included consistency and colour of stool, hair appearance and any other clinical signs. From each experimental and clean mice group, 4 animals were randomly selected and sacrificed on the last day of mycotoxin exposure (30 day post-commencement of mycotoxin administration). Weights of liver and spleen were taken at euthanasia and these weights (liver and spleen): body weight ratios calculated and recorded. Vital organs such as the heart, spleen, liver, kidneys and lungs were harvested and processed for histopathological examination employing standard methods (Drury and Wallington, 1980). Briefly, tissues were fixed in 10% buffered formalin, processed for histology using an automatic tissue processor (Histokinette®), embedded in

molten wax, sectioned at 3 or 5μm thickness using a rotary microtome (Microm HM310, Germany). Sections were stained with Haematoxylin and Eosin, mounted in DPX and examined for lesions under the microscope.

2.3.10 Determination of cure rates and curative dosages (CD values)

The response variable was the number of mice cured out of 6. Cure was considered to be achieved if mice remained aparasitaemic throughout the observation period (Sones *et al.*, 1988) of 90 days after initiation of treatment (Mdachi, Per. Comm.). For each dose of suramin, proportion of mice cured was recorded as a percentage. Suramin curative dosages (CD_{50}, CD_{75} and CD_{90}) of the control and aflatoxin-fed mice were computed by substituting estimates of regression coefficients in a logistic linear regression model.

2.3.11 Detoxification of mycotoxic waste and safety precautions

All cages housing mycotoxin-exposed animals were specifically marked and people wore facemasks while handling these animals and their wastes. The glassware that came into contact with biological materials from these animals were rinsed with methanol, immersed in 1% NaOCl solution for 2 hours and rinsed with acetone. This was left to react for 30 minutes and washed thoroughly (Scott, 1995). Droppings and waste bedding material of mycotoxin-exposed animals were put in a labeled heavy duty plastic container, drenched in diesel and incinerated (Gathumbi, Pers. Comm.).

2.4 Methods of data analysis and statistical packages used

Data collected included serum aflatoxin B1 levels, clinical, haematological, pathological and cure rates. Means ± S.E. of peripheral aflatoxin B1 levels in aflatoxin-fed 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 individual variation determined from coefficient of variation (CV). Pre-patent period, levels of second parasitaemic wave and tissue weight data were subjected to analysis of variance and mean separation using SAS and StatView statistical packages. Skewed tissue weight and haematological data (WCC, haematocrit, RCC, haemoglobin levels and MCH) were first transformed (square root +1) before further statistical analysis. These data were then subjected to a repeated measures analysis (multivariate method), analysis of variance and mean separation carried out using SAS. The means ± S.E. were determined by least square procedure employing a General Linear Model that uses a completely randomized design, $Yij = \mu + Ti + \varepsilon ij$, where Yij=Repeated response (Week 1, 2…, 6), μ =Overall mean, Ti=Treatment groups and εij=Random error.

Survival data was analysed employing the Kaplan-Meier method that uses 'proc lifetest procedure' on SAS statistical software for determination and separation of survival function estimates and plots of the treatment groups. Test of equality over the treatment groups comprising of rank tests of homogeneity (Log-Rank and Wilcoxon tests) were used to determine effects of treatments on early and larger survival times respectively (Everitt and Der, 1998). Since cure rate data had a binomial distribution, B(n,p), where, n=number of mice and p=probability of cure, a logistic linear regression model, log

$\{p/(1-p\} = ß_0 + ß_1$ rate + $ß_2$ $drug_j$ + ß3(rateXdrug interaction) + ε_i , where $ß_i$ are 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, suramin dosage X aflatoxin B1 interaction was determined.

CHAPTER THREE

RESULTS

3.1 Levels of aflatoxin B1 in mice

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

3.2 Effects of aflatoxin B1 and ochratoxin A on pathogenesis of *T. b. rhodesiense* infection in mice

3.2.1 Pre-patent period

The pre-patent period, expressed as mean days, of *T. b. rhodesiense*-infected aflatoxin-fed mice was 3.60 ± 0.1 days which was significantly ($p<0.05$) longer than that of controls (3.26 ± 0.1 days). For *T. b. rhodesiense*-infected ochratoxin-fed mice, pre-patent period was 2.58 ± 0.2 days which was significantly ($p<0.05$) shorter than that of controls (3.26 ± 0.1 days).

3.2.2 Parasitaemia patterns

The daily mean number of trypanosomes in blood in the control and *T. b. rhodesiense*-infected mycotoxin-fed mice is shown in Fig. 3.1. Although no significant ($p>0.05$) statistical difference in levels of the second parasitaemic waves between the groups was observed, aflatoxin-fed and ochratoxin-fed mice showed higher second parasitaemic waves than controls. Parasitaemia was characterized by two prominent parasitaemic waves; an initial wave observed during the first week of infection followed by a smaller

wave which remained high until death. In the controls, initial wave occurred 2 to 9 days post-infection with a peak at day 6. The second wave was observed 9 days post-infection with a peak at 20 days resulting in death. On the other hand, ochratoxin-fed mice showed an initial wave 2 to 7 days post-infection with a peak at 6 days, and a second smaller one 10 to 24 days post-infection with a peak at 20 days. The aflatoxin-fed group had the initial wave between 2 to 9 days post-infection with peak at 6 days post-infection, with a smaller second wave 10 to 31 days post-infection with a peak at 24 days (Fig. 3.1). Since very few mice survived beyond 34 days post-infection, graphical presentation of these data was misleading beyond this point.

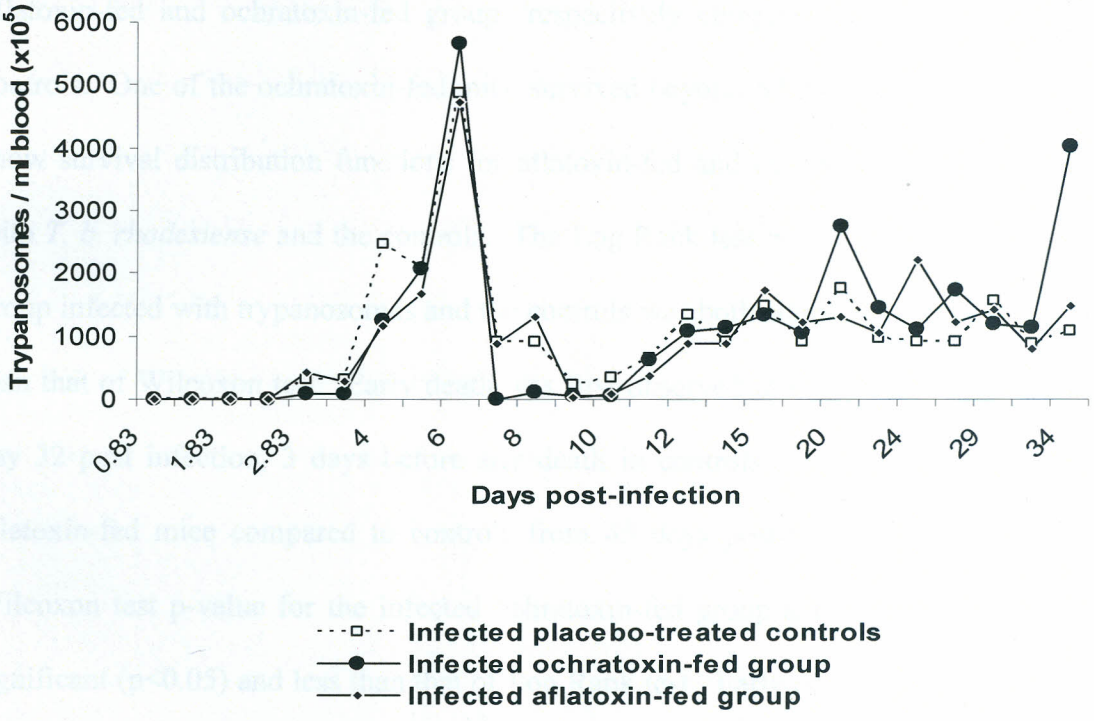

Fig. 3.1: Mean parasitaemia of control and *T. b. rhodesiense*-infected mycotoxin-fed mice

3.2.3 Clinical signs associated with mycotoxins

Clinical signs of all *T. b. rhodesiense*-infected mice included lethargy, dyspnoea, raised hair coat, facial and scrotal oedema. However, these signs were more pronounced in both the mycotoxin-fed mice than in controls, especially dyspnoea in the ochratoxin-fed mice and emaciation, raised hair coat and eyelid oedema in the aflatoxin-fed group. These signs became more severe from 24 days post-infection.

