Research paper

Sperm quality and hormonal levels in C57BL/6 mice infected with *Neospora caninum*

Somayeh Bahrami[a,*], Seyyede Sedighe Mousavi[a], Ali Reza Alborzi[a], Godratollah Mohammadi[b], Mehdi Namavari[c]

[a] *Department of Parasitology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran*
[b] *Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Iran*
[c] *Razi Vaccine and Serum Research Institute, Shiraz Branch, Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran*

A B S T R A C T

Neospora caninum is a major pathogen of cattle and dogs, and neosporosis is widespread in five continents. In this study effect of experimental neosporosis on sperm quality of C57BL/6 mice in different days was investigated. Based on the results sperm concentration was not changed in infected groups but neosporosis induced a significant decrease in epididymis sperm motility at 60 days post infection. A significant increase in the number of abnormal sperms at five, 15, 30 and 60 days post infection was found. At days 15, 30 and 60 days post infection testosterone concentrations were significantly low in infected groups and FSH level was significantly high in infected groups at five and 30 days post infection. LH level was decreased in infected groups, but the difference was significant at five, 15 and 30 days post infection. Comparison of TSH and T4 levels between groups revealed a significant decrease in infected groups at five, 15, 30 and 60 days post infection. Except 15 days post infection T3 levels decreased significantly in infected groups. GPX activity, MDA and TAC level was significantly increased in infected mice at five days post infection. In this study neosporosis is associated with hypogonadotrophic gonadal insufficiency in infected C57BL/6 male mice.

1. Introduction

Neospora caninum is an obligate intracellular parasite causing bovine abortion and may be also responsible for decrease of milk yield, increase involuntary culling and consequent decrease of genetic progress (Thornton et al., 1991). *N. caninum* DNA was detected in fresh and frozen semen from experimentally and naturally infected bulls and it is proved that *N. caninum* can infect reproductive organs (Ortega-Mora et al., 2003; Serrano-Martinez et al., 2007). The observation that most *N. caninum* DNA is found in the cell fraction and virtually no specific DNA is present in seminal fluid suggests that parasites can be associated with certain cell types (Ortega-Mora et al., 2003; Caetano-da-Silva et al., 2004). It is likely that immune cells such as mononuclear phagocytic cells have important role for parasite dissemination in blood and protect them from complement and circulating antibodies. Trafficking of leukocytes to transport intracellular parasites via a Trojan horse-type mechanism has been postulated for apicomplexan parasites (Barragan and Sibley, 2003). There are different studies about association of neosporosis and reproductive performances of female animals, but it seems that its importance in male animals is neglected.

Despite the existence of *N. caninum* in the genitalia, data about its effect on male performance and sperm characteristics are scarce. Our previous study showed that sperm concentration, motility, and viability were significantly low in bulls with natural neosporosis (Bahrami et al., 2018b). Since there is a little information about the effects of *N. caninum* on male reproductive functions, therefore, the aim of the present study was to investigate probable effects of experimental neosporosis on male reproductive system in mice.

2. Materials and methods

2.1. Parasites

In this study, tachyzoites of the *N. caninum* NC1 isolate were used for experimental infections. The tachyzoites were cultured in the Vero cell line in RPMI medium (Sigma Co, St Louis, MO, USA) with 2% fetal calf serum, penicillin (10,000U), streptomycin (100 mg), and amphotericin B (25 mg) (Invitrogen, Carlsbad, CA, USA). Tachyzoites were collected by scraping off the cell monolayer 4–5 days after infection. For preparation of the inoculum dose, the tachyzoites were pelleted by

centrifugation and the concentration was evaluated by hemocytometer (Superior, Germany) (Bahrami et al., 2017).

