

***Trypanosoma brucei*-Induced apoptosis of leucocytes as a factor of trypanosusceptibility in infected goats**

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Abstract. White blood cell apoptosis has been demonstrated and is suspected to contribute to the decrease in peripheral leukocyte count and the severity of the disease during acute *T. brucei* infection in rats. Thus, an investigation of blood and tissue leukocyte apoptosis during *T. brucei* infections in two natural hosts (Red Sokoto (RS) and West African dwarf (WAD) goats (12 per group)), with differing levels of trypanosusceptibility was conducted. Nine out of 12 animals in each breed group were infected intraperitoneally with 10⁴ parasites/mL and euthanized over time points. Blood and tissues were collected for detection and quantitation of apoptosis by three methods (DNA gel electrophoresis, light microscopy and transmission electron microscopy). *T. brucei* infected RS animals (trypanosusceptible) showed a significant increase in apoptosis of white blood cells (p=0.0092) and splenocytes (p=0.0239). The infected WAD animals (trypanotolerant) also showed a relatively lower but yet still significant (p=0.0022) increase in white blood cell apoptosis. Apart from the liver, significant increase (P<0.05) in tissue cell apoptosis was recorded in infected RS compared to non-infected controls. The mean white blood cell and splenocyte apoptosis was significantly higher (p=0.0072) in RS compared to WAD. In addition, the peak blood cell apoptosis tallied with peak parasitemia and anemia as well as with the lowest leukocyte count in RS animals. Our data support a relationship between peripheral and tissue white blood cell apoptosis and susceptibility to *T. brucei* infections. The molecular mechanisms mediating apoptosis of host cells during trypanosoma infections may reveal novel therapeutic or vaccine targets.

INTRODUCTION

Trypanosomiasis is one of the most important debilitating diseases of animals and humans in Sub-Saharan Africa. It is estimated that 60 million people and 48 million cattle are at risk of contracting African trypanosomiasis transmitted by 22 species and 33 sub-species of tsetse flies in Africa (Kristjason *et al.*, 1999). Trypanosome infection is characterized by both stimulation and suppression of the immune system at different stages of the disease.

Apoptosis or programmed cell death occurs in several pathological and non-pathological conditions and constitutes a part of the mechanism of cell replacement and

tissue re-modeling, leading to maintenance of cellular homeostasis (Kerr *et al.*, 1972, Thompson, 1995). Morphological changes that characterize the apoptotic process are cytoplasmic blebbing, cell shrinkage, chromatin condensation, margination of condensed chromatin to the nuclear wall and nuclear fragmentation.

Host cell apoptosis has been reported in the pathogenesis of various intra- and extracellular parasitic diseases with parasite-induced host cell apoptosis being exploited for parasite survival, dissemination in the host, or to evade systemic immune response. Laboratory animal experiments demonstrate that pathogens such as *Plasmodium* spp. (Toure-Balde *et al.*, 1995,

Helmby *et al.*, 2000), *T. gondii* (Khan *et al.*, 1996, Liesenfeld *et al.*, 1997), *T. cruzi* (Lopes *et al.*, 1995, Zuniga *et al.*, 2002, Silva *et al.*, 2007), *E. histolytica* (Huston *et al.*, 2003, Boettner *et al.*, 2005) and *T. brucei* (Radwanska *et al.*, 2008, Happi *et al.*, 2012), evade potentially protective host responses through increase in leukocyte apoptosis in order to establish and maintain infection.

T. brucei-induced splenic marginal zone B cell apoptosis with resultant loss of protective anti-parasite antibody responses and abolishment of vaccine-induced memory responses have been demonstrated (Radwanska *et al.*, 2008). In addition, Bockstal and colleagues (2011) reported a reduction of B cell precursors and immature B cell stages in the bone marrow during murine *T. brucei* infection. We hypothesized that trypanotolerant hosts may have a better ability to control leukocyte apoptosis than trypanosusceptible hosts, resulting in a better and more sustained immunological response and thus greater ability to cope with the disease.

MATERIALS AND METHODS

Animals, Infection and Parasitemia

Twelve adult male trypanotolerant goats (WAD) and twelve adult trypanosusceptible goats (RS) weighing between 7-10 kg were used, divided into 2 groups (3 uninfected controls and 9 infected experiments). Prior to infection, all animals were acclimatized for 4 weeks during which they were dewormed using a benzimidazole (Albendazole). Animals were also treated with antibiotics (long acting oxytetracycline and gentamycin injection for those found to be pyrexia) and Berenil® twice at 9-day interval. They were also given an endo/exo-parasiticide (ivermectin) and multivitamins, subcutaneously and intramuscularly respectively. The animals were confirmed to be free of hemoparasites (trypanosomes, babesia, anaplasma) by light microscopic examination of the buffy coat and thin blood smears before inoculation. The 9 WAD or RS animals were infected with 10⁴ *T. brucei* parasites (Federe strain) in one mL

of saline. The parasites were obtained from the Nigerian Institute for Trypanosomiasis Research (NITR, Vom, Plateau State, Nigeria).

The infected animals were further subdivided into 3 sub-groups according to the stage of infection when they were euthanized (acute, sub-acute and chronic). Three animals from each sub-group were sacrificed on the 14th (sub-group 1) or 42nd (sub-group 2) day post-infection (PI) or prior to death. Three were followed until they became moribund or weak and recumbent (sub-group 3). One non-infected control each from WAD and RS was also euthanized on the 14th, 42nd day and the one left until the end of the experiment (Day 75 for RS and Day 104 for WAD).

The levels of parasitemia were measured daily and then weekly for each animal from the first day parasites were found in peripheral blood. The trypanosomes were enumerated by phase contrast buffy coat technique and the estimate of trypanosome per milliliter of blood was determined based on a log scale (Paris *et al.*, 1982).

Ethical considerations

Ethical approval was obtained from the University of Ibadan, Animal care and Use Research Ethics Committee (ACUREC). Animals were sacrificed without inflicting pain and suffering through humane euthanasia.

Blood and tissue samples collection

A pre-infection blood sample was collected for baseline hematology. Blood smears were made for examination of apoptotic cells. After infection, blood for hematology and DNA extraction was collected every 2 weeks from each animal and before sacrifice into 5ml K2 EDTA tubes. Blood smears were also made and stained with Diff-Quik® stain (Dade Behring Inc., Newark, DE 19714, USA) and examined for apoptotic leukocytes.

After euthanasia, tissue samples were collected from the spleen, mesenteric lymph node, thymus, liver and bone marrow for cytology, electron microscopy and apoptotic DNA ladder extraction assays for the detection, quantification and comparison of apoptotic levels in the 4 groups. Blood and tissues were prepared and preserved for the

various analyses as previously described by Happi *et al.* (2012).

Apoptosis DNA purification and analysis

DNA extraction and purification from blood and the frozen sections of tissues obtained from each animal was carried out using Biovision's Apoptotic DNA ladder extraction kit, catalog # K170-50 according to the manufacture's protocol. DNA fragmentation was evaluated by gel electrophoresis using 15µl of the extracted DNA sample obtained from blood and tissues in 1.2% agarose gel containing 0.5µg/ml ethidium bromide. The ethidium bromide-stained DNA was visualized by trans-illumination with UV light at 325 nm wavelength and photographed.

Transmission electron microscopy preparation and examination

Sections of bone marrow, spleen, liver and thymus were selected for electron microscopy from non-infected control animals, infected animals at Days 14 and 42 for both breeds, and Day 104 PI for WAD animal and 75 PI for RS animal. Tissues were processed using a standard method as previously described by Dawes (1979). Three blocks from the organs selected for each animal were used for the examination and counting of apoptotic cells in per 500 nucleated cells in each block, and the average apoptotic cells calculated per 500 cells in the 3 sections. Transmission electron microscopy (TEM) of thin sections were used to confirmed apoptotic morphology. Cellular identification was based on the morphologic features of apoptosis of the cells. The average apoptotic cells counted were analyzed and compared within each group and between the two breeds.

