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Pathogenesis of Testiculopathy Induced by Malaysian Isolate of *Trypanosoma evansi*

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Abstract: The obscured pathogenesis of surra caused by *Trypanosoma evansi* with regards to reproductive failure formed the basis of this investigation. A total of 35 adult male rabbits were divided randomly into 6 groups (G1-G6) consisting of 5 animals each, based on the duration of exposure with an additional control group comprising of similar number of animals. Trypanosome-infected rabbits were inoculated intravenously with 1×10^5 trypanosomes/mL while the control group received phosphate saline glucose via. the same route. The Five rabbits were sacrificed and examined at 1 (G1)-6 (G6) months post-infection (p.i.). Apart from a decrease in packed cell volume, total erythrocyte count and hemoglobin concentration, the protozoan waspresent in blood 72 h p.i. The infection led to significant ($p<0.0001$) decreases in sperm concentration, motility and viability percentages and increases in the percentages of morphological abnormalities in the infected rabbits at one month's p.i. respectively while there was aspermia and 0% of motility, viability and morphologic parameters in the infected rabbits at 2-6 months p.i. Histopathological changes, especially aspermia were observed as early as 2 month p.i. The antigenic expression by Immuno Histo Chemistry assay (IHC) was prominent around the seminiferous tubules in the testicular blood vessels and in the testicular interstitial connective tissue. Moreover, immunopositive reaction was seen in the interstitium of the epididymis. Our findings indicated that the pathogenesis of Malaysian isolate of *T. evansi* in chronically infected laboratory animal model was associated with severe destruction of the testes and epididymides that caused sterility in the infected animals.

Key words: *Trypanosoma evansi*, testis, epididymis, rabbits, semen, sterility

INTRODUCTION

Trypanosoma evansi is the protozoal causative agent of surra (Cox, 2002) which can be mechanically transmitted via. blood sucking flies such as those of Tabanus and Stomoxys. Nevertheless, the infection is not only limited to bovine but spills over to equine, canine, feline, lapine and large murine (capybaras) and humans (Silva *et al*., 1995a, b; Herrera *et al*., 2004; Joshi *et al*., 2005; Tonin *et al*., 2011; Eberhardt *et al*., 2014). In Malaysia, *T. evansi* was isolated from deer, cattle, buffaloes, pigs and horses (Arunasalam *et al*., 1995; Elshafie *et al*., 2013; Nurulaini *et al*., 2007, 2013). Reproductive disorders caused by trypanosomes infection are those of alterations in sexual behavior and changes in the quality and quantity of semen (Al-Qarawi *et al*., 2004; Sekoni *et al*., 2010; Mbaya *et al*., 2011).

The precipitation of immune complexes beneath the basement membrane of the seminiferous tubules plus hormonal disorders following Trypanosoma infection harms the sequential divisions and differentiation of germ cells to yield normal spermatozoa (Al-Qarawi *et al*., 2004). In addition, other factors including anoxia (Habila *et al*., 2012), the toxic substances released by living parasites and dead host tissue may also contribute to testicular damage (Tizard *et al*., 1978; Morrison *et al*., 1981; Mbaya *et al*., 2012).

Rabbit is a suitable model of Trypanosomosis due to ease of blood sampling and absence of the lethally high parasitaemia (Costa *et al*., 2012). This will permit the investigation into the pathogenesis, the diagnosis, prevention and treatments with utmost goal of direct field application (Ramirez-Iglesias *et al*., 2011).

The current study was designed to study the effect of trypanosomosis on the semen quality in rabbits and the degree of damage in testis and epididymis at chronic stage of *T. evansi* infection throughout the period of 6 months which could reveal additional information

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regarding the pathogenesis of the disease has not been attempted before. The information could be of value in the continued use of trypanosome infected animals for breeding purposes.

MATERIALS AND METHODS

Experimental animals and design: The study was approved by the Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM/FPV/PS/3.2.1.551/AUP-R159). A total of 35 adult male New Zealand White rabbits weighing between 2-3 kg were purchased from local supplier (RK Northern Supplies, Malaysia). Animals were placed within individual cages in a fly-proof wire netted room with temperature ranging from 23-28°C. They were acclimatised for 14 days where a commercial diet (Gold Coin, Malaysia) and water was provided ad libitum. Haematological and biochemical examinations were performed twice during this period to ensure that the rabbits are free from trypanosome and were in perfect health. During this period the rabbits were dewormed by subcutaneous injection of Biomectin 1%.

