Original Article

Hormonal changes in *Trypanosoma evansi*-infected *Rattus norvegicus*: An approach to understand host-parasite interaction

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ABSTRACT

Background: Several studies were conducted on *T. evansi* infections in camels, but its pathogenesis is still mysterious especially in association with hormonal and infertility problems.
Objective: The present research aimed to monitor the endocrine changes associated with *T. evansi* infection and its relation to immunity.
Material and Methods: Twenty-four rats were used, four of them were considered the control group (CG), and the others were infected with *T. evansi* (IG). Blood samples were collected from CG at 0 day, while blood samples and testicular specimens were collected from IG on the 7th, 14th, 21st, 28th, and 35th day (4 rats each time period). Cytokines, hormones, minerals, and electrolytes were measured, and the testicular specimens were sectioned, stained, and microscopically examined.
Results: There was a significant increase in the pro-inflammatory cytokines accompanied by significant (P<0.05) hypercortisolemia, increased growth hormone (GH) and thyroid stimulating hormone (TSH) levels, hypothyroidism, hypoinsulinemia, decreased testosterone, minerals, and electrolytes concentrations. Pro-inflammatory cytokines positively correlated with cortisol, growth hormone, and TSH and negatively correlated with T3, T4, insulin, and testosterone. The measured hormones yielded good values of sensitivity, specificity, and accuracy rate. The histopathological examination of the testicular tissue showed degeneration and necrosis of the spermatocytes and seminiferous tubules and hyperplasia of the interstitial tissue with edema, congestion, and infiltration.
Conclusion: The recorded immunological changes during trypanosomiasis are strongly associated with the hormonal changes and infertility, and decreased minerals and electrolytes levels. Cortisol, T3, T4, and GH are proposed good indicators of trypanosomiasis.

Keywords: cytokines; histopathological; hormonal changes; sterility; *T. evansi*.

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INTRODUCTION

Trypanosomiasis is a major limitation to livestock production in the African continent. It attacks a wide section of the animal kingdom as it has several species, and *T. evansi* is one of its dangerous species. It mainly targets dromedaries, causing economic losses such as death, emaciation, poor meat and milk production, sterility in males and females, besides costly treatment[1,2]. Infection by *T. evansi* stimulates different body systems to control and restrict its harmful effect. The most affected systems during the disease course are the immune and endocrine systems. Both of them are vital for keeping body hemostasis and harmony. The immune system uses its tools (cytokines, acute phase proteins, free radicals, immunoglobulins) for destroying pathogenic enemies, while the endocrine system maintains the proper metabolism of body organs through its hormones[3,4]. Previously, the two systems were thought to work separately. Recent studies referred to their close interaction during different types of infections, especially regarding glucose metabolism regulation. Cytokines alter the peripheral organs response to the endocrine signals and changes the hormones blood levels; thus, promoting the ability of the body to fight infection. No doubt, understanding the immunoendocrine interaction during trypanosomiasis is essential for developing new drugs, finding vaccine targets, and devising new therapies for several infectious diseases[5-7].

Hence, this research aimed to assess the immune-endocrine interaction and its role in sterility pathogenesis in experimental infection with *T. evansi* in rats with special emphasis on histopathological

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alterations in the testicular tissue. Additionally, the study evaluated the diagnostic and prognostic value of hormonal assays in *T. evansi* infection.

MATERIAL AND METHODS

This experimental descriptive analytical study was conducted at the Parasitology Department, Faculty of Veterinary Medicine, Menoufia University, Egypt during the period from August, 2022 to September, 2022.

Study design: Male rats were injected with *T. evansi* trypomastigotes. Blood samples and testicular specimens were collected on the 7th, 14th, 21st, 28th, and 35th day. Cytokines and hormones were estimated. Testicular specimens were sectioned, stained, and microscopically examined.

Animals: Twenty-four male rats (*Rattus norvegicus*) with an average body weight of 200 g were kept in well-ventilated cages and fed on enough chow and water.

Preparation of *Trypanosoma* inoculum: The inoculum was immediately prepared from the serum of naturally infected camels, diagnosed and identified[8]. The inoculum was continuously passed in Swiss albino mice of mixed sexes (weighing 20-25 g) to produce parasitemia of approximately 105 parasites/ml. Mice were injected intraperitoneally with 0.01 ml of blood containing approximately 10^3 trypomastigotes. Blood films were prepared from the infected mice, and the trypomasigotes count was determined using the "rapid matching" method[9].

