Disruption of reproductive cycles in rats (*Rattus norvegicus*) acutely infected with *Trypanosoma brucei brucei* (*T.b.b*) is associated with Hyperthermia and, ovarian and pituitary gland tissue degenerations

N. J. Masimba¹, N. G. Magak², M Ndiema³, R. Odera², L. Chemwolo², M. D. Minjire², D. K. Ndungu²

1. Department of Biological Sciences, Chepkoilel University College, P.O. Box 1125–30100, Eldoret
2. Department of Medical Physiology, School of Medicine, Moi University, P.O. Box 4606–30100, Eldoret
3. Department of Anatomy, School of Medicine, Moi University, P.O. Box 4606–30100, Eldoret

**Correspondence to:** Ng’wena G. Magak; Department of Medical Physiology, School of Medicine, Moi University, P.O. Box 4606–30100, Eldoret; Email: gngwena@hotmail.com

**SUMMARY**

**Background:** Trypanosomosis causes reproductive dysfunction and reversal of sleep/wake cycles but the mechanisms are not well understood.

**Objectives:** To investigate the effects of *Trypanosoma brucei brucei* infection on the temperature, estrous cycle, and ovarian and anterior pituitary gland tissues in non–pregnant female rats.

**Methods:** This was a randomized experimental animal study in which twenty four reproductively mature non–pregnant rats were kept in cages, fed on mice pellets and exposed to 12/12 hours of dark/light cycles. Reproductive cycles of each rat were monitored twice every day by vaginal smear cell profiling for two months before randomly dividing the rats into controls (4) and experimental (20) groups. Rectal temperatures were determined daily and screening for hemoparasites done fortnightly during the two months. Then the experimental rats were each infected with 2.0 x 10⁴ *Trypanosoma brucei brucei* parasites and the vaginal smear profiling, rectal temperature and parasitaemia monitoring continued for one month. The animals were then sacrificed and the ovaries and brain tissues harvested and processed for histological examination.

**Results:** The infection caused hyperthermia (39.1°C) and disrupted cyclicity in the experimental rats. Three rats’ cycles’ period was 6–7 days, seven rats 2–3 days while the rest became acyclic with persistent estrus. In the non–infected rats the cycles were 4–5days throughout the study period. The ovaries of infected rats had necrotic areas and mostly showed corpus luteum with more theca but few granulosa cells which were atretic. Several degenerating ova and poorly developed granulosa cells were present within the follicles. The anterior pituitary gland tissues showed pyknotic cells with degenerative changes in most areas. The tissues of the non–infected animals appeared normal.

**Conclusion:** Acute *T. b. b.* infection induced hyperthermia, disrupted cyclicity, and ovarian and anterior pituitary gland tissues degeneration in non–pregnant female rats.

**Key words:** *Trypanosoma brucei brucei*, Acute trypanosomosis, Hyperthermia, Estrous cycle, Reproductive dysfunction

Introduction:

Trypanosomosis, a complex insect–transmitted tropical infection is caused by protozoan hemoflagellates of the genus *Trypanosoma* [1]. Transmitted by the tsetse flies of the *Glossina* species, the disease is a major threat to livestock development in areas where the vectors are endemic. Sporadic cases of congenital transmission of trypanosomes have also been reported in humans [2, 3, 4]. Of the parasites’ subspecies *Trypanosoma brucei brucei* is considered the most virulent form to domestic animals.

To date no successful chemotherapeutic or prophylactic methods are available for the control and cure of trypanosomosis [5, 6]. The disease causes reproductive disorders in both male and female animals [7] and available reports have shown that it is associated with deterioration of semen quality and quantity and aspermia in male goats [8, 9, 10]. Testicular atrophy has also been reported with the presence of dead spermatozoa in the seminiferous tubules. Histological studies have revealed that the testicles of infected animals are devoid of spermatozoa and show blood vessels that contain microthrombi and inflammatory cells’ infiltration [9]. The disease causes diffuse calcification with extensive deposition of compounds that contain calcium ion thus obliterating the seminiferous tubule vesicles and ducts and also the epididymal ducts.

Omeke and Onuora [11] reported the presence of *Trypanosoma brucei brucei* and *T. congolense* in the genital tract, soft organs and the brain of infected boars. They also observed severe lesions resulting in the degeneration of testes which involved the Leydig cells, basement membrane, Sertoli and germ cells, with resultant loss of libido. It has also been reported that animals that survived after infection often remained infertile [12, 13].