Five rabbits which acted as the controls where intravenously injected with 1.0 mL of Phosphate buffered Glucose (PSG). The remaining 30 rabbits were also intravenously injected with 1.0 mL of blood containing 1×10^5 trypanosomes/mL *T. evansiisolated* from bovine in 2007 (Te002, Johor, Malaysia) and designated as follows, viz infected for 1-6 months p.i. were coined as G1-G6, respectively. Rabbits from G1-G6 were all killed and necropsied at the end of the stipulated period while that of control were killed after the 6 months period. The animals were first euthanized intramuscularly with ketamine (Ketamil, Australia) and xylazine (Ilium, Australia). At this instant, blood was collected from the heart via. intracardiac puncture. The rabbits were then euthanised by intracardiac injection with Dolethal at the dose of 250 mg/kg.

Parasitaemia evaluation: Following inoculation with *T. evansi*, the level of parasitaemia was determined twice weekly by the collecting blood through the central ear artery using Micro-Hematocrit Centrifugation Technique (MHCT) (Woo, 1969) and via. hemocytometer (Morrison *et al*., 2010).

Haematology: Complete haemogram was performed immediately after blood collection where PCV, total RBC and Hb concentration were measured using automated blood cell counter (Abbott Cell DYN 1700, USA).

Semen collection: Instantaneously following euthanasia, the scrotum was incised by a sterile blade where the testicular and epididymal portion was identified and separated. Epididymal tail was dissectedusing sterile blade subsequently, testicular semen was flushed into Eppendorf tube which contains 0.9% normal saline at body temperature and samples were examined almost immediately (Slott *et al*., 1994).

Rabbit spermatozoa evaluation: Microscopic examination was done under a bright field microscope (Eclipse E200, Nikon) for motility, viability, morphology and concentration of the spermatozoa at 400× magnification, using a warm (38°C) eosin-nigrosin stain (Evans *et al*., 1987) and sperm concentration was determined using a Neubauer haemocytometer (Obi *et al*., 2013). The percentage of live sperm was determined from 10 microscope fields by identifying sperm that did not take up the stain. Moreover, the percentage of abnormal sperm cells were calculated from a total of 200 sperm cells examined under oil immersion.

Histopathology scoring: At necropsy, the procuredtestes and epididymides were sliced to a 2×2 cm in size and fixed in Bouin's solution for 48 h. Routine histological processes were done by embedding the samples in paraffin wax, sectioning with a microtome at 4 μm and stained with H and E according to the standard procedure (Luna, 1968).

The method employed by Adamu *et al*. (2007) was used to grade the degree of testicular degeneration. About 50 cross sections of the testicular tubules were observed at 100× and the scoring of the testicular lesion was done as follows:

- 0 = Normal: when the depletion of spermatogenic cells and reduction in the germinal epithelial was >5% of testicular tissue
- 1 = Mild: when the depletion of spermatogenic cells and reduction in the germinal epithelial layers of testicular tissue was between 5-25%
- 2 = Moderate: when the depletion of spermatogenic cells and reduction of germinal epithelial layers was reduced by 26-50%
- 3 = Severe: when more than 50% depletion of spermatogenic cells and reduction of germinal epithelial layers