Rats' infection and collected samples: After rats' acclimatization, blood samples (3 ml) were aspirated *via* cardiac puncture from 4 rats without *T. evansi* infection (0 day) and were considered as the control group (CG). The other twenty rats (*T. evansi*-infected group, IG) were intraperitoneally injected with 0.5 ml inoculum containing ~ $2X10^3$ trypomastigotes. Blood samples obtained by cardiac puncture and tissue specimens from the testis were collected on the 7th, 14th, 21st, 28th and 35th day post-infection (PI), i.e., from 4 rats on each time period.

Parameters assessment: Coagulated blood samples were centrifuged at 3000 rpm for 20 min at 37^{o}C, and serum was separated in clean labelled Eppendorf tubes. Estimation of cytokines [IL-1β (Cat. No. MBS825017), and TNF-α (Cat. No.: MBS355371)]; hormones [cortisol (Cat. No. MBS727040), growth hormone (GH), (Cat. No: MBS019990), thyroid hormones T3 (Cat. No. MBS261285), T4 (Cat. No. MBS580037), and TSH (Cat. No. MBS701641)]; insulin (Cat. No. MBS281388), and testosterone (Cat. No. MBS9424769) levels were performed using kits supplied by MyBioSource Company®. Minerals (Ca[10], P[11], and Mg[12]), and electrolytes (Na[13], Cl[14], and K[15]) were spectrophotometrically measured using commercial kits of Biodiagnostic Company®. All manual instructions were strictly followed.

Histopathological examination: Testicular specimens were sectioned and fixed with 10% neutral buffered formalin solution. Later, these sections were stained using Hematoxylin and Eosin (H & E)[16].

Statistical analysis: SSPS program version 24 was used to compare statistically between means of different parameters (one-way ANOVA test) and calculate the correlations between the selected parameters (Pearson's simple correlation method). A difference was considered significant at $P<0.05$. In addition, graph pad prism version 8 program was used to calculate the area under the curve (AUC) cut-off points, sensitivity, specificity, and likely-hood ratio (LR) for the estimated hormones between IG and CG. The positive predictive value (PPV), negative predictive value (NPV), and accuracy rate were calculated as the result of dividing the number of true positive or true negative or the sum of both by the number of total positive or total negative or total population respectively, then multiplied by 100.

Ethical considerations: This experiment was approved in accordance with the ethical guidelines of the Animal Care and Use Committee of Faculty of Veterinary Medicine, Menoufia University, Egypt.

RESULTS

Throughout the experiment, *T. evansi* infection induced an innate immune response represented by the significant elevation ($P<0.05$) in serum IL-1β and TNF-α concentrations in IG when compared to CG (Table 1). In parallel, cortisol, TSH, and GH levels significantly ($P<0.05$) increased in IG relative to CG. In contrast, T3, T4, insulin, testosterone, Na, Cl, K, Ca, P, and Mg concentrations significantly decreased in IG relative to CG ($P<0.05$). Table (2) shows in the IG a significant ($P<0.05$) positive correlation between the experiment duration and cortisol, TSH, and GH; and a significant ($P<0.05$) negative correlation between the experiment duration and T3, T4, insulin, and testosterone. On the other hand, a significant ($P<0.05$) positive correlation was recorded between pro-inflammatory cytokines (IL-1β and TNF-α) and cortisol, TSH, and GH; while correlation between pro-inflammatory cytokines and T3, T4, insulin, and testosterone in IG was negative ($P<0.05$).

Concerning the diagnostic and prognostic value of the estimated hormones, table (3) elucidated relative good values, i.e., more than 70% of sensitivity, specificity, PPV, NPV, and accuracy rate. However, TSH

and testosterone specificity was 50% with low LRs (lower than 5) at AUC =1 (except for insulin AUC=0.97). Thus, cortisol, T3, T4, and GH values were better than TSH, testosterone, and insulin.

Table 1. The effect of *T. evansi* experimental infection in rats on serum concentrations of pro-inflammatory cytokines, hormones, minerals, and electrolytes.