3.2.4 Effect of mycotoxins on survival of mice

Survival period for *T. b. rhodesiense*-infected mice was 30-48 and 6-47 days for aflatoxin-fed and ochratoxin-fed groups respectively compared to 33-65 days for the controls. One of the ochratoxin-fed mice survived beyond 67 days. Figures 3.2 and 3.3 show survival distribution functions for aflatoxin-fed and ochratoxin-fed mice infected with *T. b. rhodesiense* and the controls. The Log Rank test p-value for the aflatoxin-fed group infected with trypanosomes and the controls was both significant ($p<0.05$) and less than that of Wilcoxon test. Early death was first observed in the aflatoxin-fed group on day 32 post infection, 3 days before any death in controls. Death rate was higher in aflatoxin-fed mice compared to controls from 45 days post-infection (Fig. 3.2). The Wilcoxon test p-value for the infected ochratoxin-fed group and the controls was both significant ($p<0.05$) and less than that of Log Rank test. Early death was observed in the ochratoxin-fed group on day 8 compared to day 33 in controls (Fig. 3.3). The rank tests of homogeneity indicated significant ($p<0.05$) reduction of survival time in both trypanosome-infected mycotoxin-fed groups compared to controls.

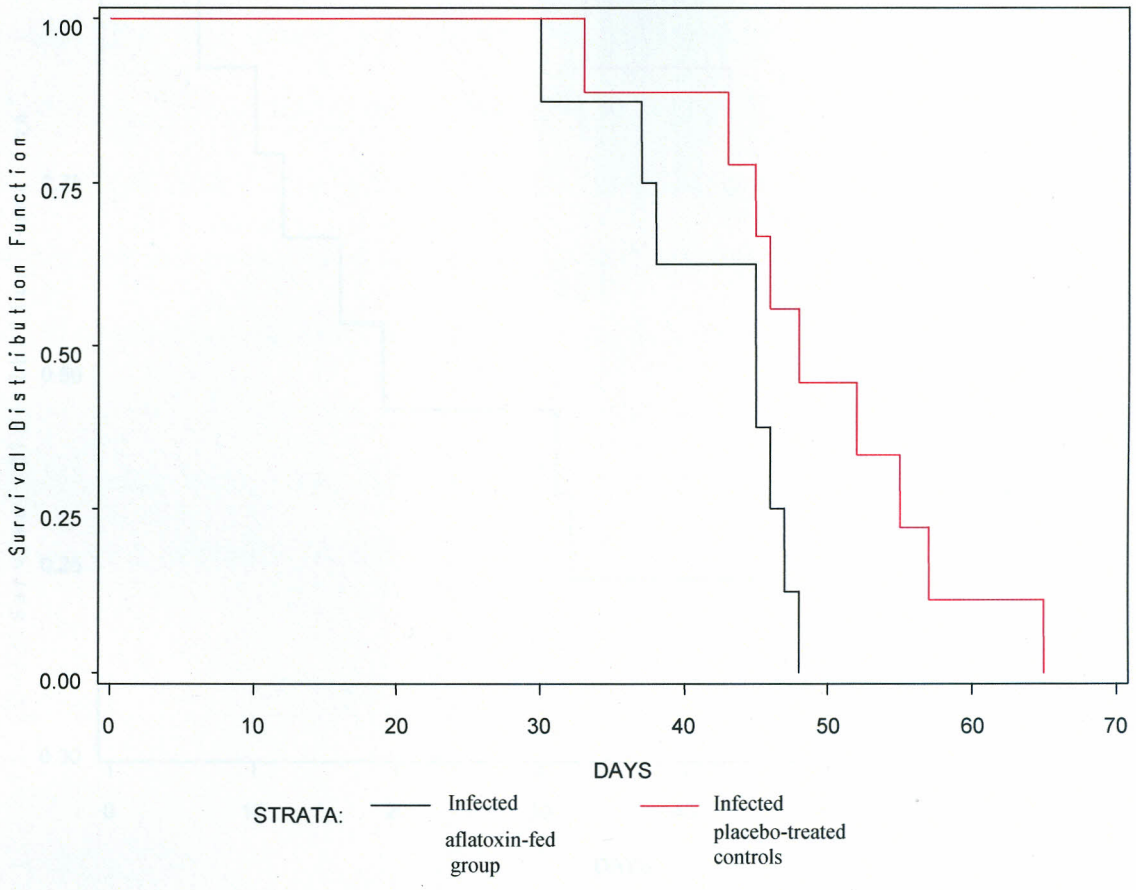

Fig. 3.2. Survival estimates plot for *T. b. rhodesiense*-infected aflatoxin-fed and *T. b. rhodesiense*-infected placebo-treated control mice. The p-values associated with tests of equality were Log-Rank= 0.037, Wilcoxon=0.095 and –2Log (LR) =0.74, indicating that the two groups differed primarily at larger survival times with significantly ($p<0.05$) shorter larger survival time in the aflatoxin-fed group compared to the controls.

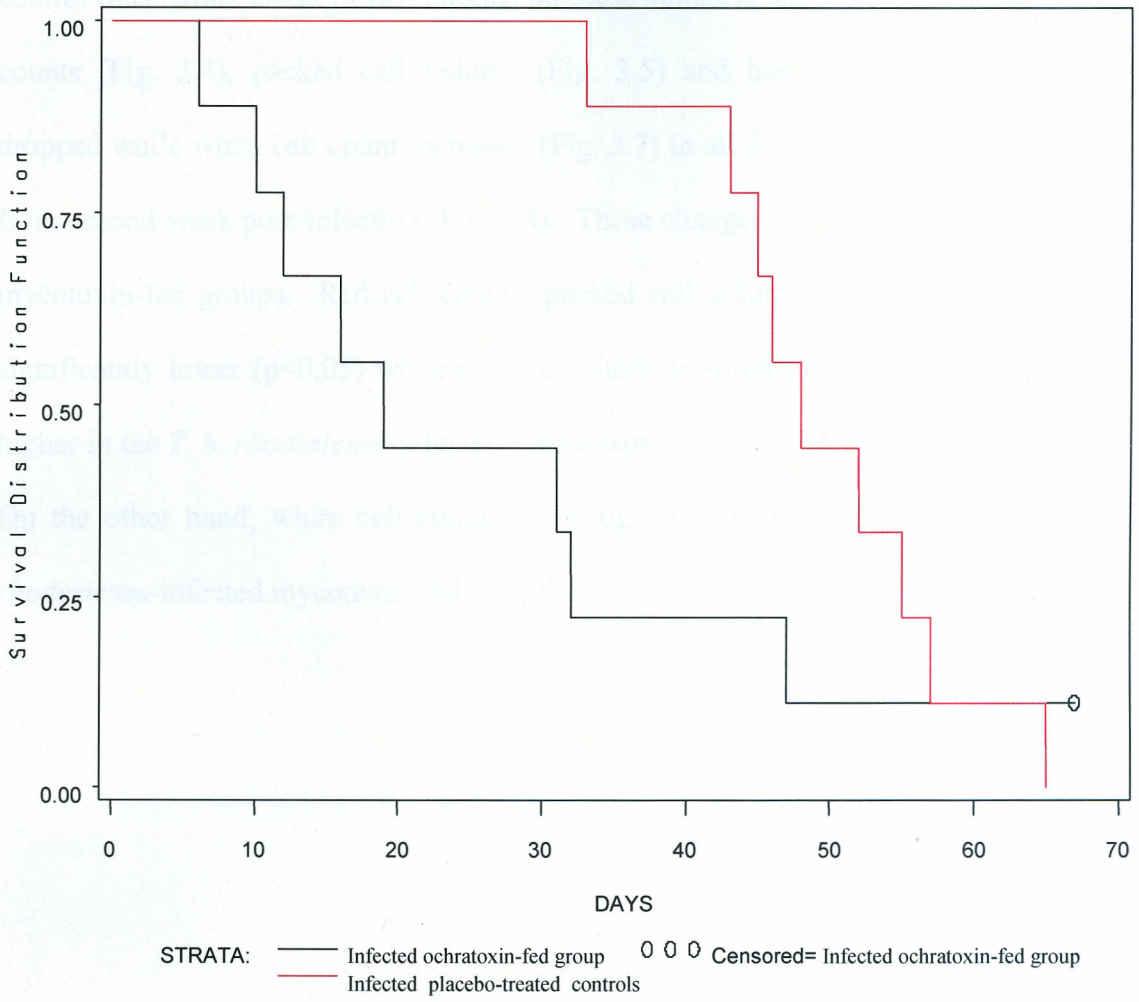

Fig. 3.3. Survival estimates plot for *T. b. rhodesiense*-infected ochratoxin-fed and *T. b. rhodesiense*-infected placebo-treated control mice. The p-values associated with tests of equality were Log-Rank= 0.15, Wilcoxon=0.01 and –2Log (LR) =0.31 indicating that the two groups differed primarily at early survival times which was significantly shorter ($p<0.05$) in the ochratoxin-fed group than in the controls.

3.2.5 Effect of mycotoxins on haematological values

There were significant ($p<0.05$) differences between groups in the white and red cell counts, haemoglobin levels, packed cell volume, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration. Figures 3.4 –3.7 show mean haeamatological values of trypanosome-infected mycotoxin-fed and

control mice while effect of mycotoxins on these values is shown in Table 3.1. Red cell counts (Fig. 3.4), packed cell volume (Fig. 3.5) and haemoglobin levels (Fig. 3.6) dropped while white cell count increased (Fig. 3.7) in all *T. b. rhodesiense*-infected mice from second week post-infection (Fig. 3.4). These changes were more pronounced in the mycotoxin-fed groups. Red cell counts, packed cell volume, haemoglobin levels were significantly ($p<0.05$) while mean corpuscular volume was significantly ($p<0.05$) higher in the *T. b. rhodesiense*-infected mycotoxin-fed mice than the controls (Table 3.1). On the other hand, white cell counts were significantly ($p<0.05$) higher in the *T. b. rhodesiense*-infected mycotoxin-fed mice than in controls (Table 3.1).