Effect of diaminazine aceturate (Berenil®) treatment on apoptosis levels in *T. brucei* infected Red Sokoto goats

Experimental animals consisting of three Red Sokoto animals were acclimatized for 4 weeks as described above. Animals were infected intraperitoneally with *T. brucei* (10⁴ parasites). From these animals, weekly samples of venous blood from the jugular vein were collected in K2 EDTA tubes. Blood

collected was used to monitor their parasitemia, PCV and apoptosis. At Day 40 post-infection (PI) when apoptotic leukocytes were observed in the peripheral blood, they were treated using 3.5 mg/kg of diaminazine aceturate (Berenil®) intramuscularly. The treatment was repeated on the eighth day despite the parasite clearance from the peripheral blood. Parasite clearance was confirmed by the absence of *T. brucei* in peripheral blood from Day 3-16 post-treatment. To confirm parasite clearance in the treated RS animals, a group of 3 rats were sub inoculated intraperitoneally each with blood from a treated animal. These rats were monitored for 8 days post inoculation to assess the presence *T. brucei* in their peripheral blood. Sixteen days post-treatment, when the parasites had been cleared and the PCV returned to pre-infection values, the treated RS animals were euthanized. Blood and tissues from thymus, lymph nodes, spleen, liver and bone marrow were harvested and analyzed (as above). Apoptotic cells were evaluated, enumerated and compared with the *T. brucei* infected RS group of animals.

Statistical analysis

For parasitemia, data were transformed to log parasites/ml for statistical analysis. Student's t-test was used to evaluate the differences in the mean parasitemia between RS and WAD animals. Variation of mean values of blood cell apoptosis over time was evaluated using one sample t-test statistical analysis in different groups within each breed. Student's t-test was used to compare the mean blood cell apoptosis between the two breeds as well as for apoptosis evaluation and comparison in hemopoietic organs by LM and TEM between the infected treated and infected non-treated RS animals.

ANOVA was used for evaluation and comparison of LM and TEM apoptotic count in hemopoietic organs in various groups of each breed. The mean apoptotic counts of both breeds were compared by Student's t-test. Correlation analysis was performed using the Pearson correlation. P values < 0.05 were considered significant for all statistical analyses. The results are presented as mean ± standard deviation.

RESULTS

Survival and parasitological findings in *T. brucei* infected WAD and RS goats

The WAD animal survived for a longer period than the RS animals. Only two RS animals survived to 73 and 75 days PI and were euthanized because they were very anemic, moribund and recumbent. Two WAD animals survived for more than 90 days PI and were euthanized in a relatively good condition.

Apart from the one RS animal showing detectable parasitemia on the fourth day PI, the prepatent period in both groups of animals was between 6 to 13 days PI. However two WAD animals showed detectable parasitemia on Days 23 and 24 PI. As shown in Fig. 1, the mean parasitemia increased steadily in the RS animals to reach its first peak on the 14th day PI and dropped slightly thereafter, whereas in the WAD animals the mean parasitemia reached the first peak at Day 28 PI. Thereafter, the parasitemia fluctuated in both breeds with no significant differences ($p>0.05$). The RS animals showed a higher parasitemia than the WAD animals. Non-infected control animals remained aparasitemic throughout the period of the experiment.

Clinical findings in *T. brucei* infected WAD and RS animals

The major clinical signs observed in both groups of animals were as follows: general weakness, pale mucous membrane of the conjunctiva, diarrhea, cachexia, alopecia, rough hair coat, decreased appetite, ocular discharge, recumbency and corneal opacity. The clinical signs were generally similar in both WAD and RS infected animals. However, RS infected animals showed more severe clinical signs than their WAD counterparts.

Hematological findings in *T. brucei* infected WAD and RS animals

Infection caused significant decrease ($p<0.01$) in PCV values of both breeds over time. However, the overall PCV level in infected RS animals was lower than that of infected WAD animals. The mean PCV in infected WAD animals decreased from $26 \pm 4.8\%$ at Day 0 to $13 \pm 1.7\%$ and $11 \pm 3.2\%$ at 35 and 42 days PI respectively. In infected RS animal, the mean PCV decreased from $28.8 \pm 8.16\%$ at Day 0 to $9.8 \pm 3.4\%$ at Day 35. In the non-infected control animals, the mean PCV fluctuated between $37 \pm 3.6\%$ and $29 \pm 4.2\%$ in WAD animals and remained stable (28.5 ± 6.3 and $28.5 \pm 0.6\%$) in RS animals.

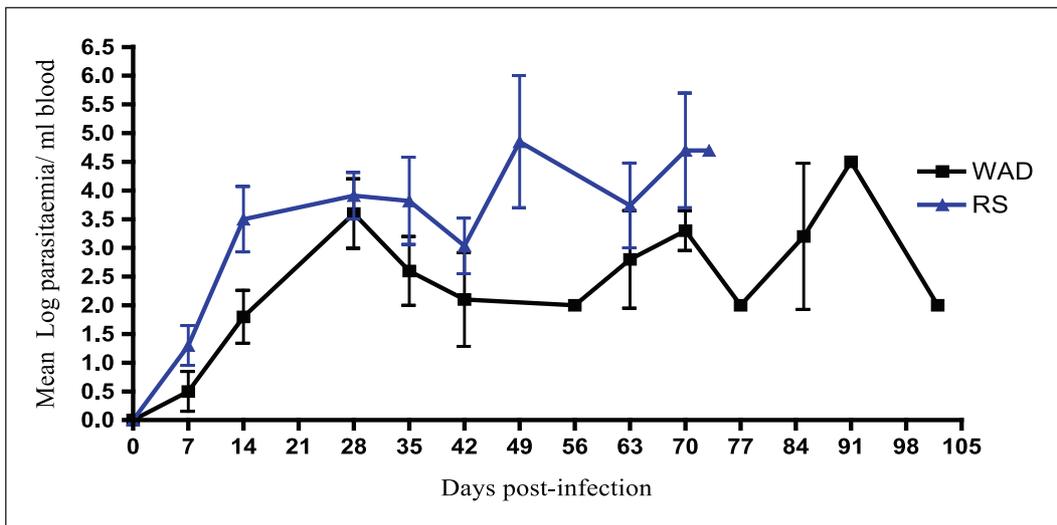


Figure 1. Mean parasitaemia in *T. brucei* infected WAD and RS goats.

Figure 2 shows the variation in the total leukocyte and lymphocyte counts in both WAD and RS *T. brucei* infected animals over the course of the experiment. The level of total WBC (TWBC) and lymphocyte counts of RS animals remained significantly ($p=0.042$) lower than that of the WAD animals. There was a significant increase in the mean TWBC count of infected WAD ($p=0.001$) and RS ($p=0.002$) animals compared to their non-infected controls. The mean TWBC of the infected WAD animal decreased from $10350 \pm 3507/\mu\text{l}$ at Day 0 to $9348 \pm 2282/\mu\text{l}$ at Day 14 PI and rose again to $13075 \pm 742.5/\mu\text{l}$ at Day 78 PI and $19275 \pm 1025/\mu\text{l}$ by the Day 85 PI. There was a slight increase at Days 45 and 65 PI in TWBC in RS infected animals with associated significant increase in lymphocytes and decrease in monocyte counts ($p=0.0005$ and $p=0.0366$) respectively (vs. non-infected controls). At the termination of the experiment in both infected breeds, there was a reduction in TWBC and lymphocytes as shown in Fig. 2 as well as in neutrophils compared to their non-infected control animals.

Light microscopic (LM) evaluation of apoptotic cells in blood smears of *T. brucei* infected WAD and RS animals

Light microscopic examination of blood smears of WAD and RS infected animals revealed scattered apoptotic leukocytes, mainly lymphocytes (Plate 1; A1 & A2) and a few neutrophils. Some of the microscopic appearance apoptotic cells are shown in Plate 1; A1 & A2). Infected WAD animals showed statistically significant ($p=0.0022$) increase in mean blood apoptotic cell counts from the 21st day PI, with a peak of $10 \pm 0/100$ WBC apoptotic blood cells at Day 91 PI compared to non-infected controls. However, as presented in Fig. 3, RS animals showed higher levels of blood leukocyte apoptosis that began to be detected by LM on the 14th day PI in 3 out of the 8 infected RS animals. The peak blood cell apoptosis detected by LM in RS animals was attained at Day 70 PI with a mean of $53 \pm 35/100$ WBC. The gradual and slightly fluctuating increase (Fig. 3) in blood leukocyte apoptosis observed in *T. brucei* infected RS animals was statistically significant ($p=0.009$) over time. There was a