Parameters	0 day	7th day	14th day	21th day	21th day	35th day
Il-1β (Pg/ml)	26.97±3.97	47.50±5.56	69.69±3.79	93.38±6.25	102.24±5.64	108.80±0.71*
TNF-α (Pg/ml)	26.01±2.92	39.84±6.47	65.75±4.69	84.99±6.53	104.01±6.68	112.90±0.01*
Cortisol (µg/dl)	0.60±0.19	1.44±0.26	1.63±0.21	2.27±0.10	2.93±0.15	3.42±0.01*
TSH (µIU/ml)	0.014±0.001	0.18±0.01	0.26±0.03	0.40±0.08	0.51±0.10	0.62±0.01*
T3 (ng/ml)	7.19±0.88	4.14±0..44	3.66±0.48	0.89±0.39	0.21±0.05	0.14±0.01*
T4 (µg/ml)	1.55±0.13	1.04±0.11	0.77±0.09	0.36±0.05	0.27±0.07	0.11±0.01*
Insulin (µIU/ml)	7.49±0.98	5.96±0.83	4.85±0.96	3.83±0.35	3.22±0.67	3.00±.00*
GH (ng/dl)	3.33±0.12	4.39±0.19	5.55±0.26	6.57±0.27	7.65±0.35	8.12±0.01*
Testosterone (Pg/ml)	1.45±0.01	1.23±0.01	1.03±0.01	0.87±0.02	0.17±0.02	0.07±0.01*
Ca (mg/dl)	9.78±0.59	9.13±0.46	8.02±0.25	7.07±0.80	6.12±0.83	6.08±0.01*
P (mg/dl)	4.89±0.40	3.75±0.14	3.50±0.08	2.85±0.05	2.34±0.13	2.29±0.07*
Na (mmol/L)	138.00±5.89	120.01±6.81	109.10±6.93	97.42±4.88	93.54±4.49	92.81±0.10*
Cl (mmol/L)	105.45±4.87	93.26 ±5.92	81.53±5.90	76.67±5.93	73.78±5.36	71.68±1.15*
K (mmol/L)	3.61±0.20	2.36±0.20	1.90±0.09	1.62±0.09	1.34±0.11	1.28±0.01*
Mg (mg/dl)	1.85±0.13	1.25±0.13	0.96±0.11	0.78±0.06	0.64±0.11	0.60±0.01*

Values represent mean±SD. ***:** Significant (*P*<0.05)

Table 2. Correlation between the infection duration, pro-inflammatory cytokines, and the estimated hormones.

Hormones	Duration	IL-1β(Pg/ml)	TNF-α(Pg/ml)
Cortisol (µg/dl)	0.976*	0.941*	0.972*
TSH (µIU/ml)	-0.942-*	-0.953-*	-0.916-*
T3 (ng/ml)	-0.959-*	-0.969-*	-0.931-*
T4 (µg/ml)	0.966*	0.963*	0.975*
Insulin (µIU/ml)	-0.900-*	-0.906-*	-0.848-*
GH (ng/dl)	0.988*	0.979*	0.995*
Testosterone (Pg/ml)	-0.969-*	-0.910-*	-0.946-*

***:** Significant (*P*<0.05)

Table 3. The AUC, cut-off points, sensitivity, specificity, LR, PPV, NPV, and accuracy rate of hormonal assays in IG compared to CG.

Diagnostic variables	Cortisol (µg/dl)	TSH (µIU/ml)	T3 (ng/ml)	T4 (ng/ml)	Insulin (µIU/ml)	GH (ng/dl)	Testosterone (ng/dl)
AUC	1	1	1	1	0.97	1	1
Cut-off points	0.76	0.015	6.43	1.45	6.66	3.43	1.45
Sensitivity%	100	100	100	100	94.44	100	100
Specificity%	75	50	75	75	75	75	50
LR	4	2	4	4	3.78	4	2
PPV%	94.74	90	94.74	94.74	94.44	94.74	90
NPV%	100	100	100	100	75	100	100
Accuracy rate%	95.45	90.91	95.45	95.94	90.91	95.45	90.91