For the estimation of Epididymal Sperm Reserve (ESR) at least 50 cross sections of epididymal tubules were observed at 100x to assess the spermatozoal content. The ESR was then expressed according to the following equations:

$$ESR\ \% = \text{Number of ETCS with normal ESR}\times 100/50$$

$$\text{Or } ESR\ \% = \text{Number of ETCS with normal ESR} \times 2$$

ETCS = Epididymal Tubule Cross Sections

Preparation of primary antibody: Antisera were produced against *T. evansi* by inoculation albino mice weighing between 20-25 g with *T. evansi*. The 5 mice were injected intraperitoneally with 1×10^5 trypanosomes/mL. After this the mice were treated with berenil (Diminazeneaceturate) 10 mg/kg body weight by intramuscular route on day 2 and 3 p.i. On day 14, mice were injected subcutaneously with 0.2 mL of emulsion consisting of 1×10^5 trypanosomes/mL in 0.2 mL of PSG, pH 7.4, mixed with 10 µL Freund's complete adjuvant. This step was repeated on day 28 with a similar emulsion containing Freund's incomplete adjuvant. Mice were sacrificed on day 42. Blood was collected from each mouse via. cardiac puncture. The serum was separated and divided into small aliquots and stored at -20°C. This hyper-immune serum was used as a primary antibody after the dilution.

Immunohistochemistry: The immunohistochemistry assay was performed using DakoEnvision™ Detection Systems-HRP, Peroxidase/DAB, Rabbit/Mouse kit (Dako K5007). The 4 µm thick sections of testes and epididymides were placed on slides poly-D-lysine (silanised, SuperFrost® Plus, Germany). Deparaffinization in xylene and re-hydration in alcohol. Antigen retrieval was done by incubated the sections in citrate buffer in microwave. Endogenous peroxidase was blocked using 3% H_2O_2, while theunspecific proteins were blocked by PBS-BSA 5%. Dilute primary antibody (1/100) was added to cover the tissue sections and incubated at 37°C for 1 h. Secondary antibody Goat anti rabbit was applied to cover the section, incubated at 37°C for 30 min. Subsequently, the reactions were revealed using DAB Chromogen for 30 sec then the sections were counterstained with Harris hematoxylin stain. Control sections were incubated without primary antibody.

Scanning Electron Microscopy (SEM) Sections from testes and epididymides were cut and fixed in freshly prepared 4% glutaraldehyde for 24 h at 4°C. Then, the samples were washed with 0.1M sodium cacodylate bufferand post-fixed in 1% osmium for 2 h at 4°C, subsequently washed with the same buffer solution for 3 changes, 10 min each. The samples after that were dehydrated in a series of acetone and then dried in the critical dryer for half an hour. Lastly, the samples coated with gold in sputter coater and examined.

Statistical analysis: All data were expressed as mean±Standard Deviation (SD) which was later subjected to statistical analysis JMP 9 (SAS, QSAS Institute Inc, Cary, NC, USA). The data were statistically analyzed by One-way Analysis of Variance (ANOVA) and the means were compared using Tukey's test whereonly $p<0.05$ is considered significant.

RESULTS AND DISCUSSION

Clinical outcome: While control rabbits did not exhibit any clinical abnormality those from the G1-G6 showed variable clinical signs of fever, anorexia, loss of condition, emaciation, pale mucous membrane of eyes, oedema of testicles and in tissues around the anus, oedema of the face and corneal opacities. Following facial and eyelid oedema the affected rabbits began to scratch leading to blepharitis.

No parasite was detected by HCT in the control group during the study period. All infected rabbits developed parasitaemia on day 3 p.i. The first peak of mean parasite count per ml of blood was noticed in all groups on day 11 p.i. after that the waves of parasitaemia in all groups fluctuated throughout the infection period of 6 months.

Haemogram: Table 1 shows the haemogram of selected blood parameters in the animals during the experimental period. Rabbits in the infected groups have significantly ($p<0.0001$) lower RBC counts, Hb concentrations and PCV values commencing from G1 until the end of the experiment.

Semen parameters: Table 2 shows the mean values for seminal parameters during the study. The spermatozoa concentration, motility, viability and normal morphology decreased significantly ($p<0.0001$) in G1that progressed until none could be detected in the G2 and G6.

Gross pathology: Figure 1 shows the gross morphology of the testes of rabbits at different stages of infection. In the testes there were large areas of adhesion between necrotic tissue and the scrotum in the left testis in G1 and both testes in G2. In addition, hemorrhage in the epididymis and surrounding soft tissue that extended into the tunica albuginea in G1. There were testicular atrophy and deformation in the shape of G3 and G4. Additionally, in G5 and G6 there are several thick fibrous bands running throughout the testicular tissue.