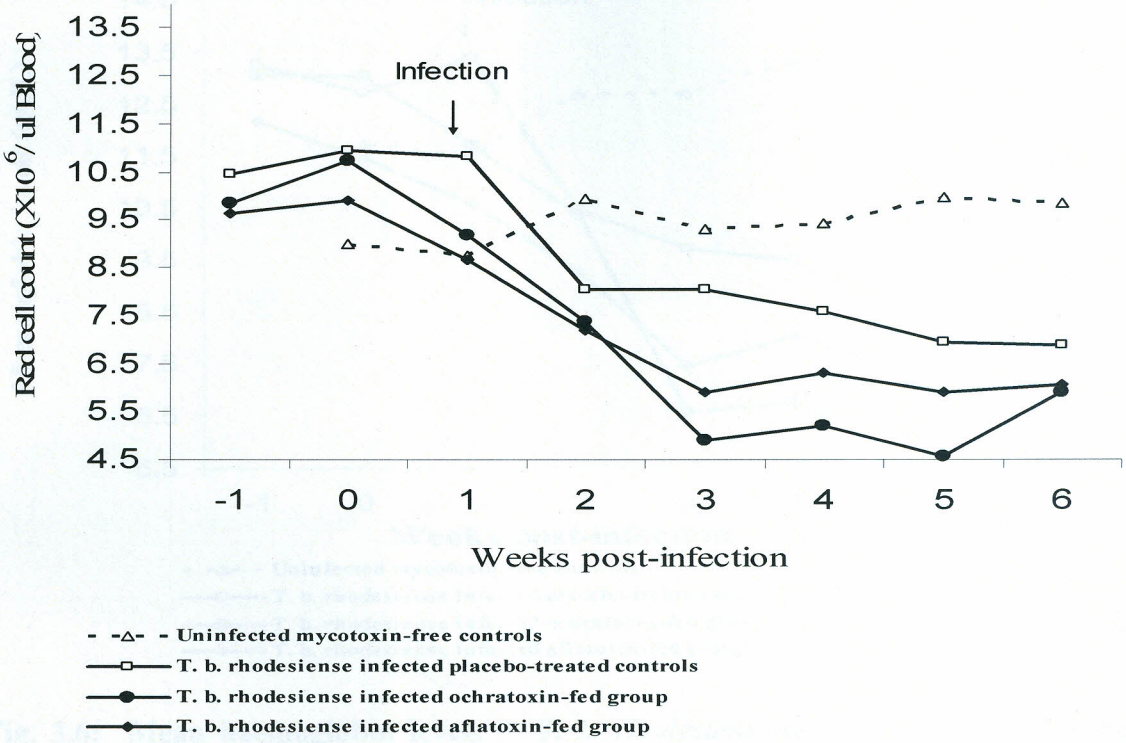

Fig. 3.4: Mean red cell counts in *T. b. rhodesiense*-infected mice at different time intervals

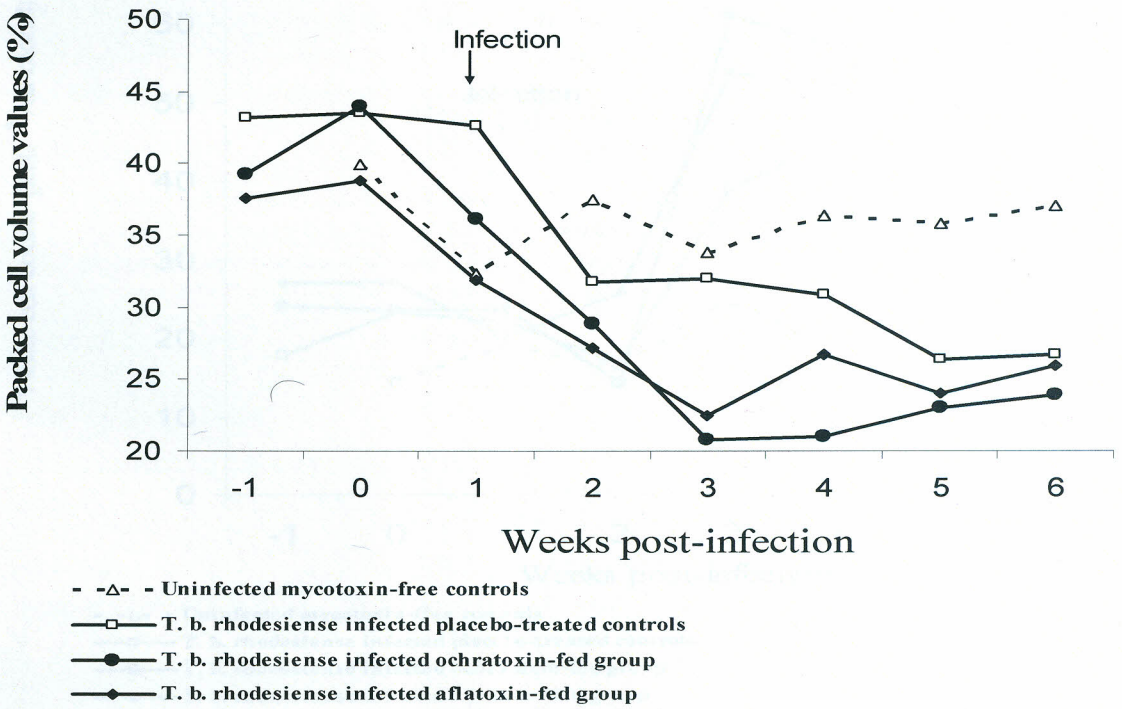

Fig. 3.5: Mean packed cell volume in *T. b. rhodesiense*-infected mice at different time intervals

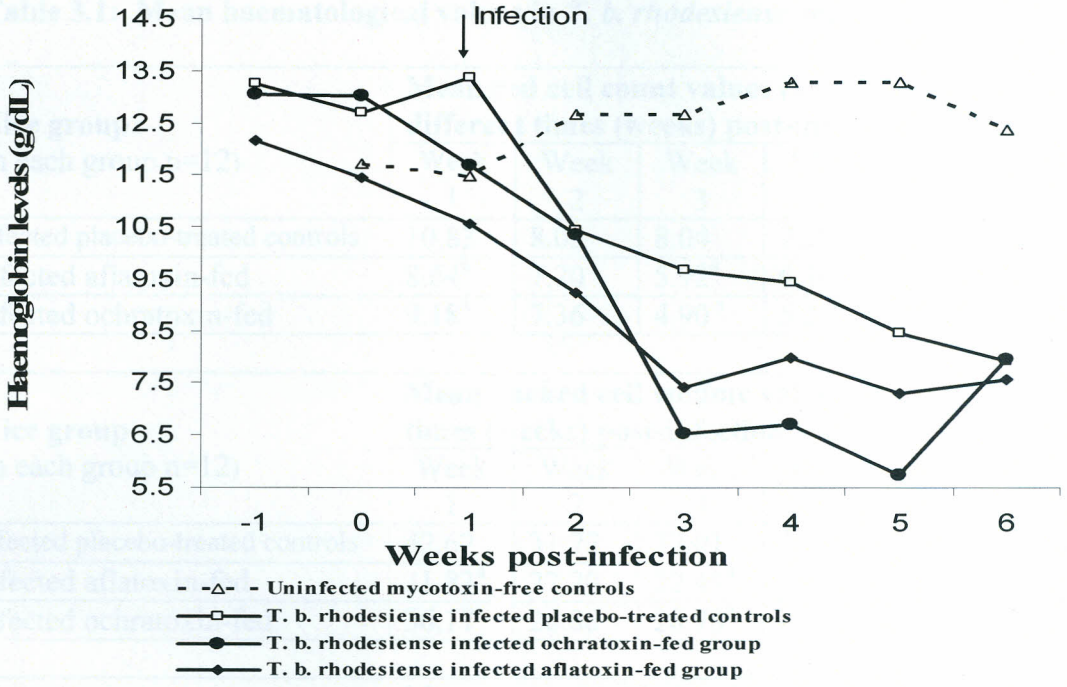

Fig. 3.6: Mean haemoglobin levels in *T. b. rhodesiense*-infected mice at different time intervals