2.2. Experimental infection

Thirty-two adult male C57BL/6 mice were prepared from laboratory animal breeding council (Jundishapur University of Medical Science, Ahvaz, Iran). All animals were housed in a certified level-three animal biosafety facility and all experiments were performed according to the requirements of the animal welfare committee of Shahid Chamran University of Ahvaz following the Iranian Veterinary Medical Association guidelines. They were housed in groups of five, kept on a 12 h light/dark cycle, relative humidity conditions (40–70%) and controlled temperature (24 ± 2 °C). During experiment, all mice had free access to standard mice chow and water. After one-week acclimation mice were divided randomly into two groups, control (n = 12) and experiment (n = 20). The infected and non- infected groups were injected intraperitoneally with approximately 10^6 tachyzoites of *N. caninum* and sterile PBS, respectively.

2.3. Blood and tissue sampling

In each sampling day, five mice from infected and three from non-infected group were euthanized in a glass desiccator jar for open-drop anesthesia with chloroform following standard animal ethics guidelines of Iran. Blood samples were obtained by cardiac puncture and collected into sterile vacuum tubes with or without EDTA and they were kept at −70 °C pending analysis. Animals were necropsied, and the left epididymis was excised immediately after anesthesia for sperm evaluation. At 15, 30, and 60 days post infection brain of each animal was collected and stored at −70 °C until DNA extraction.

2.4. Examination of mice for N. caninum infection

Polymerase chain reaction (PCR) assay was used to confirm infection. At five days post infection DNA was extracted from whole blood and at 15, 30 and 60 days post infection DNA was extracted from brain using a genomic DNA purification kit (CinnaGen, Iran). In this study primers targeting the Nc5 gene was used to detect of *N. caninum* (Kang et al., 2009). Primers (Bioneer) used in the reaction were the forward primer Np 21 with the sequence 5′−CCCAGTGCGTCCAATCCTGT AAC-3′ and the reverse primer Np 6 with the sequence 5′-CTCGCCAG TCAACCTACGTCTTCT-3′, yielding a 338 bp product. Negative control, consisting of the reaction mix and two mL of DNase/RNase-free water instead of DNA and a positive control consisting of DNA sample from the tachyzoites of *N. caninum* NC1 isolate were included in reactions. PCR reactions were performed in 25 mL consisting of 12.5 mL Taq DNA Polymerase Master Mix Red (Ampliqon, Odense, Denmark), 1 mM primer and 50 ng DNA templates. PCR cycling involved an initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 50 s, annealing at 56 °C for 50 s, and extension at 72 °C for 60 s. This was followed by a final extension at 72 °C for 5 min. Finally, PCR products were electrophoresed in 1.5% agarose (SinaClon Bioscience) in Tris–acetate–EDTA (TAE) buffer, stained with Green Safe stain (SinaClon Bioscience) and visualized under ultraviolet light.

Furthermore, *N. caninum* agglutination test (NAT) was carried out to make sure that infection happens. NAT was performed in 96-round-bottom-well microplates according to the method previously described by Desmonts and Remington (1980). Briefly, 50 μl of 0.2 M 2-mercaptoethanol in PBS was distributed in each well and sera of mice were diluted up to 128, starting at 1:2. Parasites were resuspended in alkaline buffer (7.02 g NaCl, 3.09 g H3BO3, 24 ml of 1 N NaOH, 4 g bovine plasma albumin (fraction V), and enough distilled water to bring the volume to one liter; pH 8.7) and their concentration was adjusted at $2 \times 10^4/\mu l$. After the sera had been diluted, 50-μl *N. caninum* antigen suspensions were distributed in each well. Plates were gently agitated to allow for complete mixing and were then incubated overnight at 30 °C. A complete carpet of agglutinated organisms was considered positive while a clear-cut button-shaped deposit of parasite suspension at the bottom of the well was interpreted as a negative reaction. The assay included two negative controls and one positive control. A serum sample obtained from a rabbit with an experimental neosporosis was considered as the positive control. *Neospora* agglutination test was used to confirm chronic infection at 15, 30 and 60 days post infection.