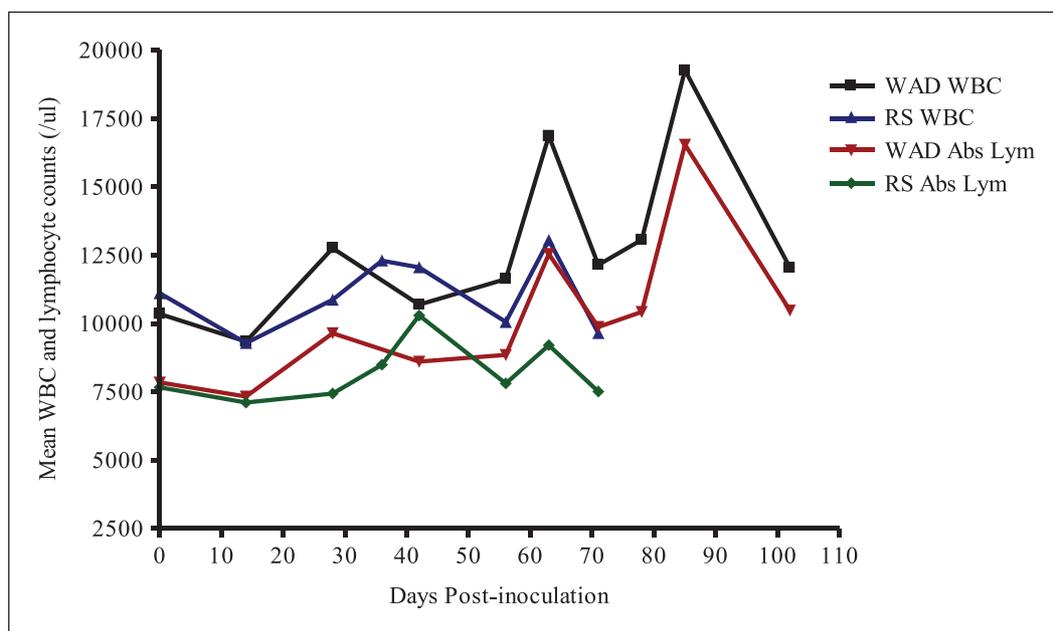


Figure 2. Mean total WBC and lymphocyte counts in *T. brucei* infected WAD and RS goats.

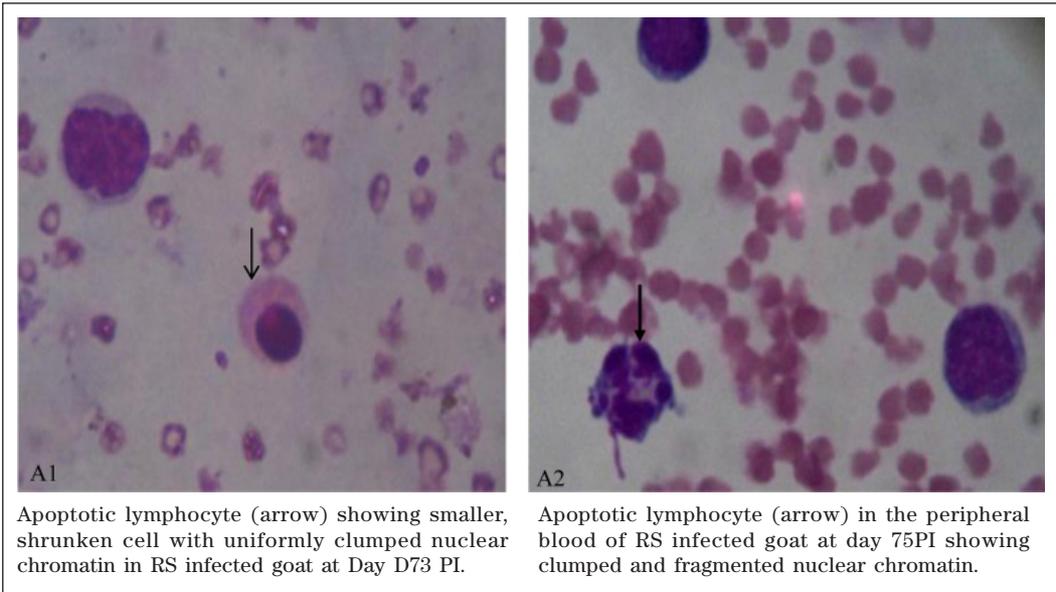


Plate 1. Apoptotic cells in peripheral blood of *T. brucei* infected goats (Diff-Quik stain, x1000 original Mag.).

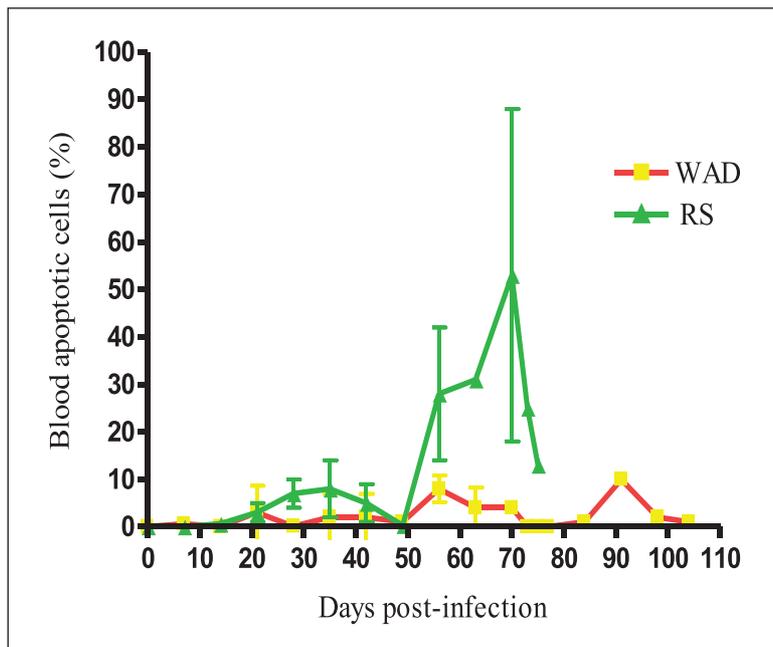


Figure 3. Blood cells apoptosis in WAD and RS *T. brucei* infected goats.

significant difference ($p=0.007$) in the mean blood cell apoptosis between WAD and RS infected animals observed by LM. Apoptotic cells were persistently observed in the blood smears of RS animal from Day 50 to 73 PI,

while in WAD animal apoptotic cells were found in blood smears only rarely.

No association was observed between parasitemia and blood cell apoptosis in both breeds of infected animals. Although both

breeds showed fluctuating parasitemia, the blood cell apoptotic counts remained very low in infected WAD animals. The peak blood leukocyte apoptosis corresponded with the highest peak parasitemia in RS infected animals as in Fig. 4.

There was no correlation between blood cell apoptosis and lymphocyte counts in either WAD ($r= 0.16962$) or RS ($r= -0.27102$) infected animals. However, the highest level of peripheral blood leukocyte apoptotic counts corresponded with the lowest level of leukocyte counts in the RS breed (Day 70 PI).

There was a significant correlation of peripheral blood cell apoptosis and duration of infection in RS animal ($p=0.0045$; $r=0.7311$). Furthermore, more apoptotic leukocytes were recorded in moribund animals or prior to death. The correlation between blood cell apoptosis and duration of infection was not significant in the WAD animal ($r= 0.2752$).

Light microscopic (LM) evaluation of apoptotic cells in tissues of *T. brucei* infected WAD and RS animals

Bone marrow smears from both WAD and RS *T. brucei* infected animals showed a few

apoptotic cells during chronic infection with no significant difference ($p>0.05$) between the 2 breeds. However, the infected WAD animals showed scanty bone marrow apoptotic cells only on Days 91 and 104 PI, while the bone marrow from RS infected animals revealed apoptotic cells from Day 42 PI with a mean of $2.0 \pm 1.7 /500$ cells. LM detected no apoptotic cell in the bone marrow of non-infected controls of both WAD and RS animals. Apoptotic cells were mostly lymphocytes.

T. brucei infection caused an increase in the number of apoptotic splenocytes in both breeds of animals, but this was only statistically significant ($p=0.047$) within the RS animals. Figure 5 shows the trend and difference in the mean apoptotic splenocytes in infected RS and WAD animals by LM during the period of experiment. Though not statistically significant ($p>0.05$), more splenocyte apoptosis was observed in RS than WAD infected animals during this study. The spleen of both breeds of *T. brucei* infected animals revealed mainly apoptotic lymphocytes and a few plasma cells by LM.