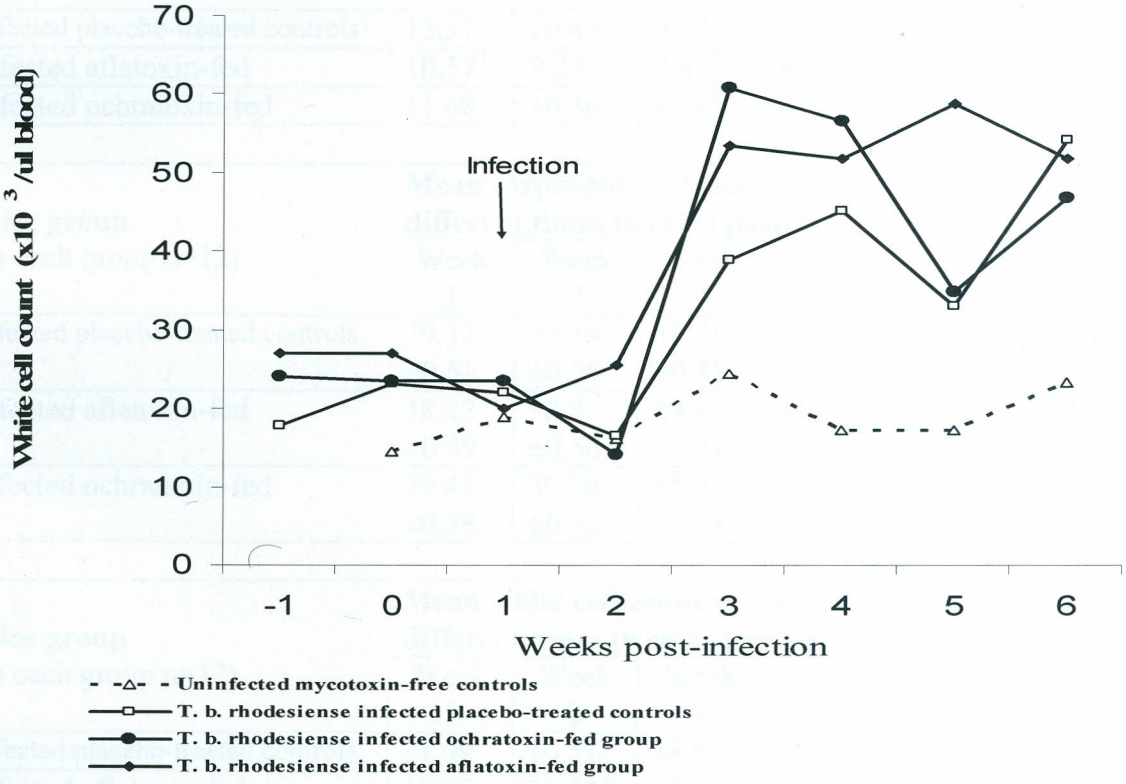

Fig. 3.7: Mean white cell counts in *T. b. rhodesiense*-infected mice at different time intervals

Table 3.1: Mean haematological values in *T. b. rhodesiense*-infected mice

Mice group (In each group n=12)	**Mean red cell count values (x10^6 cells / µl blood) at different times (weeks) post-infection**					
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Infected placebo-treated controls	10.83	8.05	8.04	7.59	6.94	6.87
Infected aflatoxin-fed	8.64[a]	7.20	5.92[a]	6.30 [a]	5.90	6.05
Infected ochratoxin-fed	9.18[a]	7.36	4.90 [a]	5.20 [a]	4.56[a]	5.90
Mice group (In each group n=12)	**Mean packed cell volume values (%) at different times (weeks) post-infection**					
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Infected placebo-treated controls	42.62	31.72	32.01	30.83	26.36	26.74
Infected aflatoxin-fed	31.83[a]	27.20	22.49[a]	26.75[a]	24.07	25.88
Infected ochratoxin-fed	36.17	28.80	20.78[a]	20.96[a]	23.03	23.95
Mice group (In each group n=12)	**Mean haemoglobin levels (g/ dL) at different times (weeks) post-infection**					
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Infected placebo-treated controls	13.37	10.45	9.67	9.43	8.47	7.92
Infected aflatoxin-fed	10.57[a]	9.22	7.41[a]	8.00[a]	7.32	7.56
Infected ochratoxin-fed	11.68	10.36	6.54[a]	6.72[a]	5.74[a]	7.97
Mice group (In each group n=12)	**Mean corpuscular volume values (fL) values at different times (weeks) post-infection**					
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Infected placebo-treated controls	39.32 ±0.51	39.39 ±0.56	39.71 ±0.83	40.63 ±0.76	37.96 ±0.66	38.73 ±0.96
Infected aflatoxin-fed	38.82 ±0.49	38.7 ±0.56	39.85 ±0.71	41.39 ±0.76	39.98[a] ±0.66	41.64 [a] ±1.02
Infected ochratoxin-fed	39.41 ±0.58	39.10 ±0.62	43.97[a] ±0.78	41.73 ±0.10	43.66[a] ±0.88	41.13 ±1.56
Mice group (In each group n=12)	**Mean white cell count values (x10^3 cells /µl blood) at different times (weeks) post-infection**					
	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
Infected placebo-treated controls	21.99	16.51	38.94	45.24	33.09	54.11
Infected aflatoxin-fed	19.97	25.50 [a]	53.46	51.80	58.88[a]	51.79 [a]
Infected ochratoxin-fed	23.43	14.23	60.88[a]	56.52	34.84	46.82

[a] *=Significant difference between infected mycotoxin-fed group and infected placebo-treated controls*

3.2.6 Gross and histopathological changes

Table 3.2 shows differences in size of liver and spleen of trypanosome-infected and uninfected mice fed on aflatoxin B1 and ochratoxin A. There were significant differences in spleen sizes between the infected aflatoxin-fed and placebo-treated groups, and the uninfected controls. Infected ochratoxin-fed mice had significantly ($p<0.05$) smaller spleen sizes compared to placebo-treated controls.

T. b. rhodesiense-infected control mice showed hepatosplenomegaly, emaciation, increased peritoneal fluid, liver capsular haemorrhages, enlarged adrenal glands, congested heart, cerebral oedema and hydrothorax. There was perivascular inflammatory cellular infiltration in the heart, liver and kidneys. Kidneys showed interstitial haemorrhages, congestion, fatty and degeneration and necrosis of tubular cells. Similar but more severe lesions were observed in the mycotoxin-fed groups that were infected with trypanosomes. The *T. b. rhodesiense*-infected ochratoxin-fed mice showed mild hepatosplenomegaly, hydrothorax, hydropericardium, congested liver and pale kidney with white pin-point areas. Compared with uninfected ochratoxin-fed controls or infected non-mycotoxin-fed controls, histological changes in infected ochratoxin-fed mice were more severe and were characterized by congestion, haemorrhages, fatty changes and infiltration with inflammatory cells (lymphocytes and eosinophils) especially around blood vessels of liver (Fig. 3.8B) and kidney (Fig. 3.9B). There was also hepatic haemosiderosis and degeneration of hepatocytes. Degeneration of tubular cells, collapsed tubules, pinkish exudate within the tubules and interstitial oedema were

also observed. In the heart, there was endocarditis, pericarditis, perivascular cuffing, necrosis and fibrosis.

Aflatoxin-fed mice presented hepatosplenomegaly (Table 3.2) with white necrotic patches on the spleen and petechial haemorrhages on the liver surface, congested heart and hydrothorax. More severe histopathological lesions were observed in the infected aflatoxin-fed mice compared to uninfected aflatoxin-fed or infected non-mycotoxin-fed controls. The liver was the most affected organ and was characterized by mononuclear cell infiltration especially perivascularly, embolism, depopulation of hepatocytes (Fig. 3.10B), fatty hepatocytes, hepatic necrosis, fibrosis and bile duct hyperplasia. Haemorrhages and thrombosis were evident in mice dying early (before 21 day post-infection). Lesions in the kidneys included massive focal perivascular infiltration mainly by mononuclear cells (Fig. 3.11B). In some areas, infiltration was several cells thick and extended between two vessels forming nodules of inflammatory cells. There was pinkish exudate within tubules (Fig. 3.11B), dilatation of Bowman's space and degeneration and necrosis of renal tubular epithelium. The heart had pancarditis characterized by infiltration by lymphocytes, fatty degeneration, embolism and necrosis. There was also fibrosis of the myocardium.

Uninfected mycotoxin-fed control groups had less severe pathological lesions than trypanosome-infected mycotoxin-fed groups. Aflatoxin-fed controls had mild ascites, congestion of the pericardium and petechial and ecchymotic haemorrhages on the kidneys capsule. The liver had severe fatty degeneration, severe necrosis, loss of normal

architecture and fibrosis while kidneys had haemorrhage, diffuse infiltration by inflammatory lymphocytes, congestion (Fig.3.11A), degeneration and necrosis of tubular epithelium. In the ochratoxin-fed controls, pathological features were liver jaundice, ulcerative gastritis and pale kidney with petechiae haemorrhage. The liver had scanty inflammatory cell infiltrations, congestion, interstitial oedema, minimal hepatocellular fatty degeneration and coagulative necrosis (Fig.3.8A). Kidneys had severe haemorrhages, congestion, renal tubular degeneration, necrosis and casts within tubular lumen and scanty inflammatory cell infiltrations (Fig.3.9A).