2.5. Evaluation of sperm characteristics

The epididymal sperm concentration was evaluated with a hemocytometer using Yokoi et al. (2003) method with some modifications. The cauda epididymis was incised and dissected on its ventral surface and incubated within warmed tyrode solution for 15 min. Then the epididymal tissue-fluid mixture was filtered via strainer to separate the supernatant from tissue particles. The supernatant fluid containing all epididymis spermatozoa was mixed with the solution included 5 g sodium bicarbonate, 1 ml formalin (35%, v/v) and 25 mg eosin per 100 ml distilled water with the ratio 1:10. The spermatozoa were counted by haemocytometer using improved Neubauer chamber described by Pant and Srivastava (2003). Eosin-nigrosin staining (0.2 g of eosin and 2 g of nigrosin were dissolved in a buffered saline, mixed for 2 h at room temperature and filtered to obtain the staining media) was used to evaluate sperm viability (Zemjanis, 1970). After thawing, one drop of the semen was placed on a tempered glass slide, which was mixed with one drop of Eosin-nigrosin solution and left to stabilize for 30 s. The mixture was smeared on the glass slide and allowed to air dry. One hundred spermatozoa were evaluated in at least five different fields in each smear under light microscope. Because of Eosin penetration in non-viable cells in they appear red and Nigrosin causes the background dark and facilitate the identification of viable, non-stained cells. In this study sperm abnormalities classified in three main groups, sperm head defects (large, small, elongated, irregular amorphous, vacuolated, detached no acrosome and small acrosome), neck and the mid-piece defects (asymmetric, thin, thick, cytoplasmic droplets, irregular, bent) and the tail defects (short, coiled, broken, hairpin duplicate and terminal droplets). For a spermatozoon to be classified as normal, the whole spermatozoon had to be normal regarding the head, mid-piece and tail. A total of 100 spermatozoa was counted and classified.

To evaluate sperm motility epididymal tissue-tyrode solution mixture was left for 10 min in 37 °C and filtered via strainer to separate the supernatant from tissue particles. The supernatant fluid containing all epididymal spermatozoa was investigated for motility and one hundred spermatozoa were evaluated in at least five various fields in each smear under light microscope.

2.6. Hormone analysis

The testosterone level was assayed using the testosterone test kits (Monobind Inc., Lake Forest, USA) based on the enzyme-linked immunosorbent assay (ELISA) technique and results expressed as ng/ml. Follicle stimulating hormone (FSH) level was analyzed by the FSH ELISA kit (MyBioSource, Inc., San Diego, USA) and for luteinizing hormone (LH) level evaluation LH ELISA kit (Reddot Biotech Inc, Kelowna, Canada) was used. The kits were based on the competitive inhibition enzyme immunoassay techniques for the in vitro quantitative measurement of FSH and LH in mouse plasma and the results were expressed as ng/ml.

For each sample, triiodothyronine (T3) serum was measured by competitive enzyme immunoassay and using T3 kit of Auto bio Diagnostic Co. with sensitivity of 0.4 (μg/dl) and thyroxine (T4) serum was gauged by competitive enzyme immunoassay utilizing T4 kit of Auto bio Diagnostic Co. with sensitivity of 0.2 (μgr/dl). Thyroid stimulating hormone (TSH) level was evaluated by TSH kit of Auto bio Diagnostic Co. that is based on the principle of a solid phase enzyme-

Table 1

Sperm concentration ($\times 10^6$/ml), motility (%) and normal spermatozoa (%) in non-infected and infected mice with *N. caninum*. Represented values are means $\pm$ SE.

Sperm quality	Days Post Infection							
	Five		Fifteen		Thirty		Sixty	
	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected
Sperm concentration ($\times 10^6$/ml)	4.1 $\pm$ 2.9[a]	3.1 $\pm$ 0.96[a]	7.8 $\pm$ 0.2[a]	5.1 $\pm$ 1.3[a]	4.2 $\pm$ 1[a]	4.3 $\pm$ 0.82[a]	6.5 $\pm$ 4.5[a]	1.3 $\pm$ 0.86[a]
Sperm motility (%)	37.1 $\pm$ 14.6[a]	54.8 $\pm$ 6.3[a]	65 $\pm$ 8[a]	38.2 $\pm$ 6.7[a]	54.05 $\pm$ 7.05[a]	40.8 $\pm$ 4.7[a]	48 $\pm$ 20[a]	14.6 $\pm$ 3.2[b]
Normal spermatozoa (%)	93.5 $\pm$ 6.5[a]	34.8 $\pm$ 6.1[b]	80 $\pm$ 3[a]	26.4 $\pm$ 3.7[b]	74.5 $\pm$ 8.5[a]	16.4 $\pm$ 2.8[b]	56.5 $\pm$ 6.5[a]	6.8 $\pm$ 4.3[b]