Following infection, there was no significant difference ($p>0.05$) in mesenteric

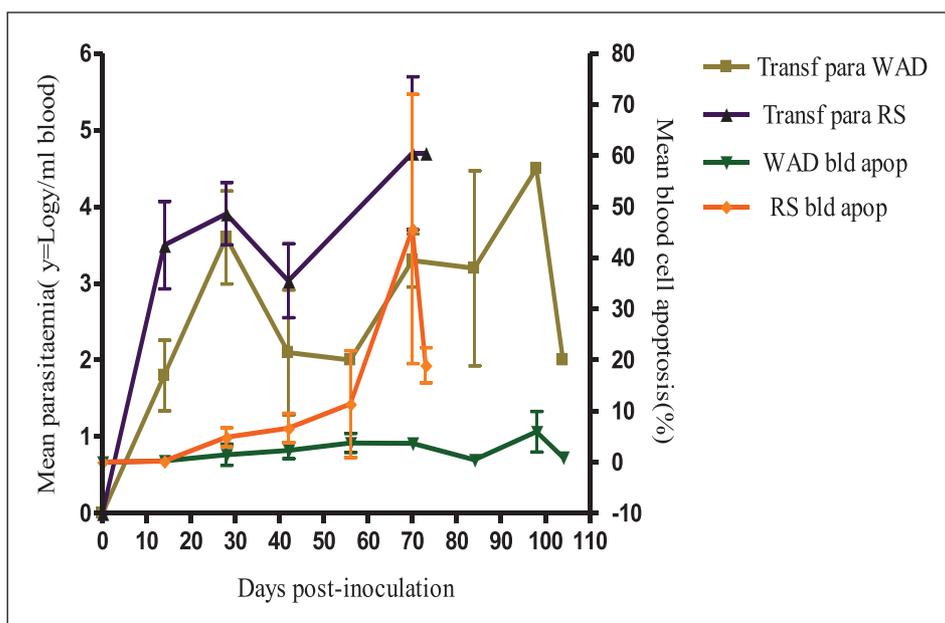


Figure 4. Relationship of mean parasitaemia and blood cell apoptosis in *T. brucei* infected WAD and RS goats.

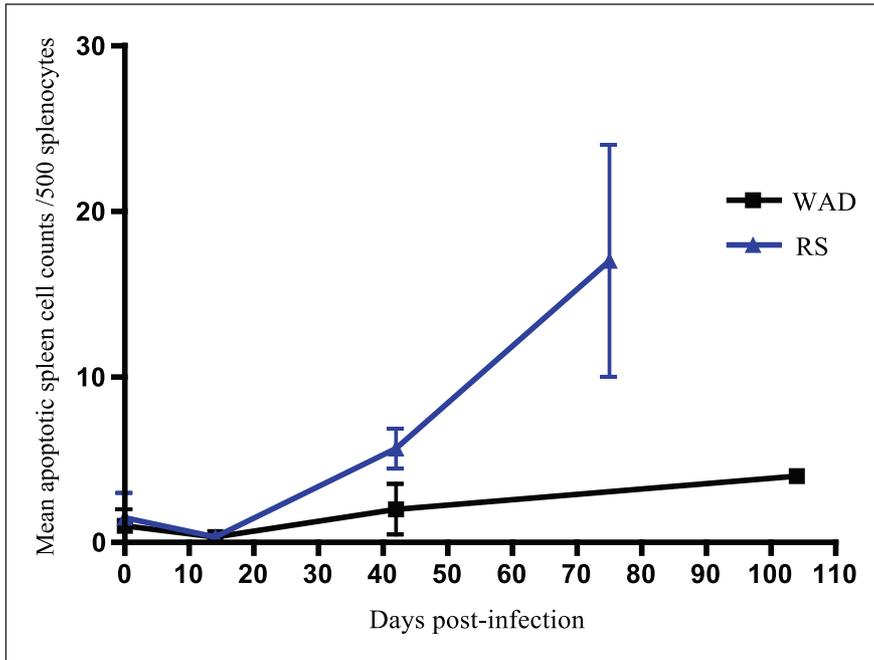


Figure 5. Mean count of spleen cell apoptosis in *T. brucei* infected WAD and RS goats.

lymph node (MLN) and thymus apoptotic cell counts in both breeds by LM. The mean value of MLN apoptotic cell counts in RS and WAD animals increased slightly from the control values.

The mean thymocyte apoptosis in infected RS increased slightly from $0.3 \pm 0.6 / 500$ cells at Day 14 PI to $3.5 \pm 0.7 / 500$ cells at Day 73 PI. On the other hand, the infected WAD thymocyte apoptotic count increased from $0.5 \pm 0.7 / 500$ cells at Day 0 to $4 \pm 5.6 / 500$ cells at Day 91 PI. However, these increase were not statistically significant compared to non-infected controls ($p > 0.05$).

The apoptotic cells identified in the liver of both RS and WAD animals were mainly lymphocytes. However, this increase in liver lymphocyte apoptotic counts was not significant statistically ($p > 0.05$) in both breeds. The non-infected control animals of both breeds showed a relatively similar mean level of liver lymphocyte apoptosis count. From Day 42 PI, the RS infected animals showed an increase lymphocyte apoptosis of $1.0 \pm 2.0 / 500$ cells to $35.0 \pm 21.2 / 500$ cells at Day 73 PI in the liver, while the mean

lymphocyte apoptotic count of the liver in infected WAD animals increased only from $5.3 \pm 5.0 / 500$ cells at Day 42 PI to $9.5 \pm 12.0 / 500$ cells at Day 91 PI.

LM examination of the bone marrow, spleen, MLN and thymus smears also revealed a few macrophages, macrophages with engulfed red cells, granulocytes (Plate 2, A1 & A2), lymphocytes, plasma cells, hemosiderosis and dividing lymphocytes, mostly in RS infected animals. Numerous plasma cells and Mott cells were also found in the spleen.

Apoptosis detection by DNA fragmentation from blood, bone marrow, spleen, thymus and liver of *T. brucei* infected WAD and RS animals

Plate 3B, Lane 6 shows faint bands of DNA fragments suggestive of apoptosis from a blood sample obtained from a RS infected animals sacrificed at Day 73 PI. Blood samples obtained from either non-infected controls or infected RS and WAD animals revealed no band of fragmented DNA (Plate 3A and B). The spleens, thymus and liver of non-infected and *T. brucei* infected WAD

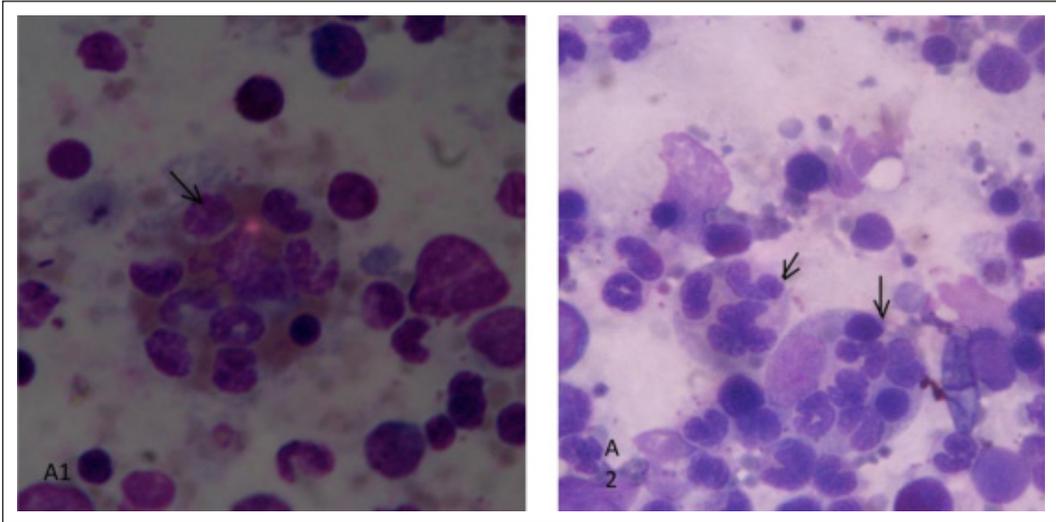
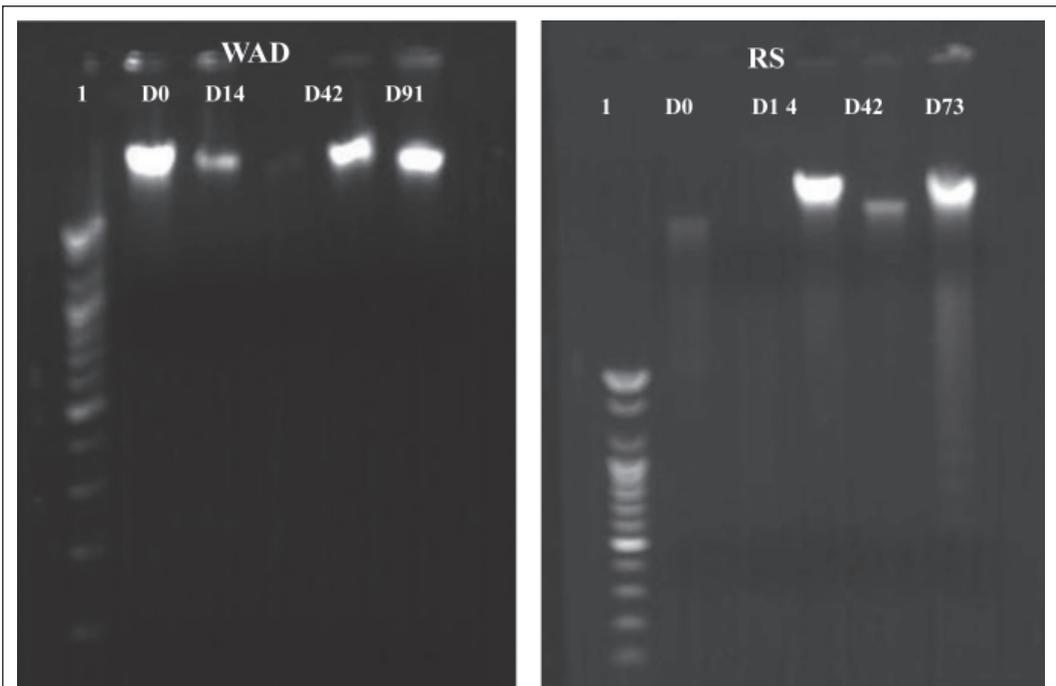