In female animals, the disease is associated with irregular cycles and high hormonal decline in susceptible goats [14, 15]. Cases of abortion, still birth and neonatal death have also been reported in goats, sheep and rats [16, 17, 18]. Acute forms of the infections have been shown to induce infertility in mice and in some cases these have lead to embryos development arrest and degeneration [19]. Absence or reduced number of corpus lutea due to a decrease in the number of primordial and primary follicles and atresia of growing follicles have also been reported in goats [20]. Trypanosomosis is thought to cause infertility perhaps due to gonadal pathology [17, 21] which may be connected to damage to the blood–ovary–barrier that was reported by Kolle and his colleagues [22]. Although the clinical and pathological manifestation of trypanosomosis have been widely reported, there is limited information on the actual mechanisms underlying its effects on reproduction especially on the linkage between the central and peripheral changes that affects multiple tissues that are involved in the regulation of the reproductive physiology. The present study was therefore carried out to investigate the effects of acute *Trypanosoma brucei brucei* infection on the rectal temperature and pituitary gland and ovarian tissues in non–pregnant female rats.

Materials and Methods:

**Study animals**

Twenty four, reproductively mature female white albino laboratory rats, acquired from Kabete Campus of Nairobi University were transported to Chepkoilel Campus of Moi University, Eldoret, Kenya. The animals were kept in cages, fed on mice pellets, exposed to 12/12 hours of
dark/light cycles and rectal temperatures monitored daily. The rats’ reproductive cyclicity was monitored twice every day for two months through vaginal smear cell profiling as described below before they were randomly divided into controls (4 rats) and experimental (20 rats) groups. After the two months the 20 experimental rats were each infected with $2.0 \times 10^4$ Trypanosoma brucei brucei parasites and the vaginal smear profiling and rectal temperature monitoring continued for one month. The animals were then sacrificed and ovaries and brain tissues harvested for histological sectioning and examination.

**Reproductive cycle determination by vaginal smear cell profiling**

A wet sterile cotton swab was inserted at approximately 45° through the vulvar lips into the vagina of each rat while being held by the tail and restrained at the neck. The swab was rotated gently through two or three revolutions to collect a smear for vaginal cells. The smear was then transferred onto a clean glass microscope slide by rolling the swab on the surface of the slide and the cells were allowed to air dry before being fixed in 70% ethanol. The smear preparations were stained in Giemsa solution and then examined under the light microscope to identify the different estrus cycle cell (epithelial cells, cornified cells and leucocytes) profiles. The cell profiles were used to determine the estrus cycles stages; Estrus (Mainly cornified epithelial cells), Metestrus (Fewer leukocytes) Diestrus (many leukocytes) and Proestrus (Nucleated epithelial cells).

**Infection of experimental rats**

Trypanosoma brucei brucei parasites obtained from Kabete Campus of Nairobi University Kenya were passaged intraperitoneally into two adult rats and delivered to Moi University, Chepkoilel Campus where the study was conducted. One of the carrier animals was anaesthetized by use of ethyl ether and 1.5ml of blood obtained through a cardiac puncture. By use of the Neauber chamber, the parasitemia of the blood was estimated and then phosphate buffered saline solution (pH 7.4) used to dilute to give a parasite concentration of $2.0 \times 10^4$ parasites per ml before one ml of the diluted blood was given intraperitoneally to each of the experimental rats. The control rats were inoculated with an equivalent volume of normal saline. The rats were then monitored for parasitaemia, weight, temperature, and vaginal smear profile changes.

**Organs harvesting, tissue processing and observation**

At the end of the experimental period the rats were anesthetized with 10% chloroform, sacrificed and the pituitary, and ovaries harvested for histological processing and examination. Each tissue was first rinsed in phosphate buffered saline, fixed in 10% formalin and then frozen at – 70º C until required for processing. The tissues were removed from the freezer, dehydrated in ascending grades (50%, 70%, 90%, 95% and 100%) of alcohol and embedded in paraffin wax before being put into the automatic tissue processor. Sections of 5µm thickness were cut in HM 310 rotary microtone and mounted on Mayer’s egg albumin coated glass slides. Each section was dewaxed in two changes of xylene for two minutes then rehydrated through descending grades of alcohol (100%, 95%, 90%, 70%, and 50%) and further washed in tap water. The sections were then stained with Harris haematoxylin and counterstained with eosin (1% for 2min) according to Mallory’s method [23]. After staining the sections were dehydrated in ascending grades of alcohol (70%, 80%, 95%, and 100%), cleared...
in three changes of xylene and mounted in DPX, covered with cover slips and examined microscopically.