Values with different lowercase superscripts are significantly different in each day (p < 0.05).

linked immunosorbent assay and its results expressed as µIU/ml.

2.7. Tissue preparation and determination of lipid peroxidation levels and antioxidant enzymes assay

Mice testes were rapidly thawed and homogenized in cold phosphate buffer (pH 7.4) with ultrasonic homogenizer (Bandelin, Germany). Debris was separated by centrifugation at 3500 *g* for 10 min. Supernatants were collected and used for protein assays and enzyme activities. Based on the generation of superoxide radicals produced by xanthine and xanthine oxidase and reaction with 2-(4-iodophenyl)- 3-(4-nitrophenol)-5-phenyltetrazolium chloride and eventually formation of a red formazon dye, superoxide dismutase (SOD) activity was evaluated. The optical density was measured at 505 nm and the SOD activity was then calculated according to the manufacturer's instruction (Ransod®-Randox Lab, Antrim, UK) and through a standard curve and expressed as unit per milligrams of protein (U/ mg protein). Activity of gluthatione peroxidase (GPX) was determined according to the GPX detection kit (Ransel®-Randox Lab, Antrim, UK) instructions. GPX catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, reduction of oxidised glutathione (GSSG) with a concomitant oxidation of NADPH to $NADP^+$ happens. The decrease in absorbance was measured spectrophotometrically against blank at 340 nm. One unit (U) of GPX was defined as l mol of oxidized NADPH per min per milligram of tissue protein. The GPX activity was expressed as unit per milligram of protein. Malondialdehyde (MDA), a toxic end product of lipid peroxidation, level in samples were measured using the thiobarbituric acid reaction method of Placer et al. (1966). Quantification of the thiobarbituric acid reactive substances was evaluated at 532 nm by comparing the absorption to the standard curve of MDA equivalents generated by acid-catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane. MDA results were expressed as nmol/mg of tissue. According to the method of Benzie and Strain (1998) total antioxidant capacity (TAC) of samples was measured. Briefly, a working solution of FRAP (ferric reducing antioxidant power) was provided by mixing buffer acetate with TPTZ solution in HCl. After that FeCl3 was added and mixed, 8 µL of samples and 240 µL of mentioned working solution were mixed and incubated for 10 min at room temperature. The absorbance of samples was measured at 532 nm. Total antioxidant capacity was expressed as µmol/L.

2.8. Protein assay

According to the colorimetric method of Lowry protein content of supernatants for enzyme analysis was determined and bovine serum albumin used as standard (Lowry et al., 1951).

2.9. Statistical analysis

The SPSS/PC program (Version 19; SPSS, Chicago, IL) was used for the statistical analysis. Data are presented as mean $\pm$ standard error of means (SE). Statistical significance was determined using student *t*-test for parametric data and Mann–Whitney test for non-parametric data, separately for each time. A *p* value of < 0.05 was considered significant.

3. Results

3.1. Molecular and serological results

At five days post infection all mice were positive for *N. caninum* DNA in their blood. At 15, 30 and 60 days post infection *N. caninum* DNA was detected in one, three and three brains of mice, respectively. Based on the NAT results all mice were positive for *N. caninum* at 15, 30 and 60 days post infection.