Table 3.2: Mean weights of liver and spleen in *T. b. rhodesiense*-infected and uninfected mice

Mice group	Mean tissue weight: body weight ratio (g/kg)	
	Spleen	Liver
Uninfected non-mycotoxin-fed (clean) (n=4)	0.007	0.046
Infected non-mycotoxin-fed (n=2)	0.046	0.064
Uninfected placebo-treated controls (n=3)	0.016	0.067
Infected placebo-treated controls (n=3)	0.070 *	0.085
Uninfected aflatoxin-fed (n= 3)	0.017	0.069
Infected aflatoxin-fed (n=3)	0.068 *	0.080
Uninfected ochratoxin-fed (n=2)	0.024	0.057
Infected ochratoxin-fed (n=2)	0.029[a]	0.076

Key: Significant difference in tissue sizes, between *T. b. rhodesiense*-infected mycotoxin-fed mice and *T. b. rhodesiense*-infected placebo-treated controls is denoted by [a], whereas that between *T. b. rhodesiense*-infected aflatoxin-fed/placebo-treated control groups and uninfected aflatoxin-fed or placebo-treated groups respectively is denoted by *.

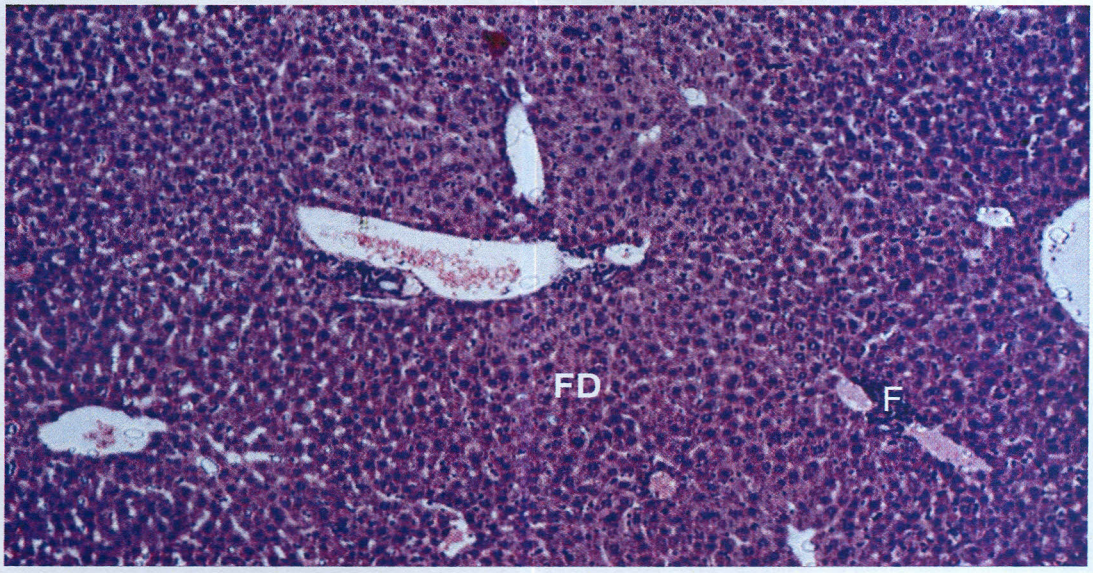

Fig. 3.8A

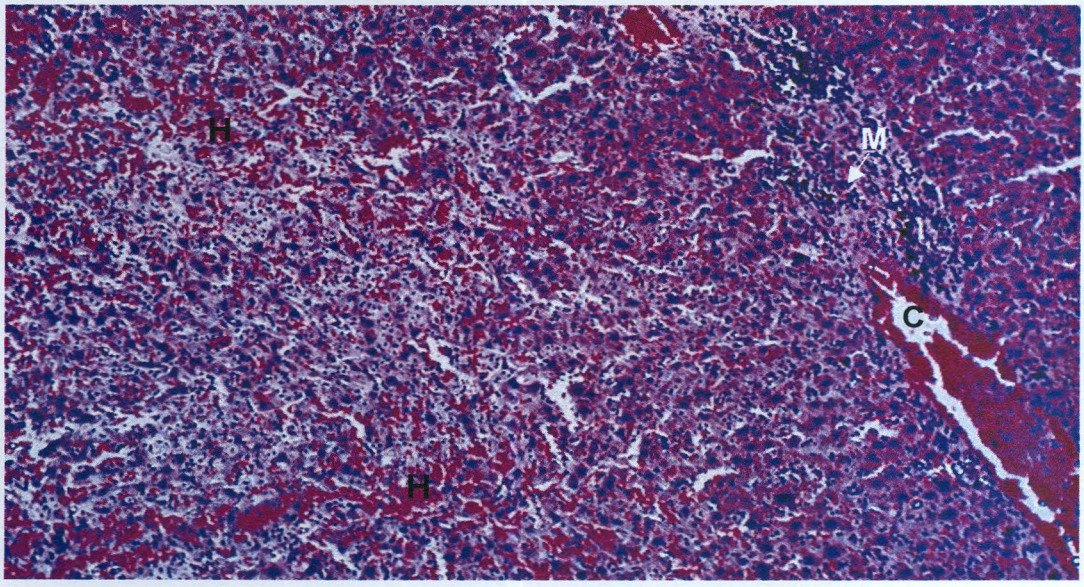

Fig. 3.8B

The liver of uninfected ochratoxin-fed control mouse at day 30 post-ochratoxin exposure (Fig 3.8A) had hepatocellular fatty degeneration (FD) and scanty inflammatory cells (F). There were more severe lesions in the liver of *T. b. rhodesiense*-infected ochratoxin-fed mouse at 47 days post-infection (Fig. 3.8B) characterized by severe haemorrhages (H), congested vessel (C) and massive infiltration with mononuclear cells (M) (Haematoxylin and Eosin x131).