Results

The effects of *T. brucei brucei* infection on the reproductive cycle pattern in non-pregnant rats

The results of the present study showed that, in all the non-infected rats’, reproductive cycles occurred every 4–5 days throughout the study periods (Table 1).

Table 1: Pre- and post-infection mean number of days per estrous cycle of non-infected and *T. b. b.*-infected non-pregnant rats *(A cycle-number of days between two consecutive estrous stages, zero days was indicated for persistent estrus)*

<table>
<thead>
<tr>
<th>Rat Group</th>
<th>Pre-infection period</th>
<th>Post-infection period</th>
<th>Most affected cycle stages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cycle days (number of rats) (n=24)</td>
<td>Cycle days (Number of rats) (n=24)</td>
<td></td>
</tr>
<tr>
<td>Group1(n=4)</td>
<td>5(n=4)</td>
<td>0 (n=2): 6 (n=2)</td>
<td>Estrus+++ and Proestrus**</td>
</tr>
<tr>
<td>Group2(n=4)</td>
<td>5(n=4)</td>
<td>2 (n=2): 6 (n=2)</td>
<td>Diestrus* and Proestrus**</td>
</tr>
<tr>
<td>Group3(n=4)</td>
<td>4(n=1); 5 (n=3)</td>
<td>0 (n=2): 6 (n=2)</td>
<td>Estrus+++; Diestrus* and Proestrus**</td>
</tr>
<tr>
<td>Group4(n=4)</td>
<td>4 (n=2); 5 (n=2)</td>
<td>0 (n=2): 3 (n=2)</td>
<td>Estrus+++ and Proestrus</td>
</tr>
<tr>
<td>Group5(n=4)</td>
<td>4 (n=2); 5 (n=2)</td>
<td>0 (n=2): 7 (n=2)</td>
<td>Estrus+++; Diestrus* and Proestrus**</td>
</tr>
<tr>
<td>Group6(n=4)</td>
<td>4 (n=2); 5 (n=2)</td>
<td>4 (n=2); 5 (n=2)</td>
<td>None</td>
</tr>
</tbody>
</table>

+++–Persistent cycle stage; *–Missing cycle stage; **–Prolonged cycle stage

The *T. b. b.*-infected rats exhibited disrupted reproductive cycles with some of the rats’ cycles occurring after six (n=6) and seven (n=2) days while others exhibited very brief cycle periods of 2 (n=2) or 3(n=2) days with specific stages of the reproductive cycles being absent in some of the infected rats (Table 1). Where there was persistent estrus (zero cycle days), the cells of other cycles stages (proestrus, diestrus and metestrus) were not observed in the vaginal smear preparations made over a period of four weeks. In the brief cycle stages the diestrus stage was not observed in most of the vaginal smears whereas in the prolonged cycles the proestrus stage was longer (2–3 days) than it is normally (brief; hours and not days) expected.
Effect of *Trypanosoma brucei brucei* infection on rectal temperatures of non-pregnant female rats

The mean rectal temperature of all the non-infected rats ranged between $36.6^\circ$C--$37.7^\circ$C (Figure 1) and there was no significant difference ($p>0.05$) in the mean rectal temperatures of the control rats in the pre-infection period as compared to the post-infection period. The *T.b.b.*-infected rats had significant ($p<0.05$) increase in the mean rectal temperatures in the post-infection period to a plateau of $39^\circ$C on the 20th day which persisted until the animals were sacrificed. There was significant ($p<0.05$) difference between the mean rectal temperatures of the control rats (range; $37.0^\circ$C--$37.7^\circ$C) and *T. b. b.*-infected rats (range; $37.0^\circ$C--$39.1^\circ$C) in the post-infection period. These findings show that *T. b. b.*-infection caused thermal dysregulation in the infected female rats.

**Histology of the ovarian tissue**

The histological examination on the ovarian section taken from the control rats revealed normal follicular development with several follicles in different stages (Fig 2A).