3.2. Epididymis sperm characteristics

To characterize the impact of neosporosis on the sperm quality, sperm concentration, motility, and morphological abnormalities were analyzed at five, 15, 30 and 60 days post infection. Based on the results there was not significant differences in sperm concentration between infected and non-infected groups on different days post infection. But neosporosis induced a significant 14.6% decrease in epididymis sperm motility in contrast to the non-infected at 60 days post infection (p = 0.03) (Table 1). Sperm morphological examination demonstrated a significant increase in the number of abnormal sperms in infected mice compared to non-infected mice at five, 15, 30 and 60 days post infection (p = 0.003, p = 0, p = 0 and p = 0.002, respectively). More pronounced morphological abnormalities were found at day 60 post infection and the main abnormalities were related to tails defects (Table 2). Fig. 1 shows abnormal sperms found in infected mice at 60 days post infection.

3.3. Hormone levels

Comparison of testosterone concentration in non-infected and infected groups showed that testosterone concentration in infected group was less than non-infected group at five days post infection although the difference was not significant (p = 0.65). At days 15 (p = 0.04), 30 (p = 0.01) and 60 (p = 0.04) post infection testosterone concentration was significantly high in infected groups (Table 3). FSH level was decreased significantly in infected groups at five and 30 days post infection (p = 0.02) but in other groups its decline was not significant (Table 3). LH level was decreased in infected groups, but the difference was significant at five (p = 0.02), 15 (p = 0.03) and 30 (p = 0.03) days post infection (Table 3).

Comparison of TSH and T4 levels between groups revealed a significant decrease in infected groups at five (p = 0.003, p = 0.01), 15 (p = 0.02, p = 0.02), 30 (p = 0.005, p = 0.04) and 60 (p = 0.03, p = 0.03) days post infection, respectively. Except 15 days post infection (p = 0.23) T3 levels decreased significantly in infected groups in other days post infection (Table 4).

Table 2

Mean percentage of normal spermatozoa and spermatozoa with head, neck and mid-piece, and tail defects in non-infected and infected mice with *N. caninum*.

Spermatozoa (%)	Days Post Infection							
	Five		Fifteen		Thirty		Sixty	
	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected
Normal spermatozoa	93.5	23	80	26.4	74.5	16.4	56.5	6.8
Head defects	0	12.6	0	3	0	0	0	1
Neck and mid-piece defects	6.5	37.9	19	22.8	23	31.8	25	24.8
Tail defects	0	26.5	1	47.8	2.5	51.8	18.5	67.4

3.4. Lipid peroxidation level and antioxidant enzyme activities

The testis antioxidant enzyme activity, MDA and TAC of experimentally infected mice with *N. caninum* were compared to the non-infected mice. In different groups activity of SOD enzyme of the infected mice were higher than the non-infected mice but the difference was not significant (Table 5). GPX activity was significantly higher in the infected mice at five days post infection (p = 0.04) but in other groups the differences were not significant (Table 5). At five days post infection MDA level was increased significantly in infected group (p = 0.04) but at 15, 30 and 60 days post infection the differences were not significant (Table 5). We found that neosporosis increased the total antioxidant capacity of the testicular tissue significantly when compared to the control group at five days post infection (p = 0.02) but in other groups the differences were not significant (Table 5).

4. Discussion

There are several reports on presence of *N. caninum* in male genitalia, but the effect of *N. caninum* on the male reproductive system is neglected and there is scarce information about the effect of neosporosis on sperm quality. Therefore, this study investigated the effect of experimental neosporosis on hormone levels and sperm quality (sperm count, motility, and morphology) in male mice. Results of the current study showed that mean concentrations of serum testosterone were significantly lower in mice infected by neosporosis compared to that in the control group at all time points post infection. The low testosterone levels in infected mice at five days post infection may reflect suppression of gonadal function because of stress and sickness related to the acute phase of infection. Immune challenge generally diminishes testosterone levels. At 15 and 30 days post infection, FSH, LH, and testosterone were significantly lower in the infected mice. Therefore, hypogonadotrophic hypogonadism likely occurred after infection with *N. caninum*. At 60 days post infection, the level of testosterone remained low in infected mice while the levels of FSH and LH increased in infected mice to levels that were not significantly different from non-infected mice. We propose that FSH and LH increased due to the decreased negative feedback of low testosterone levels on the hypothalamus. In response to low circulating levels of testosterone, the hypothalamus may secrete gonadotropin-releasing hormone (GnRH), which acts on gonadotropes in the anterior pituitary to stimulate the secretion of the gonadotropin FSH and LH (Veldhuis et al., 2009).