Plate 2. BM smears showing macrophages with phagocytosed RBC (A1), lymphocytes and some progenitor of granulocytic cells (A1 & A2) in *T. brucei* infected RS goat at days 72 and 45 PI (arrows). (Diff-Quik stain, x1000 original Mag.).



A: No DNA fragmentation is observed in blood obtained from both non-infected control and *T. brucei* infected WAD goat (Lanes 2, 3, 4, 5 and 6).

B: DNA fragmentation is observed in blood obtained from *T. brucei* infected RS goat at Day 73 PI (Lane 6). The non-infected control (Lane 2) and infected RS blood from Days 14, 42 and 75 PI (Lanes 3, 4 and 5) showed no DNA fragmentation.

Plate 3. Agarose (2%) gel electrophoresis of DNA extraction from whole blood of *T. brucei* infected goat. Lane 1 shows 100bp DNA ladder.

and RS animals showed bands of DNA fragmentation indicative of apoptosis. However, bands of DNA fragments of infected RS breed were thicker and stronger than those of WAD infected animals and non-infected controls of both breeds (Plate 4).

Transmission electron microscopic (TEM) evaluation of apoptosis in tissues of *T. brucei* infected WAD and RS animals

Table 1 reveals that there was a significant increase in cell apoptosis in bone marrow ($p=0.0188$), spleen ($p=0.0286$) and thymus

($p=0.0221$) of *T. brucei* infected RS animals compared to non-infected control by TEM evaluation. However, in WAD animals, the mean apoptotic cell counts in the above mentioned organs increased, but not significantly ($p>0.05$) in infected animals compared to non-infected controls. A non-significant ($p>0.05$) difference in mean apoptotic cell counts between the two breeds was recorded in all the organs. There was also a general and marked decrease in bone marrow cell population in the RS animals during the chronic stage of infection.

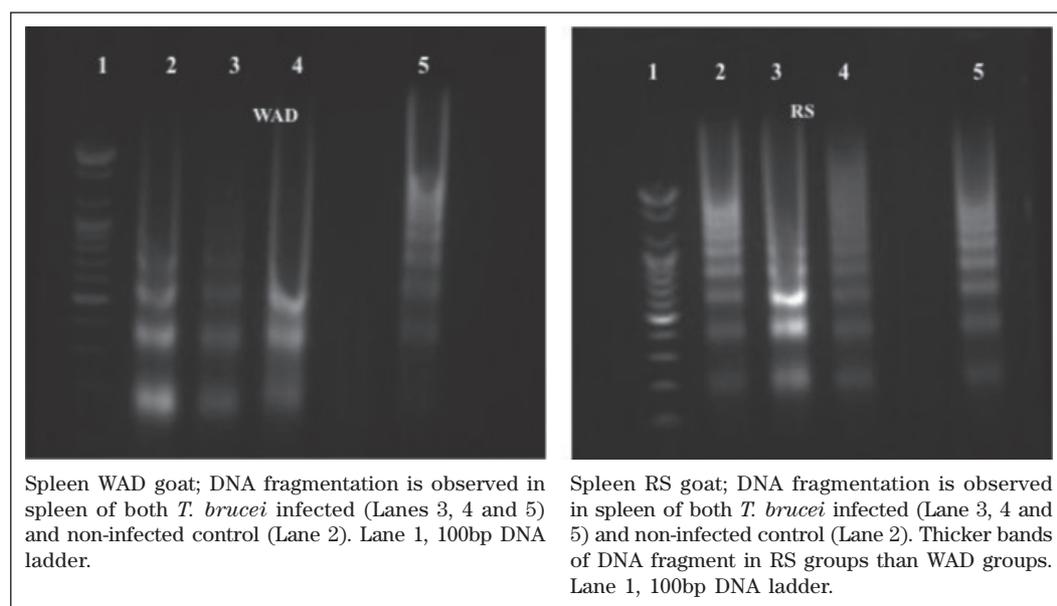


Plate 4. Agarose (2%) gel electrophoresis of DNA extraction from the spleens of *T. brucei* infected WAD and RS goats.

Table 1. TEM evaluation of apoptotic cells of tissues of WAD and RS *T. brucei* infected animal

DPI	Thymus (/500 cells)		Spleen (/500 cells)		Liver (/500 cells)		BM (/500 cells)	
	WAD	RS	WAD	RS	WAD	RS	WAD	RS
Controls	1.66±0.57	11.00±2.64	0.66±0.57	1.33±2.30	0.00±0.00	0.33±0.57	0.00±0.00	0.00±0.00
D14	3.00±2.00	6.66±3.21	0.66±1.15	5.00±1.00	0.00±0.00	0.00±0.00	1.33±1.52	6.67±0.57
D42	4.00±3.00	25.66±8.96	1.66±1.52	38.67±16.50	0.00±0.00	0.66±0.57	0.00±0.00	4.67±1.52
D73		3.00±1.00		3.00±1.00		1.00±1.00		2.66±1.15
D104	9.33±1.52		3.33±1.52		0.00±0.00		1.00±1.73	
P value	0.0713	0.0221*	0.1403	0.0286*	**	0.3059	0.2451	0.0188*

* Significant difference ($P<0.05$); BM= Bone Marrow, EM= Electron Microscopy

** All the values in the column are the same

Plate 5; A2 illustrates an apoptotic lymphocyte (a) and plasma cell (b) in the spleen of *T. brucei* infected RS goat by TEM.

Table 1 also shows that infected animals of both breeds developed a non-significant change in liver lymphocyte apoptotic count by TEM evaluation compared to non-infected controls with no significant ($p > 0.05$) difference between WAD and RS animals.

Comparison of level of apoptosis between the 2 breeds of *T. brucei* infected animals

In general, the RS animal showed more blood and tissue cell apoptosis during *T. brucei* infection compared to their tolerant WAD counterpart. However a significant difference ($p = 0.0072$) in mean apoptotic count was observed only in blood between the two breeds by LM. The TEM examinations showed more apoptosis in RS infected animals in the spleen, thymus and bone marrow (Table 1). The LM and TEM examinations of apoptotic cells of the organs confirmed the findings obtained by gel

electrophoresis. In addition, both LM and TEM results of apoptotic counts revealed a significant increase in splenocyte apoptosis in the infected RS breed.