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**Figure 1**: Pre- and post-infection mean rectal temperatures of *T. b. b.*-infected and non-infected non-pregnant female rats.
The mature follicles consisted of a single oocyte surrounded by stratified layers cuboidal or rounded granulosa cells separated from the stromal cells exhibited normal maturation features. The sections showed the development of the antrum, the presence of eccentric oocyte and typical features of a secondary follicle. The ovarian follicles exhibited clear zona pellucida region, and normal oocyte. In some sections fluid–filled spaces appeared to be separating the granulosa cells. These usually enlarge and finally coalesce to form a single large antrum as follicular maturation reaches its final stage before it is ovulated. The ovary of the *T. b. brucei*–infected rats on the other hand showed extensive atresia and degeneration of several follicles (Fig 2B). The process of atresia is usually characterized by cessation of mitosis in the granulose cells, detachment of granulosa cells from the basal lamina and degeneration of the oocyte. The follicular cellular layers appeared completely disrupted, and the theca interna cells appeared severely degenerated, and the granulosa cells which are supposed to be cuboidal were shrunken with extensive pyknosis (Fig 2B). The massive atresias observed in the ovarian sections were associated with completely disrupted degenerated oocyte. The ovarian sections of the infected rats did not exhibit clear maturation features of the follicle involving the antrum, zona pellucida and cumulus oophorus. The results in this part showed that *T. b. brucei*–infection was associated with disruption of the ovarian cellular structures and possibly the functions which include regulation of reproductive cycles.

**Histology of the adenohypophyseal tissue**

The histological examination of the adenohypophyseal part of the pituitary gland showed normal acidophil, basophil and chromophobe cells with clearly visible large nuclei and nucleoli in non–infected rats (Fig 3A & B).

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Figure 2: Histological changes in the ovarian tissues of Control rats (A) and Experimental rats acutely infected with *T. b. brucei* parasites (B).
Figure 3: Adenohypophyseal tissues histology of control rats (A) and T. b. b. infected experimental rats (B).

The cells were clearly distinguishable from one another and extensive vascularization was evident in this region. These results were indicative of a normal tissue structure that would synthesize, release and secrete the relevant chemical messengers (hormones) from this region of the pituitary gland in the control rats. The adenohypophyseal tissues of the T. b. brucei-infected rats showed shrunken pyknotic cells with extensive cellular degenerative changes (Fig 3B). Acute T. b. brucei infection caused structural disruption of the adenohypophysis, an outcome which may also be disruptive to the endocrine processes of this region of the pituitary gland.

Discussion

This study has demonstrated that reproductive dysfunction in T. b. brucei–infected non-pregnant rats was characterized by disrupted estrous cycles, hyperthermia and degenerative ovarian and pituitary tissues. The infected animals exhibited irregular (short or long cycle periods) cyclicity and/or acyclicity as compared to the regular, 4–5 day cycles in all the non-infected reproductively mature non-pregnant female rats that were used in the present study. These findings have provided evidence that the trypanosomosis–induced reproductive dysfunction is multifactorial and is associated with disruption of the hypothalamic–pituitary–gonadal axis functioning.

Reproductive processes in female mammals are characterized by cyclic alterations in the female gonads, tract and in sexual receptivity [24, 25]. The metabolic processes that take place in the reproductive system require an optimal temperature of between 36.6°C and 37.7°C. Therefore, normal tissues and cellular integrity of the hypothalamus, pituitary and the ovaries are indicators of fertility in all mammalian females. The cyclic events are regulated by Gonadotropin releasing hormone (GnRH) from the hypothalamus, luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary and, progesterone and estrogen from the ovaries. In each reproductive cycle, follicular development and maturation of oocyte normally occur in the ovaries. Any factors acting centrally in the brain, or peripherally on gonadal tissue, that may affect the integrated functioning of the hypothalamus, pituitary and the ovaries, have the potential of causing infertility due to disrupted follicular development, failure of oocyte maturation and altered uterine tissue changes. Trypanosomosis has been reported to release substances that may cause their effects either centrally or peripherally.