Histopathological results: Testicular tissue samples from infected rats after one week of infection showed that most seminiferous tubules were normal with a few numbers of affected tubules. The affected tubules showed degeneration of primary and secondary spermatocytes with slight edema in the interstitial tissue (Figs. 1 A, and B). Testicular tissue sample from infected rats after two weeks of infection showed degenerated and vacuolated spermatocytes with loss of spermatids in some seminiferous tubules. Edema and slight inflammatory cells infiltration in the interstitial tissue also occurred (Figs. 1 C, and D). Testicular tissue sample from infected rat after three weeks of infection showed degeneration and necrosis of spermatocytes with desquamation in the lumen of seminiferous tubules, edema, congestion, and infiltration in the interstitial tissue by inflammatory cells (Figs. 1 E, and F). Samples taken after four weeks of infection, showed increased severity of lesions with evident loss in numbers of spermatocytes, and wavy walls of seminiferous tubules. Edema and congestion with infiltration by inflammatory cells persisted in the interstitial tissue (Figs. 1 G, and H). Testicular tissue sample from infected rat after five weeks of infection showed shrinkage of affected seminiferous tubules and increased size of interstitial tissue (Figs. 1 I, and J).

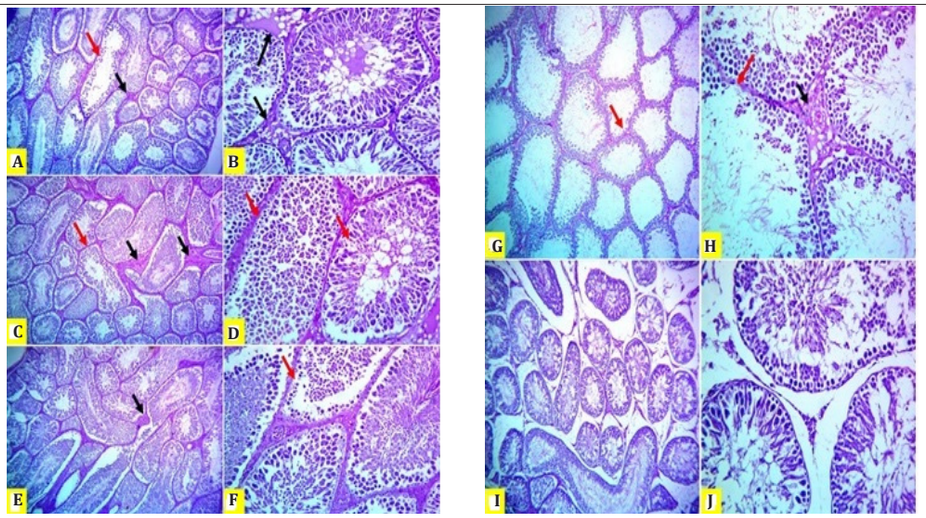

Fig. 1. Testicular tissue samples from infected rats from the first to the fifth week of infection **(A-J)**. Degenerated spermatocytes (red arrow), edema and inflammatory cells infiltration (black arrow). [H&E: A, C, E, G, and I (X10), B, D, F, H, and J (X40)].

DISCUSSION

The immune system is the army concerned with the body's protection against microbial invaders. Its major generals are small glycoproteins pro-inflammatory cytokines. They are responsible for regulating the interaction and communication between different immune cells. In our research as well as previous ones, trypanosomiasis *evansi* stimulated cytokines production to start an immune response. Generally, the protozoal presence, survival, migration, and multiplication in the animal body and related destructed tissues activate the pro-inflammatory cytokines generation from different immune cells, mainly IL-1β and TNF-α. They work synergistically to initiate, propagate, and exaggerate the inflammatory immune response against *T. evansi*. This is orchestrated through induction of innate immunity (neutrophilia, hepatic acute phase response, free radicals, matrix metalloproteinases), activation of the complement system, and humoral immunity (immunoglobulins production)[1,2].