Despite the low level of testosterone in infected mice, the sperm count was normal. We can deduce that this amount of testosterone is enough to maintain normal sperm counts. However, sperm motility was significantly lower at 60 days post infection and percentage of sperm abnormality was higher in all the infected mice at all time points.

TSH is a hormone synthesized and secreted by thyrotrope cells in the anterior pituitary gland which regulates the endocrine function of the thyroid gland. Upon stimulation by TSH, thyroid gland secretes thyroid hormones: T3 and T4 (Laposata, 2010). To assess whether thyroid disease is primary or secondary, the TSH must be evaluated in
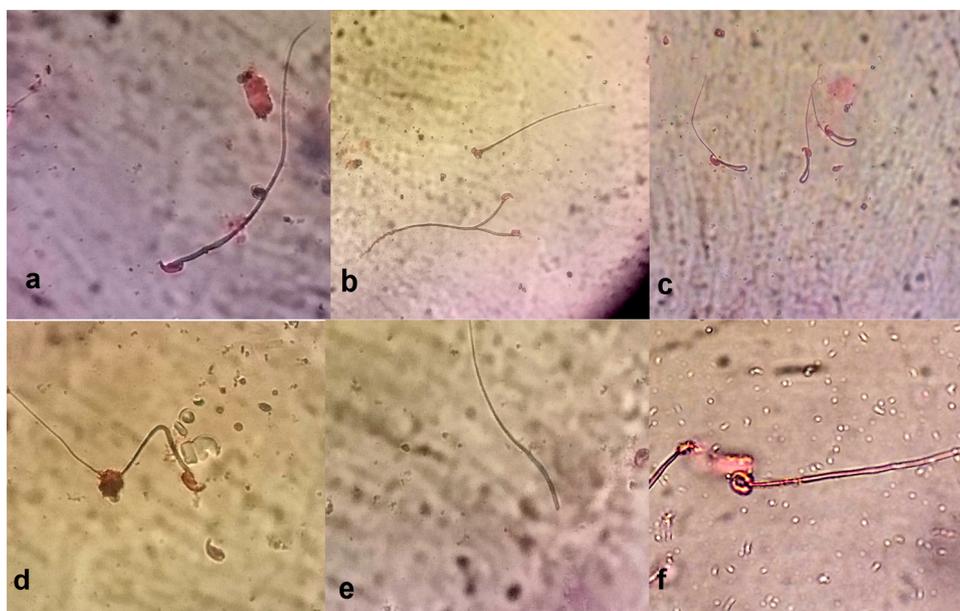
Fig. 1. Abnormal spermatozoa in mice infected with *N. caninum* at 60 days post infection. a: normal sperm; b: double headed; c: bent tail; d: coiled tail and no tail; e: no head; f: double headed spermatozoa.

Table 3

Testosterone, FSH, and LH concentration (ng/ml) in non-infected and infected mice with *N. caninum*. Represented values are means ± SE.

Hormones (ng/ml)	Days Post Infection							
	Five		Fifteen		Thirty		Sixty	
	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected
Testosterone	6.7 ± 5.3[a]	2.6 ± 1.1[a]	5.3 ± 1.05[a]	2.5 ± 0.52[b]	7.3 ± 1.1[a]	3.6 ± 0.5[b]	7.1 ± 3.3[a]	3.3 ± 0.8[b]
FSH	21.1 ± 4.9[a]	6.3 ± 2.3[b]	28.4 ± 5.2[a]	16.4 ± 3.1[a]	18.2 ± 4.6[a]	6.2 ± 1.2[b]	23.4 ± 7.2[a]	15.8 ± 3.1[a]
LH	2.8 ± 1[a]	0.81 ± 0.21[b]	3.1 ± 1.1[a]	0.9 ± 0.3[b]	2.54 ± 0.6[a]	1.1 ± 0.2[b]	2.59 ± 0.7[a]	1.7 ± 0.4[a]