Studies have reported that elevated core body temperature or hyperthermia which leads to heat stress disrupt reproduction [26]. Hyperthermia resulting from trypanosomosis, through homeokinesis, may lead to redistribution of blood flow from the body core to the periphery or inhibition of the feeding center. Beside, high temperatures may disrupt hormone biosynthesis and release from the hypothalamus, pituitary and ovaries. Furthermore, heat stress leads to homeokinetic system failure in regulating reproductive physiology [26]. When redistribution of blood flow occurs, substances that are supposed to flow to the reproductive structures may be diverted to other structures thus disrupting the synchrony of tissues responses in the female reproductive system. This may also be a cause of the disrupted reproductive cycle. The homeostatic redistribution of blood may affect hormones, electrolytes and soluble enzymes all of which are required at specific concentrations in order to maintain cyclicity. It has been demonstrated that
hyperthermia occurring in cows 10 days before estrus was associated with low fertility [27]. Hyperthermia has also been reported to affect the endocrine profiles of reproductive hormones. High effective ambient temperature (HEAT) and a change in an animal’s physiological core temperature is a major factor responsible for low reproductive performance in domestic animals during hot season [28]. Reproduction–related endocrine profiles may also be affected by HEAT, which some studies have associated with decline in progesterone and estrogen levels [29, 30]. These factors could directly or indirectly modify female hormonal profile and in turn alter reproductive physiology [29, 31]. Heat stress can alter follicular growth [32, 33], steroid secretion [31, 34, 35], LH secretion [36] and gene expression [37]. Studies carried out in goats demonstrated that in addition to heat stress reducing plasma estradiol concentration it also reduced aromatase activity and LH receptor levels and delayed ovulation [35, 38]. Effects of heat stress on follicular functions therefore could involve changes at the level of the follicle or the secretion of the pituitary hormones that control development of follicles. Studies have also reported that process of oocyte maturation is disrupted at elevated temperatures [39, 40, 41, 42]. Elevated rectal temperatures observed in the present study may have had a direct or indirect role in the disruption of reproductive cyclicity. It is highly possible that the observed hyperthermia induced heat stress which probably disrupted the endocrine profiling. The findings of the present study are in agreement with the findings of Ochiogu who reported that rats trypanosomosis caused by *T. brucei brucei* has very serious negative implication on female reproduction [43]. He reported that the disease affects all the indices of reproduction and the general productivity of infected rats. Sekoni also reported that trypanosomosis was among the important diseases which cause various reproductive disorders in both males and females [7].

Hyperthermia causes oxidative stress in cells and tissues by generating the superoxide anion (O$_2^-$ or hydrogen peroxide H$_2$O$_2$) [44]. It is highly likely that this may be one of the mechanism underlying trypanosomosis–induced reproductive dysfunction. These factors may be associated with degenerative changes that were observed in the present study in both the pituitary and the ovaries of the infected female rats. Mutayoba and others also reported degenerative changes in the gonads of goats [14, 45]. Available reports from other studies have shown that damage to the oocyte during the preovulatory period by heat shock appears to involve the generation of reactive oxygen species and antioxidants have been shown to reduce the effects of heat stress *in vivo* and *in vitro* [46, 47]. Apoptosis which damages cells has been implicated in effects of thermal stress on the maturing oocyte [48, 49, 50]. The degenerative changes observed in the ovarian tissues of *T. b. brucei* infected rats of the present study may also account for the observed disrupted reproductive cycles. Functionally the ovaries are responsible for oocyte development and hormone production and these processes are regulated by the anterior pituitary gland hormones. Follicular growth and maturation is stimulated by follicle–stimulating hormone and luteinizing hormone from the adenohypophysis. Luteinizing hormone receptors are located on the theca cells of the follicles while granulosa cells synthesize receptors for FSH and estrogens. The degenerative changes in the pituitary probably disrupted the synthesis and release of the LH and FSH thus leading to disrupted reproductive cycles in the *T. b. brucei*–infected rats. The degeneration of ovarian tissues may have caused LH and FSH receptor down regulation
or desensitization of the cells to circulating adenohypophyseal hormones and hence lack of stimulation. The probable adenohypophyseal-ovarian receptor disruption may have led to a disruption of ovarian endocrine functions which have been observed in some previous studies. Ovarian degeneration may have also affected the theca and granulosa cells’ formation and functioning, oocyte growth, maturation and ovulation, and corpus luteum development and functioning which are all prime prerequisites for a normal reproductive process.

The pituitary glands of infected rats were investigated in order to assess trypanosomosis’ effects on the central regulatory mechanisms. Due to the gland’s vital hormonal controls, disorders of the pituitary gland can cause wide spectrum of symptoms. The adenohypophysis of the T. b. brucei-infected rats used in the present study showed shrunken cells with extensive degenerative changes. This structural disruption of the adenohypophysis may have caused a disrupted endocrine process and hence impaired regulation of the gonadal activities in the female rats. Trypanosomosis-induced pituitary dysfunction has been reported by several studies [15, 20]. When degenerative changes occur in the pituitary gland, there could be total inhibition of hypothalamic hormone activity or decline in the gonadotrophes population that would result in a decline in the levels of LH and FSH. The degenerative changes may have also affected the vasculature in addition to the endocrine cells and if this occurred then the transport of hormones may also become impaired.

The findings of the present study have confirmed that trypanosomosis causes reproductive dysfunction and has provided evidence that the dysfunctions are directly or indirectly caused by hyperthermia. The study has also shown that T. b. brucei infections causes degenerative changes in the pituitary and ovaries and that these changes disrupted the pituitary–gonadal axis functioning with resultant irregularity in reproductive cycles and/or acyclicity in non-pregnant female rats.

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