Histopathology

Testes: Figure 2 shows the morphology of the testis of rabbits during the experimental period. While the tissues

Table 1: Selected haemogram of rabbits during the experimental period (mean±SD)

Groups	PCV (%)	RBC ($\times 10^6$/L)	Hb (g/L)
Control	37.0±1.00[a]	5.6±0.23[a]	118.0±3.39[a]
G1	27.4±1.34[b]	4.0±0.27[b]	85.4±6.19[b]
G2	25.3±1.89[b]	4.0±0.49[b]	82.5±5.00[b]
G3	22.5±5.32[b]	3.5±1.04[b]	73.2±17.00[b]
G4	23.3±1.26[b]	3.8±0.32[b]	75.0±3.83[b]
G5	24.0±1.16[b]	3.7±0.10[b]	78.0±5.00[b]
G6	24.0±1.00[b]	3.8±0.27[b]	78.0±7.21b

[a, b]Values within column bearing different superscript significantly differ at p<0.0001

Table 2: Semen parameters of rabbits infected with *T. evansi* (mean±S.D)

Parameters	Control	G1	G2-G6
Sperm concentration ($\times 10^6$/mL)	395.2±67.67[a]	88.0±17.07[b]	0.0±0.00[c]
Motility (%)	88.4±2.70[a]	11.4±2.19[b]	0.0±0.00[c]
Viability (%)	78.0±8.37[a]	22.0±5.70[b]	0.0±0.00[c]
Normal morphology (%)	83.2±2.78[a]	31.0±4.24[b]	0.0±0.00[c]
Abnormality (%)	16.8±2.78[a]	67.0±5.75[b]	0.0±0.00[c]
Abnormal head (%)	2.60±0.55[a]	8.0±2.00[b]	0.0±0.00[c]
Abnormal mid piece (%)	2.80±0.84[a]	12.0±3.08[b]	0.0±0.00[c]
Abnormal tail (%)	5.60±1.14[a]	14.6±2.30[b]	0.0±0.00[c]

[a-c]Values within rows bearing different superscript differ at p<0.0001

Table 3: Scores of testicular damage in *T. evansi*-infected rabbits (mean±SD)

Groups	No. of STCS examined	No. of STCS affected	STCS affected (%)	Grades
G1	50	28.0±1.87[a]	56.0±3.74[a]	Severe
G2-G6	50	50.0±0.00[b]	100.0±0.00[b]	Severe

[a, b]Values within column bearing different superscript differ at p<0.0001

Table 4: Epididymal sperm reserve in *T. evansi-infected* rabbits groups

Rabbits groups	No. of ETCS	No. of ETCS with normal ESR	ESR %
G1	50	7.0±1.58[a]	14.0±3.16[a]
G2-G6	50	0.0±0.00[b]	0.0±0.00[b]

[a, b] Different superscript significantly different (p<0.0001)

of rabbits in the control were within normal limits those of the G1-G6 showed varying degrees of testicular degeneration, spermatogenic depletion and germinal atrophy with or without fibrosis.

Table 3 shows the testicular lesion score of the infected rabbits during the experimental period. There is a markedly significant (p<0.0001) progression of lesions from G1 there after although, those changes between G2-G6 after this period has remained comparable.

Epididymides: Table 4 and Fig. 3 show lesion score and epididymal morphology of the rabbits during the infective stage. Rabbits sacrificed at G1 had moderate epididymal degeneration with approximately 14% epididymal sperm reserve. However, as time advanced (G2-G6), epididymal degeneration became much more severe along with inflammatory response, fibrosis, cystic dilatation and an epididymal sperm reserve of 0% (Table 4 and Fig. 3).