Values with different lowercase superscripts are significantly different in each day (p < 0.05).

comparison to T3 and T4 levels. Primary thyroid disease occurs when TSH increases and thyroid hormones decrease or TSH decreases and thyroid hormones increase. Secondary hyperthyroidism or secondary hypothyroidism occur when TSH and thyroid hormones increase or decrease together, respectively. Secondary hypothyroidism occurs when the hypothalamus produces insufficient thyrotropin-releasing hormone (TRH) or the pituitary produces insufficient TSH (Jannin et al., 2019). In this study, TSH, T4, and T3 levels were significantly lower in infected mice, therefore, secondary hypothyroidism occurred during neosporosis.

Thyroid hormones have a central role in controlling basal metabolic rate, growth, as well as the development and differentiation of many cells in the body. Also, their effect on spermatogenesis is proved (Wagner et al., 2008). Singh et al. (2011) claimed that abnormal thyroid profile could affect testis development, spermatogenesis, semen quality, and may lead to infertility. Different studies showed that hypothyroidism can show a decline in progressive forward motility of the sperm. Furthermore, hypothyroidism can lead to lower proportion of morphologically normal sperm (Krassas et al., 2002, 2008). Zamoner et al. (2008) reported that phosphorylation, and the immunoreactivity of cytoskeleton-associated vimentin protein was increased in Sertoli cells of hypothyroid rats, and this results in loss of Sertoli cell cytoskeleton integrity. Therefore, in this study besides low level of FSH, LH, and testosterone effects of thyroid hormones on sperm cytoskeleton can be the reason of high proportion of morphologically abnormal sperm. Based on Stahl and Kaneda (1998) study mice infected with *Toxoplasma gondii* reveal a rapid decrease in serum T4 levels. The integrity of the pituitary-thyroid axis of infected mice was evaluated by a TRH assay to locate the locus of the hypothyroxinaemia. Their observations indicate that the locus of thyroid dysfunction is in the hypothalamus, not the pituitary or thyroid, and apparently involves impairment of the pulsatile release of TRH. Our previous study showed that serum T4 of bulls with natural neosporosis was decreased significantly but changes of serum T3 was insignificant (Bahrami et al., 2018a).

Different investigations have detected *N. caninum* DNA in the semen of animals following parasitemia (Ortega-Mora et al., 2003; Serrano-Martinez et al., 2007). Therefore, the present study investigated the probable incidence of oxidative stress in the testis in neosporosis. Results of the present study indicated that SOD, GPX, MDA, and TAC were

increased in some infected groups. Elevated levels of GPX, MDA, and TAC were significant in the infected group only at five days post infection, indicating that oxidative stress occurred in the testis. Recent studies demonstrated that hosts with enhanced generation of reactive oxygen species (ROS) have high tolerance to parasitic infections (Boczoń et al., 1996; Sánchez-Campos et al., 1999). ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and nitric oxide (NO^-), are chemically reactive molecules resulting from oxygen consumption. At certain concentrations, ROS are of extreme importance to sperm function (Agarwal et al., 2003; Ford, 2004). Some studies show that low levels of NO^- induce capacitation and that high levels block sperm motility (Zini et al., 1995; Herrero et al., 1999). Also, low O_2^- levels are required for the capacitation process while low H_2O_2 levels act as an inductor of the acrosome reaction. High O_2^- levels are deleterious for sperm function and high H_2O_2 levels adversely affect sperm motility parameters (O'Flaherty et al., 1999; Du Plessis et al., 2010). In addition, spermatozoa are susceptible to oxidative stress-induced injury because their plasma membranes contain large amounts of polyunsaturated fatty acids and their cytoplasm contains low concentrations of antioxidant enzymes (Shen and Ong, 2000). Sharma et al. (1999) demonstrated that mitochondria are susceptible to oxidants and free radicals. When mitochondria are under oxidative stress, mitochondria cannot produce ATP, which are required for the movement of the flagella of sperm cells. Hence, reduced or impaired mitochondrial function will interfere with sperm motility as observed in the infected groups of this study.