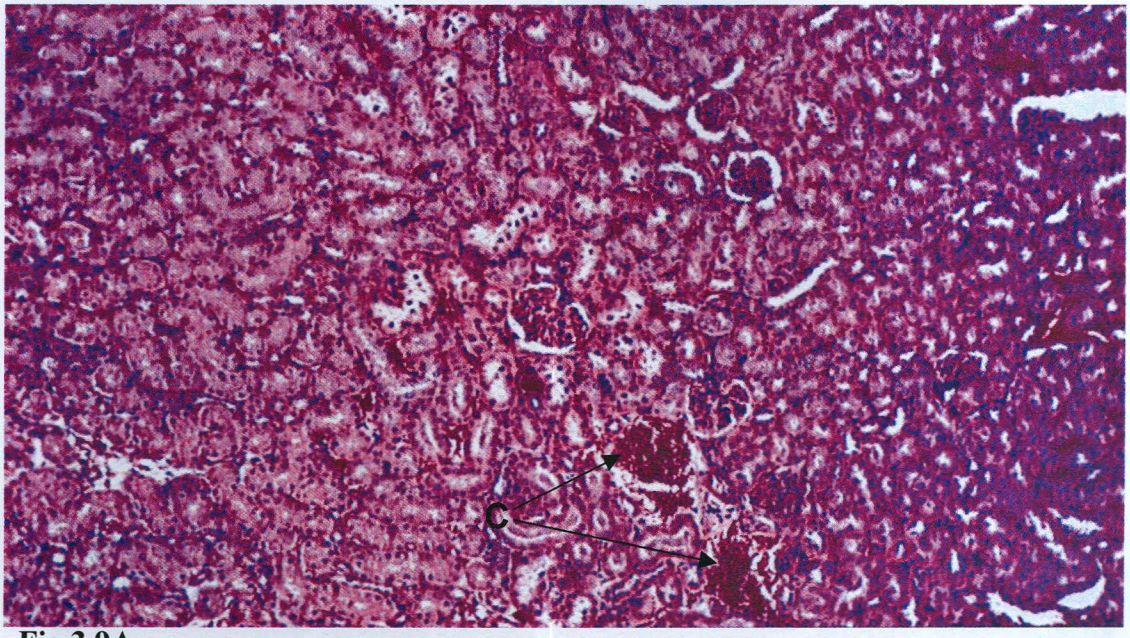

Fig 3.9A

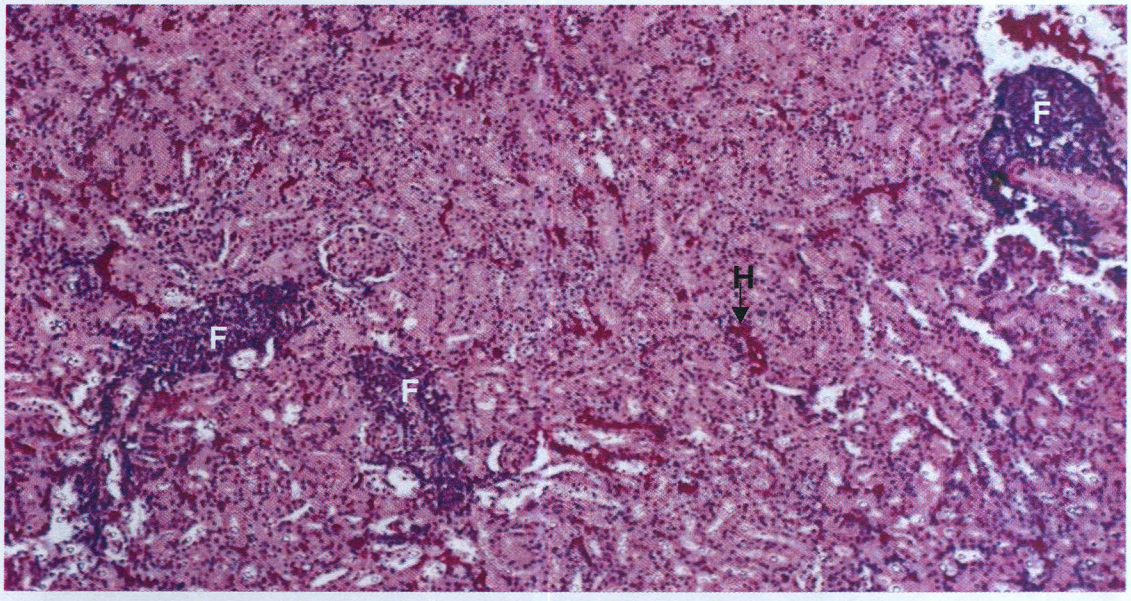

Fig 3.9B

The kidney of uninfected ochratoxin-fed control mouse at day 30 post-ochratoxin exposure (Fig 3.9A) showing congestion (C) and few inflammatory cells. Kidneys of *T. b. rhodesiense*-infected ochratoxin-fed mouse at 47 days post infection (Fig. 3.9B) had massive perivascular inflammatory cell infiltration (F) and haemorrhages (H) and pinkish exudate in renal tubules (Haematoxylin and Eosin x131).

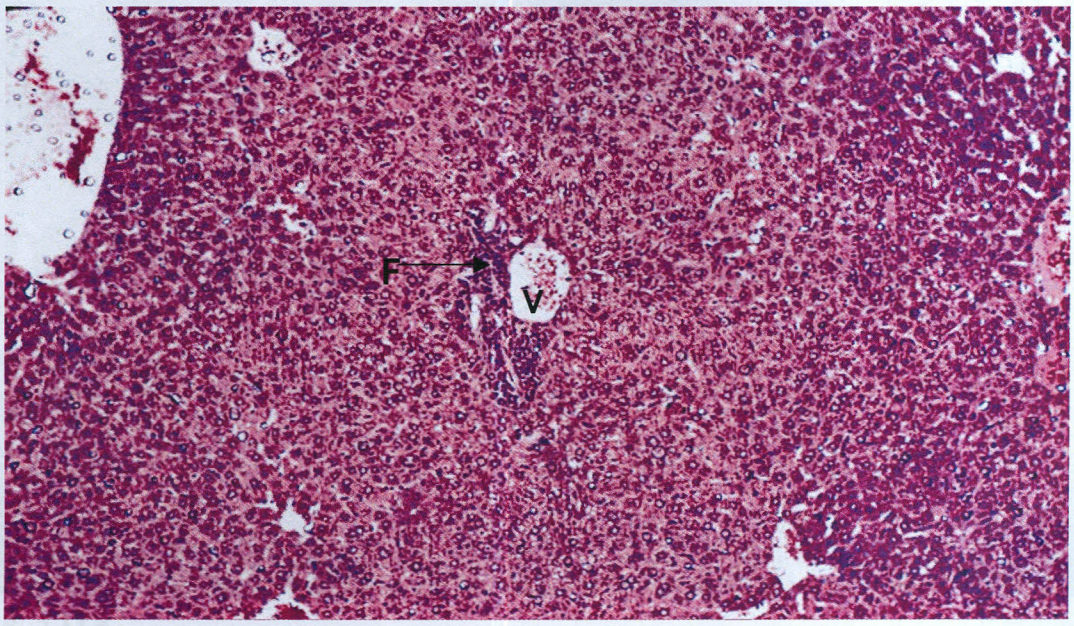

Fig. 3.10A

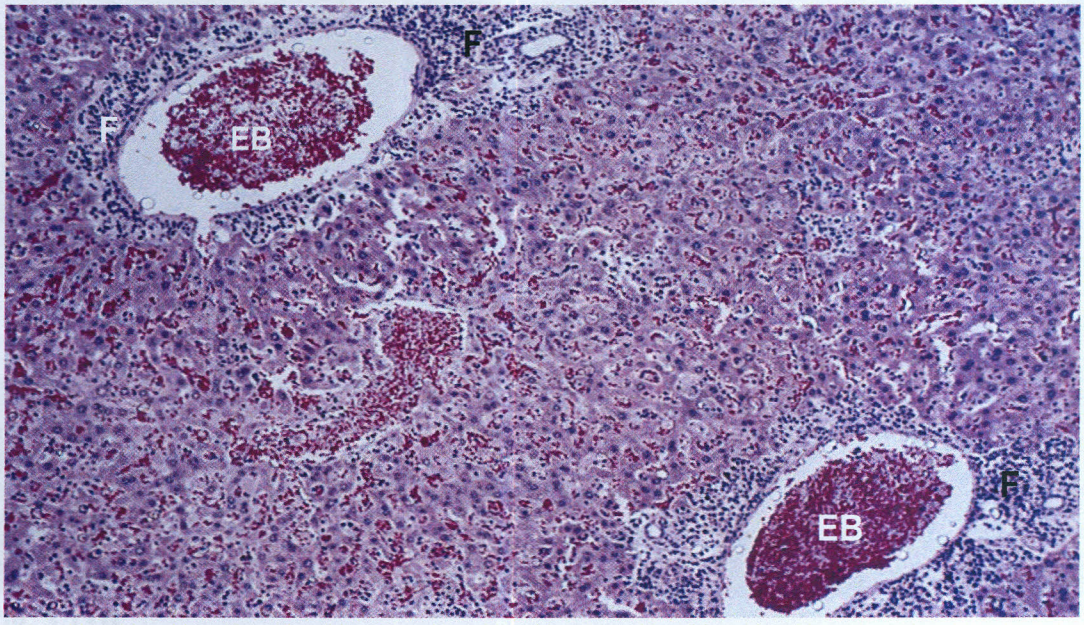

Fig. 3.10B

Liver of *T. b. rhodesiense*-infected non-mycotoxin-fed control mouse at 23 day post-infection (Fig. 3.10A) showing fatty degeneration of hepatocytes, central vein (V) and perivascular infiltration by inflammatory cells (F). Fig. 3.10B shows a more severely affected liver of *T. b. rhodesiense*-infected aflatoxin-fed mouse at 35 day post-infection with perivascular infiltration by inflammatory cells (F), embolism (EB) and depopulation of hepatocytes (Haematoxylin and Eosin x131)