Comparison of apoptosis in *T. brucei* infected RS animals treated with diaminazine aceturate (Berenil®) and infected RS controls

In Fig. 6, the parasitemia increased but fluctuated slightly in *T. brucei* infected RS animals to reach its highest peak of 5×10^6 /ml at Day 29 PI. After treatment with Berenil® at Day 40 PI, no parasites were detected in the peripheral blood from Day 43 PI until the end of experiment (Day 56 PI). Figure 6 also shows the mean blood leukocyte apoptotic count in *T. brucei* infected RS animals prior to treatment at Day 39 PI and after treatment with Berenil®. Three days after treatment (Day 43), the leukocyte apoptotic level dropped to 0/100 WBC till the end of the experiment. Parasite clearance in the peripheral blood due to Berenil® treatment coincided with the disappearance of

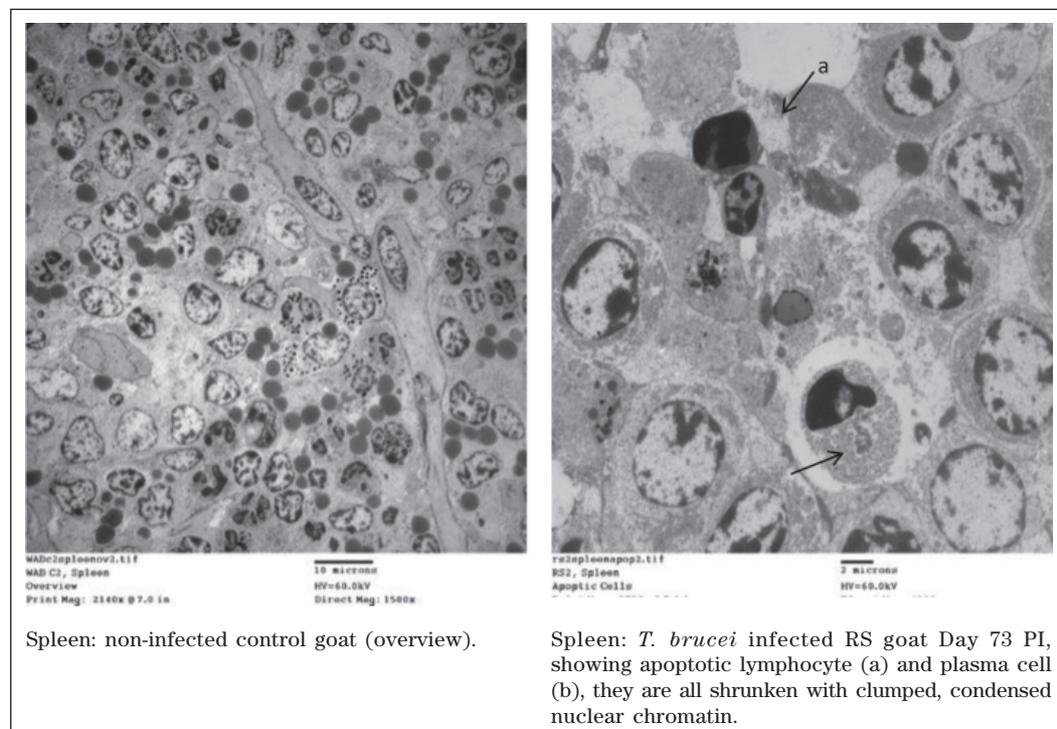


Plate 5. TEM of spleen of non-infected control (left) and *T. brucei* infected goats showing apoptotic lymphocyte and plasma cell (right).

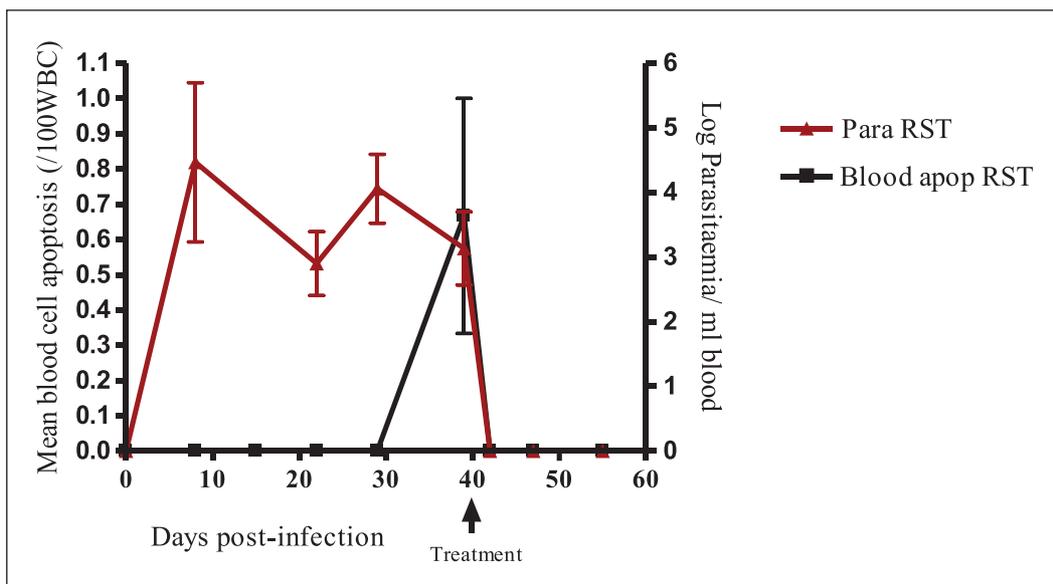


Figure 6. Parasitemia and peripheral blood leukocyte apoptosis in *T. brucei* infected and treated RS goats.

apoptotic leukocyte in circulation (Fig. 6). The PCV showed a decrease from the pre-infection value ($21.4 \pm 3.2\%$) to a mean of $14.67 \pm 1.52\%$ at Day 29 PI, and remained below the pre-infection value until Day 39 PI. After the Berenil® treatment on the 40th day PI, the PCV began to rise again above the pre-infection value from Day 8 post-treatment ($24.33 \pm 4.04\%$) until Day 16 post-treatment ($28.33 \pm 2.8\%$) when they were euthanized.

Blood and tissue (liver, thymus, lymph node and bone marrow) smears of Berenil® treated RS goats (RST) revealed no apoptotic cells at Day 16 post-treatment. However, the spleen showed a decrease in apoptotic splenocytes from $17 \pm 9.9/500$ cells in infected RS to $2.33 \pm 1.15/500$ by LM cells in RST at Day 16 post-treatment (i.e Day 56 PI).

In the RST animals, apoptotic cell counts by TEM decreased significantly in all the organs examined, compared to the infected non-treated RS group. In the bone marrow, the mean apoptotic cell counts significantly ($p=0.0200$) decreased from $1.44 \pm 0.46/500$ cells in non-treated RS to $0.39 \pm 0.14/500$ cells in RST animals. The spleen also showed a significant ($p<0.0001$) decrease in mean apoptotic cell counts from $2.28 \pm 0.18/500$ cells in infected RS to $0.32 \pm 0.08/500$ cells

in RST animals. A significant decrease in apoptotic cell count was also recorded in the thymus and liver of RST animals compared to infected RS animals ($p=0.0412$ and $p=0.0030$ respectively). The apoptotic cell counts in the liver and thymus of infected RST animals also showed a non-significant decrease compared to the untreated control.

DISCUSSION

Recent data from experimental rodent trypanosomiasis demonstrated blood and tissue leukocyte apoptosis, and it was suggested to represent a putative mechanism for the severity of leukopenia and the disease (Happi *et al.*, 2012). Thus, an investigation of apoptosis in blood and tissues leukocytes during *T. brucei* infections in natural hosts with different levels of susceptibility (WAD and RS goats) was carried out in the present study.

As expected, infected RS animals had a shorter prepatent period, sustained a higher degree of parasitemia, developed more severe anemia and showed more mortality than the infected WAD animals. The ability of the WAD animals to partially control the

infection was shown by leukocytosis and lymphocytosis, while only neutrophils decreased. On the contrary, infected RS animals showed reduced TWBC, lymphocyte and neutrophil counts especially during the chronic and end stage of the infection. The underlying mechanisms for the reduction in different cell populations are not clear, and it is possible that more than one mechanism may be involved. The reduction of certain cell populations in the peripheral blood has been described in different trypanosomiasis. For instance, erythrocytes and neutrophils have been reported to be destroyed by cells of the mononuclear phagocytic system in trypanosome-infected cattle (Murray *et al.*, 1988).