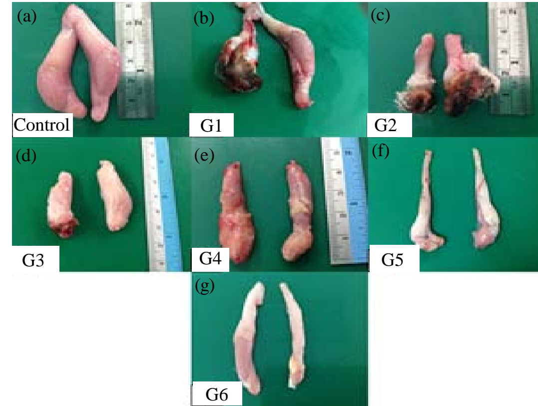

Fig. 1: a-g) Photograph of the testes of rabbits infected at different stages of the experimental period. Varying degrees of lesions comprising from necrosis at the early stages (G1 and G2) which later progresses into fibrosis (G3-G6)

Immunohistochemistry: In the testes, the antigenic expression appeared in the interstitium, blood vessels and surrounding the seminiferous tubules as a dark brown coloration (Fig. 4 and 5). Also, epididymides show expression of antigens represented in the interstitial tissue between epididymal tubules (Fig. 6).

Figure 7 shows an ultrastructure image of an extravascular *T. evansi* nearby abnormal sperm within epididymis tubule at G1.

The *T. evansi* used in this study caused clinical trypanosomosis in all infected rabbits, characterized by the reduction in body weight throughout the 6 months of the study period compared to the controls which incessantly gained a weight. Similar, findings of weight lost were also reported in rats infected with *T. brucei* (Eghianruwa, 2012), goats and sheep infected with *T. congolense* (Mutayoba *et al.*, 1989; Katunguka-Rwakishaya *et al.*, 1995) and goats infected with *T. evansi*, *T. brucei* and *T. vivax* (Dargantes *et al.*, 2005; Adeiza *et al.*, 2008). Those findings along with that of the present study denote that infection by trypanosome can be considered that of a wasting disease. The decrease in the body weight during trypanosomosis in the study reported here may be related to the anorexia most likely initiated via. pro-inflammatory cytokines (Pathak, 2009). Similarly, an increase in cytokine was seen in all infected rabbits further endorsing the possible role of cytokines in inducing weight loss following trypanosomosis. The deleterious effects of the released cytokines IL-1, IFN-γ and TNF-α has a directly or indirect reduction of feed intake. Their effect on glucose-sensitive neurons of the hypothalamus and through the stimulation

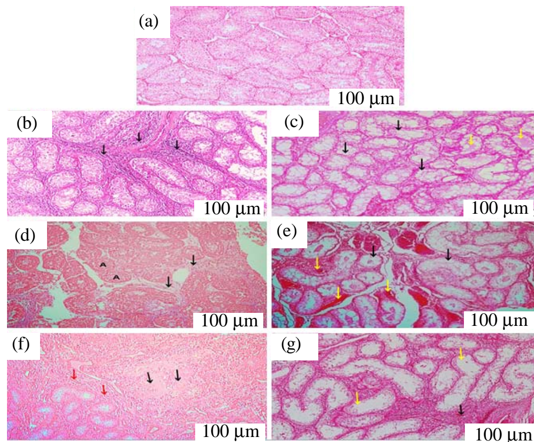

Fig. 2: Photomicrographs of testes in rabbits; a) control group showing normal seminiferous tubules containing full complement of spermatogenic cells; b) at G1: spermatogenic cells depletion in the seminiferous tubules and severe infiltration by mononuclear cells (arrows); c) at G2: severe necrosis and vacuolation of the seminiferous tubule (black arrows) leaving an intraluminal homogenous eosinophilic exudate (yellow arrows); d) at G3: severe coagulative necrosis of seminiferous tubules (A) with fibrosis (black arrows) and mononuclear cell infiltration; e) at G4: vacuolation of the wall of the seminiferous tubules (black arrows) with likely microthrombi (yellow arrows) and fibrosis; f) at G5: fibroplasia (red arrows) and ongoing coagulative necrosis of seminiferous tubules (black arrows) with infiltration of mononuclear inflammatory cells; g) at G6: degeneration and necrosis of seminiferous tubules (yellow arrows) with microthrombi (black arrows) with dilation of interlobular blood vessels (H&E)