In addition, cytokines promote significant endocrine alterations. It was observed that IL-1β and TNF-α directly enhance hypothalamic pituitary adrenal axis activity, resulting in corticotropin-releasing hormone production which is issued from the hypothalamus to induce Adrenocorticotropic hormone (ACTH) secretion from the pituitary gland. Notably, ACTH stimulates the adrenal gland to produce more glucocorticoids (mainly cortisol)[11,17]. Cortisol triggers hepatic gluconeogenesis, increases fatty acids transfer from adipose tissue to the liver, and suppresses thyroid activity and pancreatic β cells activity[3,7]. These actions are important to decrease cellular glucose uptake and reverse the hypoglycemia usually observed with the disease and save energy required for the immune response and host survival. Besides, the anti-inflammatory action of cortisol protects the host from the inflammatory immune response exacerbation. The activation of the hypothalamus-pituitary unit also results in the release of GH which is capable of improving the immune response and counteracting the cortisol-driven immunosuppression[17,18]. In agreement with this theory, our current data revealed a marked hypercortisolemia and increased GH levels in IG (correlating positively with the infection duration) as well as hypothyroidism and hypoinsulinemia (correlating negatively with the infection duration). While the increased TSH that positively correlated with infection duration, may be attributed to the pituitary response to the resulting hypothyroidism. Similar hormonal changes were reported before in *Trypanosoma* infected animals and human[3-7].

Interestingly, cytokines-mediated hypoglycemia restricts nutrient access to the parasite, maximizing the above-described endocrine changes[19,20]. Therefore, the immune response was involved indirectly and directly in the resulting hormonal changes as previously mentioned in the current work. In this aspect, a positive correlation was recorded between the measured pro-inflammatory cytokines and cortisol, GH, and TSH, as well as a negative correlation was noticed between the pro-inflammatory cytokines and T3, T4, and insulin.

Additionally, the obtained hypercortisolemia in IG impaired the gonadotrophin-releasing hormone (GnRH) secretion from the hypothalamus and subsequently suppressed LH and FSH production from the pituitary gland. This consequently resulted in a pronounced drop in testosterone levels in IG, (negative correlation with the infection duration). Similar results

were noted before in *T. gambiense*-infected human[21], *T. b. brucei* and *T. evansi*-infected rams[22], and *T. evansi*-infected camels[23]. On the other hand, cytokines-induced pituitary inflammation, pyrexia, anorexia, and anemia, and the trypanosomal proteases may be other causes of GnRH depletion and hypothalamus-pituitary-gonadal axis dysfunction. Thus, the immune response modulates the sex hormones in different ways and participates in infertility usually accompanying trypanosomiasis[23]. This hypothesis was further confirmed by the negative correlation recorded between the estimated pro-inflammatory cytokines and testosterone levels.

Another possible explanation for the recorded decline in the testosterone levels in IG, is the testicular damage usually observed in *Trypanosoma* infections. Notably, about 90% of blood testosterone is of testicular origin[22,23]. In parallel, histopathological examination of the testicular tissue of IG showed ascending testicular damage. It started in the 1st week with the degeneration of a few numbers of seminiferous tubules and ended by losing the seminiferous tubules wall structure, increased thickness of interstitial tissue, and loss of a high number of spermatocytes. Edema and congestion with inflammatory cells infiltration in the testicular tissue appeared in the examined tissue in the 5th week. As reported, the testicular damage clarified the low libido and poor semen quality usually reported with *Trypanosoma* infection. Similar lesions were reported before in *Trypanosoma*-infected rams and camels[22 23].

The cytokines-induced pyrexia and dependent anorexia also contributed to outstanding hyponatremia, hypokalemia, hypocalcemia, hypophosphatemia, hypochloremia, and hypomagnesemia recorded in IG. While the high parasite needs for Ca to survive and facilitate host muscle penetration is a more specific cause for the recorded hypocalcemia here. Clinically, these minerals and electrolytes deficiencies are displayed as edema due to hyponatremia and hypokalemia, coagulopathy due to hypocalcemia, and convulsions because of hypomagnesemia[24]. Regarding the evaluation of the estimated hormones as biomarkers for the disease, the data illustrated in table (3) suggested cortisol, GH, T3, and T4 as good markers for trypanosomiasis. This agreed with previous results that referred to cortisol as a biomarker for parasitic infection (mange) in rabbits and for tracking its treatment[25,26].

In conclusion, the interactions between the immune and endocrine system during *T. evansi* infection is critical for the maintenance of homeostasis within the host body. The immune response is widely implicated in the sterility and decreased minerals and electrolytes associated with trypanosomiasis. Hormones may be good biomarkers for trypanosomiasis but for more accuracy, this study should be applied to naturally infected camels.

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Author' contribution: All authors equally contributed in proposal of the study topic, study designing, performing practical studies, writing the manuscript. All authors accepted the authorship and the final version before publication.

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