In the present study, *T. brucei* infection of two different breeds showed increase in nucleated cell apoptosis in blood. The apoptotic cells were mostly lymphocytes and neutrophils but some cells in an advanced stage of apoptosis could not be identified properly with LM. In most parasitic disease conditions, apoptotic blood cells are very rare to find with light microscopy because adjacent cells such as monocytes rapidly engulf them. However, an increase in blood cell apoptosis has been reported in cancer (Yoshino *et al.*, 2000; Lima *et al.*, 2002), viral infections (Carrero and Unanue, 2006), bacterial diseases (Holznage *et al.*, 1998), parasitic infections (*P. falciparum* and *T. cruzi*) (Thompson, 1995; Toure-Balde *et al.*, 1996; Pino *et al.*, 2003; Silva *et al.*, 2007) and AIDS (Helmbly *et al.*, 2000; Sanchez-Toures *et al.*, 2001; Kapoor, *et al.*, 2011). Blood cell apoptosis has also been shown to be induced by drugs (Solary *et al.*, 1996; Stahnke *et al.*, 2001).

Our result showing an increase in blood cell apoptosis especially at the later or chronic stage of infection suggests that trypanosome induced Programmed Cell Death (PCD) contribute to the reduction in leukocyte count during trypanosome infection especially as the peak as apoptotic cell count corresponded with the lowest leucocyte count. This further confirms the earlier work on *T. brucei* infection in rats (Happi *et al.*, 2012). In addition, the correlation between the apoptotic blood cell

count and duration of infection in RS on none hand, together with the low leukocyte count and high level of apoptotic leukocytes suggests exhaustion of the bone marrow to replace the lost cells at the later and moribund stage of infection. This study also demonstrated that the more susceptible breed (RS) showed earlier and higher levels of blood leukocyte apoptosis especially during the chronic phase and moribund state of the infection than the tolerant WAD. This increase in apoptotic blood cells also corresponded with the increase in parasite count, suggesting that leukocyte PCD during trypanosomiasis may allow parasites to escape the host immune response and facilitate parasite replication. Barcinski and DosReis, (1999) in their study described a mechanism whereby *T. cruzi* parasite takes advantage of T-cell apoptosis to replicate and spread in host tissues despite vigorous immune responses against it. It has also been reported that parasite-triggered apoptosis of immune cells allow parasites to survive and eventually persist in immunocompetent hosts by abolishing anti-parasitic immune responses (Schaumburg *et al.*, 2006). In the tolerant host, leukocyte apoptotic counts remained low with adequate leukocytic response and consequently a lower parasitaemia, suggesting that leukocyte apoptosis in trypanosoma infection is involved in the pathogenesis of the disease and contributes to host susceptibility to infection especially as they survive longer and are in a relatively good condition. Elucidation of the mechanism for control of blood cell apoptosis in the tolerant host may be of importance in the control of the disease.

Bone marrow (BM), liver, thymus and mesenteric lymph node did not show significantly different levels of apoptosis between RS and WAD animals and non-infected controls. This could be due to any of the following reasons: the small number of animals (three) examined in each group; the fact that both breeds were relatively vulnerable to trypanosomiasis though with differing degrees of severity; the quantitative methods used (LM and EM) were not very sensitive, given that detection of cells in the early stage of apoptosis with no visible

morphologic changes may not be evident with these methods.

The present study showed more apoptotic cells in bone marrow, spleen and thymus during in moribund RS infected animals compared to WAD animals and non-infected controls of both groups. In addition, macrophages were observed to be actively phagocytic in these organs. These findings also suggest that the voracious phagocytosis observed in the RS animals could be due in part to trypanosome-induced apoptotic or early apoptotic cells. It has been demonstrated that adjacent cells or macrophages quickly engulf cells primed to undergo programmed-cell-death (PCD). The termination of apoptotic event occurs in most cases via recognition and clearance by phagocytes, such as macrophages and immature dendritic cells (Rovere-Querini and Dumitriu, 2003. Schlegel and others (1996) reported that apoptotic cells release phosphatidylserine in the outer membrane that acts as a tethering molecule, allowing the firm binding of apoptotic cells to macrophage surfaces, and stimulate rapid clearance of apoptotic cells from peripheral blood and tissues by phagocytosis. The phosphatidylserine and/or other factors released may then stimulate proliferation and activation of macrophages for phagocytosis of apoptotic cells, and by so doing may inhibit the innate response to infection in the susceptible animals. Since the susceptible animals had more apoptotic cells, it is then possible that numerous phagocytic cells are stimulated to clear the dead cells, thereby digressing the macrophage attention for release of cytokines and consequently down regulation of inflammatory response.

In this study, an increase in bone marrow cell, splenocyte and thymocyte apoptotic death by LM and TEM was recorded in infected RS and WAD animals during the chronic stage of the infection compared to non-infected controls. Increase in splenocyte apoptosis during *T. brucei* infection has been reported (Radwanska *et al.*, 2008 and Happi *et al.*, 2012). A significant increase in apoptotic bone marrow, thymus (with TEM) and spleen (with LM and TEM) cells was observed in RS animals during the

terminal and chronic stage of *T. brucei* infection compared to the more tolerant WAD animals suggesting a connection between susceptibility to *T. brucei* infection and splenocyte apoptosis as well as thymus and bone marrow cell apoptosis to a lesser extent. This might also explain why the apoptotic loss of lymphocytes and plasma cells was recorded more in the susceptible animal and especially during the terminal stage of the disease. This result leads to the suggestion that trypanosome induced PCD of lymphocytes and plasma cells contributes to the susceptibility to infection. Additional evidence directly showing the detrimental effect of lymphoid cell death on pathogen handling comes from *T. cruzi* and the cecal ligation and puncture model of induced sepsis (Carrero and Unanue ER, 2006), as well as the abrogation of vaccine-induced protective response to non-related human pathogen such as *Bordetella pertussis* during *T. brucei* infection (Radwanska *et al.*, 2008).

Although during this study no direct correlation was observed between bone marrow, nor thymus, nor spleen cell apoptotic death and parasitemia, it was however observed that the highest number of apoptotic splenocytes coincided with the highest level of parasitemia (by LM at Day 73 PI) in RS breed. This result corroborates the work of Bockstal and others who suggested a direct link between levels of parasitemia and the kinetics of induction of splenic B-cell apoptosis (Bockstal *et al.*, 2011). Consequently, it can be extrapolated that trypanosusceptibility also correlates positively with induction of blood leukocyte as well as splenocyte apoptosis and parasitemia. This however raises the question of how tolerant hosts maintain lower levels of apoptosis of lymphocytes during infection. This question can only be addressed when the mechanism of *T. brucei* induced leukocyte apoptosis as well as trypanosome apoptotic factor(s) has been fully understood. A trypanosome apoptotic factor (TAF) has been identified, characterized, and shown to mediate apoptosis of mouse-brain and human-brain vascular endothelial cells and therefore could enable the parasites to cross the blood brain barrier (Stiles *et al.*, 2004).

Such a factor could also contribute to induction of leukocyte PCD. Recently, Bockstal and colleagues (2011) demonstrated *T. brucei*-induced B cell apoptosis through contact dependent mechanism (Bockstal *et al.*, 2011), suggesting that more than one mechanism is involved in *T. brucei*-induced host cell apoptosis during infection.

Treatment of *T. brucei* infected RS animals with diaminazine aceturate (RST) resulted in parasites clearance, which correlated with the loss of peripheral blood leukocyte apoptosis. This finding indicates a cause and effect relationship between parasites or parasite factors and leukocyte apoptosis during infection. More interestingly, the RST showed a significantly lower level of cell apoptosis in spleen, thymus, bone marrow, lymph node and liver compared to the infected and non-treated RS animals by LM and TEM. This further demonstrates *T. brucei*, a tissue-invading parasite, induces apoptosis of host cells during infection. These findings agree with previous observations of Bockstal and others (2011) who found that living *T. brucei* induce cell death of transitional B cells *in vitro* through a contact dependent mechanism.

The findings in this study indicate that apoptosis of lymphocytes and possibly other leukocytes contribute to leukopenia and depression of lymphoid cells in hemopoietic organs thereby assisting parasite escape of immune clearance. Leukocyte apoptosis may also contribute to the increase hemophagocytic activity as well as depression of inflammatory response and hemopoiesis.

In conclusion, this study has shown the likely involvement of leukocyte apoptosis in susceptibility to trypanosomiasis and control of trypanosome-induced leukocyte apoptosis could help to ameliorate the severity of the disease.