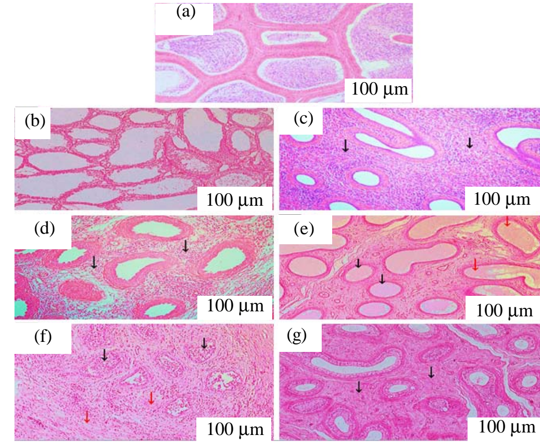

Fig. 3: Photomicrograph of epididymides in rabbits; a) control group showing an almost normal tubule with normal epididymal sperm reserve; b) at G1: a high degree of reduction in epididymal sperm reserve; c) at G2: severe degree of mononuclear infiltration (arrows) and absence of epididymal sperm reserve; d) at G3: beginning of fibroblast deposition (arrows), infiltration of mononuclear cells and absence of epididymal sperm reserve; e) at G4: apart from the absence of spermatozoa and ongoing fibroplasia, cystic dilatation of seminiferous tubules (black arrows) containing proteinous intraluminal fluid in intra-seminiferous tubules (red arrows) are present; f) at G5: fibroplasia (red arrows) and loss of architecture due to necrosis of seminiferous tubules with infiltration of mononuclear inflammatory cells and degenerated spermatids with vacuolated nuclei (black arrows); g) at G6: fibrosis (arrows) with infiltration of mononuclear cells and absence of epididymal sperm (H&E)

of the hypothalamic prostaglandin E2 synthesis which in turn stimulates the release of Corticotrophin Releasing Factor (CRF) from the hypothalamus (Dinarello *et al*., 1986; Plata-Salaman *et al*., 1988; Uehara *et al*., 1989).

Emergence of parasitaemia also differs between species where in goats infected with 5×10^3 and 5×10^4 trypanosome/mL, it appeared 2-7 days p.i. (Dargantes *et al*., 2005) while in cats infected with 1×10^8, an earlier appearance is seen, i.e., at 1-2 days p.i. (Aleksandro *et al*., 2009). In the current study, emergence of parasitaemia at 3 days p.i. is slightly longer than that of cats (Silva *et al*., 2010) but earlier than those of goats (Dargantes *et al*., 2005). The differences in the prepatency period may per chance be related to the differences in the dose size, strain virulence of *T. evansi* and animal species (Dargantes *et al*., 2005). Nevertheless, in the present study, the waves of the parasitaem ia followed by the initial detection of the parasite in the blood could probably be initiated to permit the parasite to evade the host's immune system via. antigenic variation of variable surface glycoprotein (Van der Lngh *et al*., 1976; Taylor and Rudenko, 2006).

Anaemia was detected to be the main clinical manifestation in cattle, sheep, goats, camel, horses, dogs, donkeys, buffaloes and cats infected with *T. evansi* (Payne *et al*., 1992; Goossens *et al*., 1998; Chaudhary and Iqbal, 2000; Marques *et al*., 2000; Aquino *et al*., 2002; Cadioli *et al*., 2006; Hilali *et al*., 2006; Silva *et al*., 2010). In the current study, there was a decline in the mean values of PCV, RBC and Hb from the second week p.i. onwards

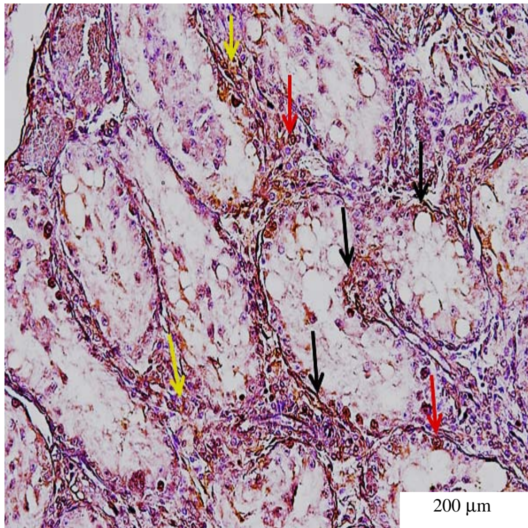

Fig. 4: Photomicrograph of testis at G4: antigenic reaction around the seminiferous tubules (black arrows), blood vessels (red arrows) and interstitium (yellow arrows) (IHC)