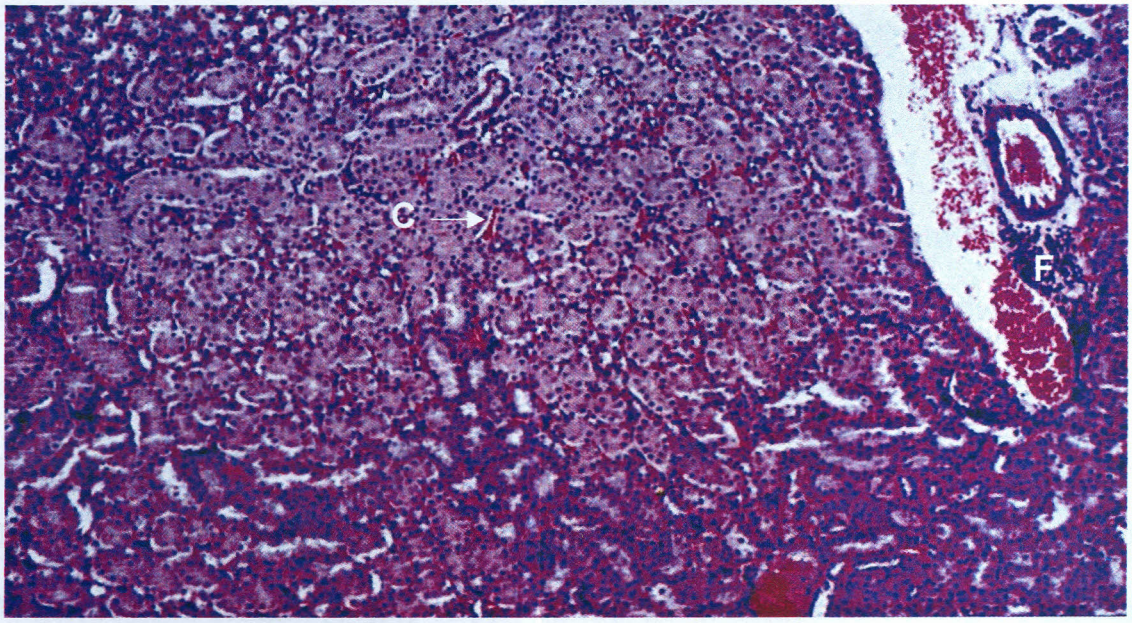

Fig. 3.11A

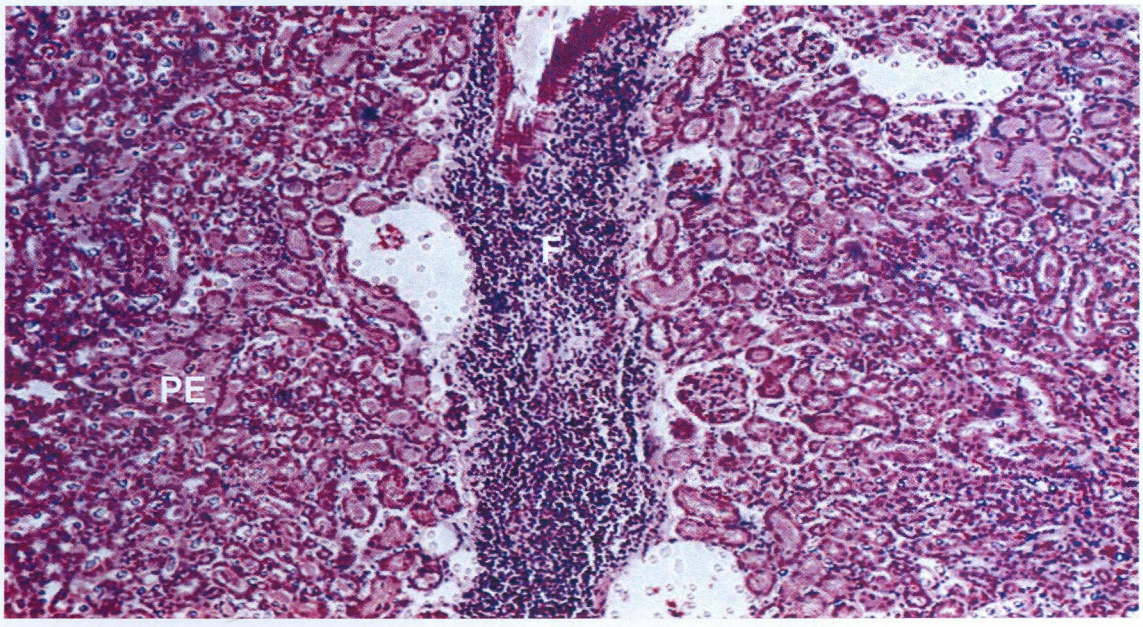

Fig. 3.11B

Kidney of uninfected aflatoxin-fed control mouse at day 30 post-aflatoxin B1 exposure showing congestion (C) and diffuse infiltration by inflammatory cells (F) (Fig. 3.11A). Kidney of *T. b. rhodesiense*-infected aflatoxin-fed mouse at 48 day post-infection (Fig. 3.11B) had more severe lesions characterized by pinkish exudate (PE) in a renal tubules, focal massive perivascular cellular infiltration (F), degeneration and necrosis of tubular epithelium (Haematoxylin and Eosin x131).

3.3 Effects of aflatoxin B1 on efficacy of suramin

Table 3.3 shows curative dosage (CD) values of suramin in *T. b. rhodesiense* infected aflatoxin B1-fed mice and placebo-treated controls while Table 3.4 shows values of logistic linear regression model for suramin efficacy in treatment of the two groups. The CD values of suramin were derived from regression coefficients for both the control and aflatoxin-fed groups as follows: (1) For placebo-treated controls, Logit (p)= Log {p/(1-p}=-5.03 + 1.1178Dosage. Hence, suramin dosage= [Log {p/(1-p} + 5.03]/ 1.178. (2) For the aflatoxin-fed mice, Logit (p)= Log {p/(1-p}=-5.03 + 1.1178Dosage- 0.58 + 0.238 Hence, suramin dosage= [Log {p/(1-p} + 5.372]/ 1.178. The curative dosages of suramin were consistently higher in aflatoxin-fed group compared to controls, but interaction between aflatoxin and suramin dosages was not significantly demonstrated.

Table 3.3: Regression coefficients of suramin efficacy in treatment of *T. b. rhodesiense*-infection in aflatoxin-fed mice and placebo-treated control mice

	Estimate	S.E.	t (*)
Constant (Overall mean)	-5.03	2.72	-1.85
Dosage	1.178	0.55	2.14 >1.86 (p<0.01)
Aflatoxin B1	-0.58	4.19	-0.14
Dosage.aflatoxin B1	0.238	0.866	0.28

Table 3.4: Curative dosages of suramin in control and *T. b. rhodesiense*-infected aflatoxin-fed mice

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

CHAPTER FOUR

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 DISCUSSION

This study has shown that mycotoxicosis alters pathogenesis of *T. b. rhodesiense* infection in mice. These included alteration of pre-patent period, aggravation of pyrexia, dyspnoea, emaciation, oedema, ascites, hydrothorax, elevation of total white cell counts, and pronounced anaemia with histopathological lesions suggestive of hypercoagulative state and vasculopathy. In addition mycotoxins significantly reduced host survival. Other exacerbated histopathological lesions included severe vacuolization, degeneration, necrosis and fibrosis of cells, and massive multi-organ peri-vascular cellular infiltrates. In addition, aflatoxin B1 induced a consistent increase in curative dosages of suramin.

Significant extension of pre-patent period in the *T. b. rhodesiense*-infected mice by aflatoxin B1 in the present study agrees with findings obtained by other scientists working with protozoan parasites. Aflatoxicosis decreased morbidity and prolonged survival time in mouse-*Plasmodium berghei* model (Young *et al.,* 1988; Hendrickse *et al*., 1986), and reduced *P. falciparum* parasitaemia in children (Hendrickse *et al*., 1986). The results in this study suggest a transient protection of the host by aflatoxicosis from *T. b. rhodesiense* infection. On the other hand, ochratoxin A rendered the host more susceptible to *T. b. rhodesiense* infection by significantly reducing the pre-patent period and producing a more virulent infection. This agrees with findings of Sacks *et al.* (1980) who found a correlation between pre-patent period and virulence of trypanosomes and that the virulence correlates with parasite intrinsic immunosuppressive activities

especially depression of IgM responses. The shortened pre-patent period by ochratoxin in the present study may be attributed to immunosuppressive effects of ochratoxin A leading to increased host susceptibility. Antibody response, an important defence mechanism against trypanosome invasion (Lejon *et al*., 2003; Njiru *et al*, 2000), has been shown to be depressed by ochratoxin A in various animals (Al-Anati and Petzinger, 2006; Bondy and Pestka, 2000; Dwivedi and Burns, 1984). Primarily, IgM production (Bondy and Pestka, 2000; Dwivedi and Burns, 1984) is reduced by blocking protein synthesis through inhibition of phenylalanyl t-RNA synthetase (Pier and McLoughlin, 1985). These results indicate that chronic ochratoxicosis may, most likely render humans and animals more susceptible to trypanosome infections or promotes disease transmission and increases infection incidence in the field.

Survival time of *T. b. rhodesiense*-infected mice has been estimated between 6-9 weeks (Fink and Schmidt, 1979) consistent with survival time of 3-7 weeks observed in control mice in this study. However, mycotoxicosis significantly reduced the host survival times with higher death rates in the early phase of infection in ochratoxin-fed mice, and during the late phase of infection in aflatoxin-fed group. Mycotoxicosis therefore caused a more acute trypanosome infection in mice, an effect that was more remarkable in the ochratoxin-fed group. This differed with results of similar studies on malarial parasites. In *Plasmodium berghei*-infected mice, aflatoxicosis was shown to increase host survival time, an effect that was attributed to direct toxicity of aflatoxin B1 on the parasite (Young *et al.,* 1988; Hendrickse *et al.,* 1986). This difference may be due to species differences.

In this study, trypanosome-infected mycotoxin fed mice showed aggravated clinical signs such as pyrexia, emaciation, facial and scrotal oedema among others. These signs have been reported in other studies. Pyrexia, common in trypanosomiasis (Kagira *et al.* 2007; Moulton and Sollod, 1976), has been attributed to a wide range of pyrogens such as antibody-antigen complexes and TNF-α (Stephen, 1986). There is up-regulation of TNF-α during *T. b. rhodesiense* infections (Naessens *et al.*, 2005; Maina *et al.* 2004), ochratoxicosis (Al-Anati, 2006) and aflatoxicosis (Thrasher, 2007). Severe anaemia observed in the *T. b. rhodesiense*-infected mycotoxin-fed groups, and possible concerted action of infection-mycotoxicosis interaction leading to TNF-α up-regulation and massive haemolysis may explain aggravation of pyrexia in these mice. Similarly, severe dyspnoea observed in *T. b. rhodesiense*-infected mycotoxin-fed mice could be due to mycotoxin-induced decline in red blood cells and lung pathology that characterized these animals. Inhibition of protein-synthesis by mycotoxins (Smith and Moss, 1985; Pier and McLoughlin, 1985) probably aggravated muscle wasting in *T. b. rhodesiense*-infected aflatoxin-fed mice leading to enhanced host energy deficit in this study.