In conclusion, neosporosis is associated with hypogonadotrophic gonadal insufficiency and affect male reproductive performance in mice. But several questions about the causes of hypogonadotrophic gonadal insufficiency in neosporosis in mice remain unanswered. First, does neosporosis cause hypothalamus defect and affect the hypothalamic–pituitary-gonadal axis? Second, does a defect in the pituitary secreting process happen during neosporosis? Finally, does degradation of the circulating FSH, LH and TSH occur after their secretion from the pituitary gland?

Further studies are needed to answer the mentioned questions and investigate the pathophysiology of *N. caninum* to explore the mechanisms by which abnormalities of the sperm and hormonal changes develop in neosporosis.

Table 4

TSH (μIU/ml), T3 (ng/ml), and T4 (μg/dl) concentration in non-infected and infected mice with *N. caninum*. Represented values are means ± SE.

Hormones	Days Post Infection							
	Five		Fifteen		Thirty		Sixty	
	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected
TSH (μIU/ml)	2.84 ± 0.5[a]	0.58 ± 0.2[b]	2.21 ± 0.6[a]	0.69 ± 0.1[b]	2.12 ± 0.4[a]	0.66 ± 0.1[b]	2.34 ± 0.5[a]	0.97 ± 0.2[b]
T3 (ng/ml)	1.2 ± 0.2[a]	0.57 ± 0.1[b]	1.4 ± 0.3[a]	0.52 ± 0.2[b]	1.02 ± 0.4[a]	0.56 ± 0.1[b]	0.94 ± 0.1[a]	0.56 ± 0.1[b]
T4 (μg/dl)	54.4 ± 9.5[a]	22.1 ± 4[b]	63.6 ± 14.5[a]	25.1 ± 5[a]	56.1 ± 11.9[a]	30.7 ± 3.3[b]	61 ± 11.5[a]	27.6 ± 6.9[b]

Values with different lowercase superscripts are significantly different in each day (p < 0.05).

Table 5

SOD and GPX activities (U/mg protein), MDA concentration (nmol/mg) and TAC level (µmol/L) in non-infected and infected mice with *N. caninum*. Represented values are means ± SE.

Antioxidant capacity	Days Post Infection							
	Five		Fifteen		Thirty		Sixty	
	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected
SOD (U/mg protein)	75.03 ± 13.6[a]	126.1 ± 20.4[a]	110.7 ± 25.8[a]	119.2 ± 14.9[a]	117.3 ± 34.7[a]	135.5 ± 16.3[a]	134.2 ± 13.7[a]	157.1 ± 18.7[a]
GPX (U/mg protein)	147.9 ± 18.3[a]	251.3 ± 27[b]	184.4 ± 16.6[a]	168.6 ± 26.1[a]	175.1 ± 14.3[a]	194.7 ± 31[a]	170.1 ± 46.4[a]	182.6 ± 36.6[a]
MDA (nmol/mg)	21.9 ± 8.5[a]	55.7 ± 9[b]	24 ± 6.1[a]	29.3 ± 10.2[a]	19.5 ± 7.5[a]	20.4 ± 3.6[a]	21.3 ± 6.7[a]	25.3 ± 9.5[a]
TAC (µmol/L)	2.1 ± 0.6[a]	4.7 ± 0.5[b]	2.4 ± 0.7[a]	1.9 ± 0.3[a]	2.2 ± 0.6[a]	2.5 ± 0.5[a]	2.3 ± 0.6[a]	2.1 ± 0.3[a]

Values with different lowercase superscripts are significantly different in each day ($p < 0.05$).

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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