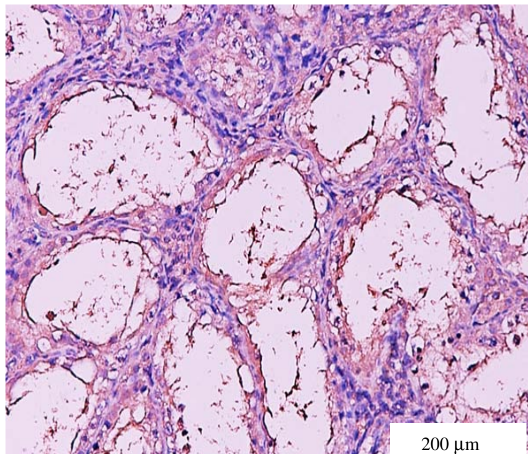

Fig. 5: Photomicrograph of testis at G6: positive reaction at the interstitium and basement membranes of the seminiferous tubules (IHC)

which was associated with the onset of parasitaemia. The reduction of erythrocyte indices continue in spite of the decrease in the parasitaemia during the study period. Severe anaemia in horse (Silva *et al*., 1995a, b) and cattle (Taylor, 1998) is regarded as the main cause of death in trypanosomosis. However, as shown in this study, rabbits probably were able to sustain life despite anaemia over a much longer period as compared to horses or cattle.

The deterioration in semen parameters was observed in natural and experimental trypanosomes

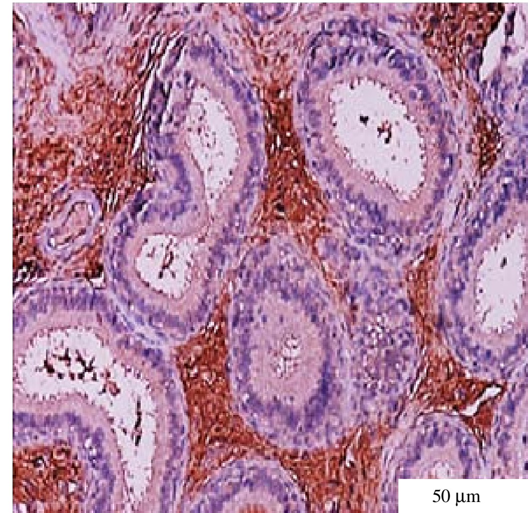

Fig. 6: Photomicrograph of epididymis at G6: antigenic expression exhibited by IHC in the interstitium

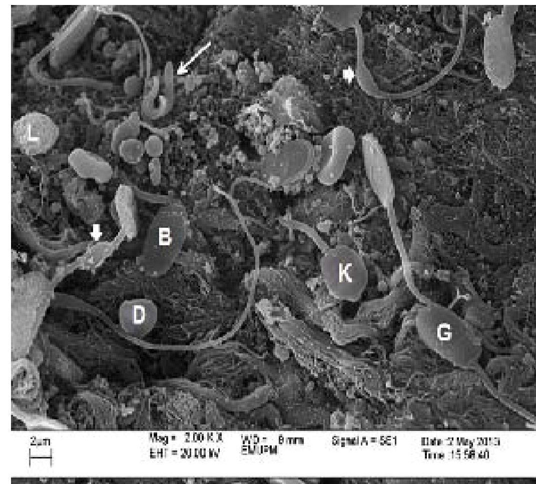

Fig. 7: Electromicrograph of Epididymis at G1, extravascular *T. evansi* (arrow) nearby abnormal sperms and leukocyte (L), distal droplet (head arrows), bent neck (B), detached head (D), knobbed acrosome defect (K), giant head with abnormal mid piece (G) (SEM)

infection in many speciesinfected with *T. evansi* and *T. brucei* (Al-Qarawi *et al*., 2004; Mbaya *et al*., 2012; Obi *et al*., 2013; Pradhan *et al*., 2013). Likewise, seen at G1 and progressed to aspermia at G2-G6 which may result from hyperthermia resulting from orchitis. Moreover, testicles become atrophied and movedcloser to the body cavity and the scrotum becomes necrotized; all these events later deteriorated spermatogenesis.