Since mycotoxins are immunosuppressive (Bondy and Pestka, 2000; Pier and McLoughlin, 1985), elevation of total white cell counts in *T. b. rhodesiense*-infected mycotoxin-fed mice was unusual. However, mycotoxins can be immunostimulatory rather than immunosuppressive (Bondy and Pestka, 2000) depending on the route of administration (Pier and McLoughlin, 1985) and a critical exposure window of dose and time (Hinton *et al.*, 2003). Nevertheless, reduced leucocytosis in aflatoxin-fed mice during the late phase of *T. b. rhodesiense* infection, may indicate aflatoxin-induced

suppression of lymphoproliferative responses (Williams *et al.,* 2004) similar to observations by other workers in rats (Raisuddin *et al.*, 1993), mice (Reddy and Sharma, 1989) and sows (Silvotti *et al.*, 1997).

Aggravated anaemia observed in trypanosome-infected mycotoxin-fed animals in this study was expected since anaemia in trypanosomiasis, ochratoxicosis and aflatoxicosis is well documented in various animal species. There are previous investigations on the effects of immunosuppressive molecules on development of pathogen-induced anaemia. These results are similar to findings on aflatoxin-mediated aggravation of anaemia in swine salmonellosis (Miller *et al.*, 1981) but differ with attenuation of anaemia by immunosuppressive corticosteroids, dexamethasone and hydrocortisone in *T. brucei*-infected mice (Balber, 1974; Halliwell and Gorman, 1989). Erythrophagocytosis, an important mechanism for development of anaemia in trypanosomiasis (Murray *et al.*, 1974) is blocked by immunodeppressants leading to attenuation of trypanosome-induced anaemia (Balber, 1974). Lack of evidence of significant mycotoxin-induced immunosuppression in *T. b. rhodesiense*-infected mice in this work explains the different effects on anaemia observed in the two studies.

On the contrary, the present results indicate that chronic mycotoxicosis is capable of complicating pathogenesis of trypanosome-induced anaemia in mice. This mycotoxin-induced aggravation of anaemia could also take place in humans. The mechanism(s) by which mycotoxins aggravate pathogenesis of anaemia in the present study could be multi-factorial perhaps involving down-regulation of erythropoietin activity (Naessens *et al.*,

2005) or disruption of metabolism of essential cofactors (Anyanwu *et al.*, 2004). Severe nephritis in *T. b. rhodesiense*-infected mycotoxin-fed mice perhaps reduced production of erythrogenin thus reducing erythropoietin activity (Anderson, 1985). Consistently mice fed on ochratoxin A, the most potent nephrotoxin of the two mycotoxins (Smith and Moss, 1985), had more severe anaemia in the present study. This aggravation of anaemia by mycotoxins was supported by evidence of exacerbation of some histopathological lesions in the trypanosome-infected mice. Haemorrhages and congestion have been reported before in trypanosomiasis (Ndung'u, 1990; Stephen, 1986). In this study however, hepatic and renal haemorrhages and hepatic congestion observed in trypanosome-infected ochratoxin-fed mice were massive and may have been caused by thrombocytopaenia (Anderson, 1985) previously reported in ochratoxicosis (Gupta *et al.*, 1983), trypanosomiasis (Kagira *et al.*, 2007), or ochratoxin A-induced consumptive coagulopathy-like syndrome (Albassam *et al.*, 1987). Similarly, infected aflatoxin-fed mice showed severe hepatic haemorrhages with massive hepatic thrombosis and cardiac thrombo-embolism that has not been reported before in trypanosomiasis, but was consistent with severe pancarditis and hepatitis in these mice.

Heart congestion could be attributed to severe obstructive coagulopathy that characterized the infection in aflatoxin-fed mice. These lesions suggest a mycotoxin-mediated aggravation of anaemia in trypanosome-infected mice probably through trapping or emigration of erythrocytes into extra-vascular space due to coagulopathy and vasculopathy respectively (Stephen, 1986; Anderson, 1985).

Other histopathological evidence suggested exacerbation of inflammation in trypanosome-infected mycotoxin-fed mice in this study. Mycotoxicosis aggravated nephritis, hepatitis, myocarditis, endocarditis, pericarditis and pancarditis characterized by vacuolization, degeneration, necrosis and fibrosis of cells, inflammatory cellular infiltrates and oedema. These lesions have been reported before in trypanosomiasis (Maina *et al.*, 2003; Stephen, 1986; Anosa and Kaneko, 1984), aflatoxicosis and ochratoxicosis (El-Arab *et al.*, 2006; Azziz-Baumgartner *et al.*, 2005; Williams *et al.*, 2004; Carlson and Ensley, 2003; JECFA 47, 2001; Albassam *et al.*, 1987; Ngindu *et al.*, 1982; Smith and Moss, 1985). Both aflatoxin B1 and ochratoxin A are potent hepatotoxins and nephrotoxins (Smith and Moss, 1985) and could have promoted vacuolization and necrosis of cells in this study as has been observed by other workers (El-Arab *et al.*, 2006).

It is likely that severe inflammatory cell infiltration in trypanosome-infected mycotoxin-fed mice could have resulted from increased numbers of necrotic cells (Anderson, 1985). Inflammatory eosinophils in ochratoxin-fed mice observed here suggests acute liver and kidney inflammation (Stephen, 1986) indicative of regulatory host responses to modulate underlying intense type I hypersensitivity reactions (Anderson, 1985). This agrees, with findings that mast cell activation and histamine production occur in trypanosomiasis (Ben-Rashed *et al.*, 2003). Ochratoxicosis- trypanosomiasis interaction is likely to have promoted interstitial trypanosome invasion through ochratoxin A-mediated blocking of antibody responses (Bondy and Pestka, 2000; Dwivedi and Burns, 1984) that are important in controlling waves of new VAT parasitaemia (Barry and Turner, 1991).

Trypanosome-induced oedema was also aggravated by mycotoxicosis in this study. Interstitial renal, facial and eyelid oedemas in all mycotoxin-fed mice were probably secondary to the observed acute renal tubular cell injury while severe pancarditis with cardiac thrombo-embolism and nephritis could be the underlying condition for prominent scrotal oedema in aflatoxin-fed mice (Anderson, 1985). The likely predisposing factors to exacerbation of generalized oedema characterized by ascites, hydrothorax in all mycotoxin-fed mice and hydropericardium in the ochratoxin-fed group were possible trypanosome-induced hypoalbuminaemia (Orhue *et al.*, 2005), and the severe starvation, kidney pathology, liver damage, heart inflammation observed in the present study (Anderson, 1985). Severe inflammation of liver, kidney and heart in trypanosome-infected mycotoxin-fed mice were probably major complicating factors consistent with reduced host survival. Mycotoxicosis and trypanosomiasis could have worked in synergy to exacerbate these lesions in this study. Budovsky *et al.* (2006) reported similar cyclophosphamide-mediated aggravation of pathological lesions in rats infected with *Trypanosoma lewesi*.

The consistent increase in curative dosages of drugs observed in *T. b. rhodesiense*-infected aflatoxin-fed mice in this study have been demonstrated in other studies using different immunosuppressive protocols. Osman *et al.* (1992) demonstrated that x-irradiation in mice considerably reduced efficacy of trypanocides and induced rapid development of high levels of stable chemo-resistance in immunocompetent mice. The reduced suramin efficacy resulting from the 7-day aflatoxin exposure period may indicate early development of drug failure. Since immunosuppressive effects of mycotoxins depend on a critical exposure window of time and dosage (Hinton *et al.*, 2003; Muller *et*

al., 1999; Pier and McLoughlin, 1985), a more significant effect would have been impacted if the aflatoxin exposure period was extended as is the case in the field.

4.2 CONCLUSIONS

- Ochratoxicosis decreased pre-patent period in *T. b. rhodesiense*-infection in mice and could possibly render humans and animals more susceptible to trypanosome infections by enhancing disease transmission and incidence in the field.
- Ochratoxicosis and aflatoxicosis aggravated anaemia in *T. b. rhodesiense*-infected mice. This was characterized by enhanced decline in red cell counts, packed cell volume, haemoglobin levels, increase in mean corpuscular volume with exacerbated histopathological features such as massive haemorrhages, thrombosis, embolism and congestion.
- Both ochratoxicosis and aflatoxicosis reduced host survival, and aggravated pyrexia, emaciation, dyspnoea and exacerbated inflammation of the liver, kidney and heart. This was characterized by generalized oedema, oedemas of the serous cavities, interstitial oedema, multi-organ focal perivascular cellular infiltration, and vacuolization, degeneration, necrosis and fibrosis of cells in *T. b. rhodesiense*-infected mice. Severe hepatitis, nephritis and heart inflammation were the major complicating factors.
- Aflatoxicosis was associated with consistent reduction in suramin efficacy for treatment of *T. b. rhodesiense* infection in mice.

These results suggest that mycotoxins aggravate pathogenesis of *T. b. rhodesiense* infection in mice, and that aflatoxin B1 was associated with reduction in efficacy of suramin. It was hypothesized that mycotoxins aggravate pathogenesis and compromise efficacy of therapeutic agents during *T. b. rhodesiense* infection in mice and therefore the hypothesis is accepted.

4.3 RECOMMENDATIONS

- It is important to consider effects of ochratoxicosis and aflatoxicosis when mounting control programs for trypanosomiasis and other tropical parasitic diseases in the affected areas.

- The biological mechanisms involved in the exacerbation of pathogenesis of trypanosomiasis by both ochratoxicosis and aflatoxicosis need to be investigated.

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