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REFERENCES

- Barcinski, M.A. & DosReis, G.A. (1999). Apoptosis in parasites and parasite-induced apoptosis in the host immune system: a new approach to parasitic diseases. *Brazilian Journal of Medical and Biological Research* **32**(4): 395-401.
- Bockstal, V., Guirnalda, P., Caljon, G., Goenka, R., Telfer, J.C., Frenkel, D., Radwanska, M., Magez, S. & Black, S.J. (2011). *T. brucei* Infection Reduces B Lymphopoiesis in Bone Marrow and Truncates Compensatory Splenic Lymphopoiesis through Transitional B-Cell Apoptosis. *PLoS Pathogen* **7**(6): e1002089. doi:10.1371/journal.ppat.1002089.
- Boettner, D.R., Huston, C.D., Sullivan, J.A. & Petri, W.A. Jr. (2005). *Entamoeba histolytica* and *Entamoeba dispar* Utilize Externalized Phosphatidylserine for Recognition and Phagocytosis of Erythrocytes. *Infection and Immunity* **73**(6): 3422-3430.
- Carrero, J.A. & Unanue, E.R. (2006). Lymphocyte apoptosis as an immune subversion strategy of microbial pathogens. *TRENDS in Immunology* **27**(11): 497-504.
- Dawes, C.J. (1979). Biological techniques in electron microscopy. Barnes and Noble, New York.
- Happi, N.A., Danny, A.M. & Antia, R.E. (2012). Blood and Tissue Leukocyte Apoptosis in *Trypanosoma brucei* Infected Rats. *Journal of Neuroparasitology* **3**: doi:10.4303/jnp/N120101.
- Helmby, H., Nsson, G.J. & Troye-Blomberg, M. (2000). Cellular Changes and Apoptosis in the Spleens and Peripheral Blood of Mice Infected with Blood-Stage *Plasmodium chabaudi chabaudi*. *AS Infection and Immunity* **68**(3): 1485-1490.

- Holznagel, E., Hofmann-Lehmann, R., Leutenegger, C.M., Allenspach, K., Huettner, S., Forster, U., Niederer, E., Joller, H., Willett, B.J., Hummel, U.R.S., Rossi, G.L., Schupbach, J.O. & Lutz, H. (1998). The Role of In Vitro-Induced Lymphocyte Apoptosis in Feline Immunodeficiency Virus Infection: Correlation with Different Markers of Disease Progression. *Journal of Virological Methods* **72**(11): 868-874.
- Huston, C.D., Boettner, D.R., Miller-Sims, V. & Petri, W.A. (2003). Apoptotic killing and phagocytosis of host cells by the parasite *Entamoeba histolytica*. *Infection and Immunity* **71**: 964-72.
- Kapoor, G.I., Bagai, U. & Banyal, H.S. (2011). *Plasmodium berghei* induces apoptotic changes in splenic and peripheral blood cells. *Tropical Biomedicine* **28**(1): 119-124.
- Khan, I.A., Matsuura, T. & Kasper, L.H. (1996). Activation-mediated CD41 T cell unresponsiveness during acute *Toxoplasma gondii* infection in mice. *International Immunology* **8**: 887-896.
- Kerr, J.F., Wyllie, A.H. & Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer* **26**: 239-257.
- Kristjanson, P.M., Swallow, B.M., Rowlands, G.J., Kruska, R.L. & De Leeuw, P.N. (1999). Measuring the costs of African animal trypanosomiasis, the potential benefits of control and returns to research. *Agricultural Systems* **59**: 79-98.
- Liesenfeld, O., Kosek, J.C. & Suzuki, Y. (1997). Gamma interferon induces Fas-dependent apoptosis of Peyer's patch T cells in mice following peroral infection with *Toxoplasma gondii*. *Infection and Immunity* **65**: 4682-4689.
- Lima, R.G., Van Weyenbergh, J., Saraiva, E.M., Barral-Netto, M., Galvao-Castro, B. & Bou-Habib, D.C. (2002). The replication of human Immunodeficiency virus type 1 in macrophages is enhanced after phagocytosis of apoptotic cells. *Journal of Infectious Diseases* **185**: 1561-1566.
- Lopes, M.F., da Veiga, V.F., Santos, A.R., Fonseca, M.E.F. & DosReis, G.A. (1995). Activation-induced CD41 T cell death by apoptosis in experimental Chagas disease. *Journal of Immunology* **154**: 744-752.
- Naessens, J. (2006). Bovine trypano-tolerance: A natural ability to prevent severe anemia and hemophagocytic syndrome? *International Journal for Parasitology* **36**: 521-528.
- Paris, J., Murray, M. & McOdimba, F. (1982). A comparative evaluation of the parasitological techniques currently available for the diagnosis of African trypanosomiasis in cattle. *Acta Tropica* **39**: 309-316.
- Pino, P., Vouldoukis, I., Kolb, J.P., Mahmoudi, N., Livage, I.D., Bricaire, F., Danis, M., Dugas, B. & Mazier, D. (2003). *Plasmodium falciparum*-infected erythrocyte adhesion induces caspase activation and apoptosis in human endothelial cells. *Journal of Infectious Diseases* **187**: 1283-1290.
- Radwanska, M., Guirnalda, P., De Trez, C., Ryffel, B., Black, S. & Magez, S. (2008). Trypanosomiasis Induced B Cell Apoptosis Results in Loss of Protective Anti-Parasite Antibody Responses and Abolishment of Vaccine-Induced Memory Responses. *PLoS Pathogen* **4**(5): e1000078. doi:10.1371/journal.ppat.1000078.
- Rovere-Querini, P. & Dumitriu, I.E. (2003). Corpse disposal after apoptosis. *Apoptosis* **8**(5): 469-79.
- Sanchez-Torres, L., Rodriguez-Rippon, A., Aguilar-Medina, M. & Favila-castillo, L. (2001). Mouse splenic CD4+ and CD8+ T cells undergo extensive apoptosis during *P. chabaudi chabaudi AS* infection. *Parasite Immunology* **23**: 617-626.
- Schaumburg, F.D., Vutova, H.P. & Lüder, C.G.K. (2006). Pro- and anti-apoptotic activities of protozoan parasites. *Parasitology* **132**: S69-S85.

- Schlegel, R.A., Callahan, M., Krahling, S., Pradhan, D. & Williamson, P. (1996). Mechanisms for recognition and phagocytosis of apoptotic lymphocytes by macrophages. *Advances in Experimental Medicine and Biology* **406**: 21-28.
- Silva, E.M., Guillermo, L.V., Ribeiro-Gomes, F.L., De Meis, J., Nunes, M.P., Senra, M.B., Dosreis, G.A. & Lopes, M.F. (2007). Caspase inhibition reduces lymphocyte apoptosis and improves host immune responses to *Trypanosoma cruzi* infection. *European Journal of Immunology* **37**(3): 738-746.
- Solary, E., Dubrez, L. & Eymen, B. (1996). The role of apoptosis in the pathogenesis and treatment of diseases. *European Respiratory Journal* **9**: 1293-1305.
- Stahnke, K., Fulda, S., Friesen, C., Strauß, G. & Debatin, K.M. (2001). Activation of apoptosis pathways in peripheral blood lymphocytes by *in vivo* chemotherapy. *BLOOD* **98**(10): 3066-3073.
- Stiles, J.K., Whittaker, J., Sarfo, B.Y., Thompson, W.E., Powell, M.D. & Bond, V. (2004). Trypanosome apoptotic factor mediates apoptosis in human brain vascular endothelial cells. *Molecular & Biochemical Parasitology* **133**: 229-240.
- Thompson, C.B. (1995). Apoptosis in the pathogenesis and treatment of disease. *Science* **267**: 1456-1462.
- Toure-Balde, A., Sarthou, J.L., Aribot, G., Michel, P., Trape, J.F., Rogier, C. & Roussilhon, C. (1996). *Plasmodium falciparum* induces apoptosis in human mononuclear cells. *Infection and Immunity* **64**: 744-750.
- Yoshino, N., Ryu, T., Sugamata, M., Ihara, T., Ami, Y., Shinohara, K., Tashiro, F. & Honda, M. (2000). Direct Detection of Apoptotic Cells in Peripheral Blood from Highly Pathogenic SHIV-Inoculated Monkey. *Biochemical and Biophysical Research Communications* **268**: 868-874.
- Zuniga, E., Motran, C.C., Montes, C.L., Yagita, H. & Gruppi, A. (2002). *Trypanosoma cruzi* infection selectively renders parasite-specific IgG+ B lymphocytes susceptible to Fas/Fas ligand-mediated fratricide. *Journal of Immunology* **168**: 3965-3973.