Testicular lesions including aspermia has been documented in bucks infected with *T. evansi* (Ngeranwa *et al.*, 1991; Dargantes *et al.*, 2005) and in bulls infected with *T. vivax* (Adamu *et al.*, 2007). Similarly, seen as a progression from G1-G6 the aspermia, interstitial infiltration, microthrombi are possibly direct sustained damage incurred by trypanosome may have contributed to infertility (Dargantes *et al.*, 2005). In the present study, the infected rabbits had severe testicular degeneration following G1 that has continued throughout the experimental period amounting to an epididymal sperm reserve of 0% (G2-G6). This indication is highly suggestive of complete cessation of spermatogenesis. Testicular and epididymal degeneration observed in the infected rabbits are similar to those reported by Adamu *et al.* (2007) in which the lesions were present in bulls infected with *T. vivax* between 14 and 56 days p.i.

The *T. evansi* can cause infertility commencing at an early stage (seen as early as G1 in this study) signifying that a long standing cases of infection is not a prerequisite for infertility. This finding also is in contrast to that documented by Sekoni *et al.* (1990) but corresponds to that by Anosa and Isoun (1980). Furthermore, in the study reported here at G2 the total epididymal sperm reserve is already at 0% contradicting findings by Sekoni *et al.* (1990) that claimed for this to happen after a period of 30 weeks. It appears that the mechanism to testicular damage at an early stage denotes that the changes that happened at G1 are irreversible. Similar finding that is in agreement to that concluded here is documented by Akpavie *et al.* (1987) and Leigh and Fayemi (2010).

Trypanosomosis can result in a decrease testicular steroidogenesis following destruction of Leydig cells which in turn is seen as subsequent depletion of spermatogenic cells (Adamu *et al.*, 2007). In the development of testicular and epididymal damage, many pathophysiological mechanisms were incriminated including pyrexia, anoxia related to anaemia, the ability of trypanosomes to produce biologically active toxins and immunological factors (Morrison *et al.*, 1981; Sekoni, 1988). All the signs and lesions were seen in the study presented here which partially imply that the likelihood of trypanosomosis-induced infertility to be multifactorial in nature.

Serum from mice infected with *T. evansi* was used in the current study as a source of polyclonal antibodies to detect trypanosome antigens in the tissues by IHC. The method has been used successfully to detect trypanosome antigens in tissues of water buffalo and rat infected with *T. evansi* (Sudarto *et al.*, 1990) in the brains of hog deer (Tuntasuvan *et al.*, 2000) and in the brain of horses which were naturally infected with *T. evansi* (Rodrigues *et al.*, 2009). Trypanosomes of the Brucei group are known to invade interstitial spaces of tissues causing infiltration of mononuclear cells and vasculitis (Losos and Ikede, 1972; Ikede and Losos, 1972).

Therefore, in the present study the detection of the parasitic antigen extravascularly was harmonic with the previous reports that was done on *T. brucei* and with *T. evansi* belonging to the same group (Losos, 1980). As *T. evansi* as a connective tissue parasite, thus IHC is the most reliable method for the detection of trypanosomes antigens in the tissue (Sudarto *et al.*, 1990). The parasites number in the peripheral blood was very low during the chronic stage of Trypanosoma infection and the parasites inhabited deep blood vessels in the case of low parasitaemia (Mahmoud and Gray, 1980). Therefore, this explained the immunopositive antigenic expression of *T. evansi* appears in blood vessels and tissue of testes and epididymides in the current study.

CONCLUSION

In conclusion, the morphological changes in testicles of *T. evansi* infected rabbits arises either as an outcome of cellular damage because of the toxicants released by the parasite, tissue responsesor due to its immunological reactions. It also confirms that the damage in testicles is irreversiblydestructive with lethal effect and that the infection by the Malaysian isolate of *T. evansi* can cause testicle and epididymal damages resulting in infertility or sterility of the affected rabbits.

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