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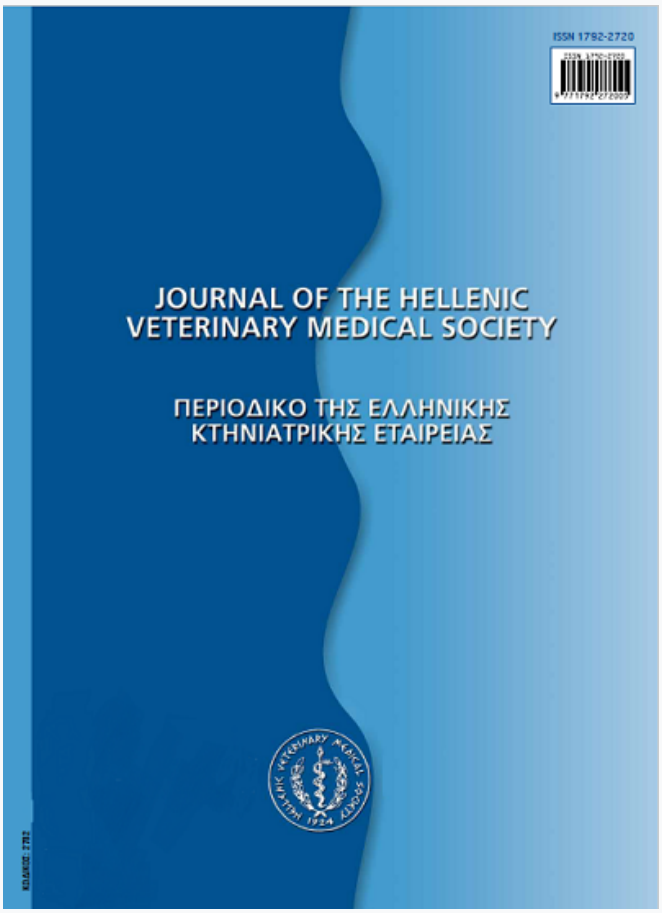

Επίδραση της φυσικής νεοσπόρωσης στα επίπεδα τεστοστερόνης και θυρεοειδικών ορμονών στους ταύρους

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Research article
Ερευνητικό άρθρο

Effect of natural neosporosis on levels of testosterone and thyroid hormones in bulls

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ABSTRACT. Present study was aimed to investigate the effect of *Neospora caninum* seropositivity on testosterone and thyroid hormones levels of Iranian bulls. Two groups containing bulls with neosporosis and bulls without neosporosis were considered. To achieve bulls with neosporosis, *N. caninum* agglutination test (NAT) and ELISA was performed. Testosterone, thyroxin (T4) and tri-iodothyronine (T3) levels were measured and compared between infected and non-infected bulls. Testosterone of bulls with neosporosis was decreased but the difference was not significant. Serum T4 of bulls with neosporosis was decreased significantly but changes of serum T3 was not significant. The hypothalamic–pituitary–adrenal axis (HPA axis) impairment, direct effect of *N. caninum* and its antigens on testis function, interrelationships between thyroid dysfunction and hypogonadism, adaptive response of infected animals to *N. caninum* and occurrence of oxidative stress in testis due to the presence of *Neospora* may be the reasons of hormonal changes. The effect of *N. caninum* on hormonal changes remains incomplete at present and is an area for future research so as to better characterize the effects and mechanisms.

Keywords: *Neospora caninum*; Testosterone; Thyroxin; Tri-iodothyronine, Bull

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INTRODUCTION

The coccidian parasite *Neospora caninum* is close to *Toxoplasma gondii* (Dubey and Lindsay, 1996) and it causes abortions and economic losses in cattle (Dubey, 1999). Dogs have been proved to be both intermediate and definitive hosts and cattle and other animals to be its natural intermediate hosts (Dubey, 1999). Routes of *Neospora* transmission include transplacental infection through tachyzoites, ingestion of tissues harbouring cysts and oral uptake of sporozoite-containing oocysts. Transplacental transmission seems to be very efficient for *N. caninum* in naturally infected cattle and plays a major role in the maintenance and spread of the disease (Davison et al. 1999). Other sources of vertical transmission, such as cow to calf transmission via pooled colostrum or milk could also be possible (Uggla et al. 1998). Previous reports showed that the seroprevalence in cattle is high in many countries, and that 12–42% of aborted fetuses from dairy cattle is infected with *N. caninum* (Dubey, 2003). Due to its high prevalence and association with abortion in cattle worldwide, neosporosis has emerged as an economically important disease for the livestock industry (Dubey et al. 2003). More recently, it has been shown that *N. caninum* is detectable in semen from infected bulls but there is no evidence to suggest that this is an important route of transmission (Ortega-Mora et al. 2003). Despite the existence of *N. caninum* in genitalia its effect on testosterone levels has not been studied. Testosterone and their derivatives (dihydrotestosterone and dehy droepiandrosterone) are androgens produced mainly in male gonads, adrenal glands and the brain. Testosterone can act directly as a ligand of androgen receptors (AR) found in several target tissues. Androgens stimulate the development of the secondary sexual characters in males; participate in reproduction and maturation of fetal testes (O'Shaughnessy and Fowler, 2014).

Thyroid gland is an essential gland in the body of cows that produces essential hormones regulated by the hypothalamic-pituitary-thyroid axis (Laposata, 2010). The main function of thyroid gland is to secrete thyroxin to regulate basal metabolic rate mostly this hormone acts through nuclear receptors that are transcripted by numerous genes and these genes regulate a number of critical physiological functions in development and metabolism (Boelaert and Franklyn, 2005). Male reproduction is adversely affected by both thyrotoxicosis and hypothyroidism. Thyrotoxicosis induces abnormalities in sperm motility, whereas hypothyroidism is associated with abnormalities in sperm morphology; the latter normalize when euthyroidism is reached (Poppi et al. 2007). Based on the absence of documented data about testosterone and thyroid hormones levels of bulls with neosporosis the present study aimed to compare testosterone, thyroxin (T4) and tri-iodothyronine (T3) levels in non-infected and *N. caninum* infected bulls.

MATERIALS AND METHODS

Study design and samples preparation

In the present study samples were collected from the Ahvaz slaughterhouse (center of Khuzestan province, southwest of Iran) from January 2016 to April 2016. To reduce the impact of temperature and season sampling was restricted to winter. Khuzestan province has a border of about 64236 km2, between 47 degree and 41 minutes to 50 degree and 39 min of eastern longitude from prime meridian and 29 degree and 58 min to 33 degree and 4 min of northern latitude from equator (Statistical book of Khuzestan province, 2006). The province has hot and wet summers, mild spring and cold winters. The bull's population mainly comprises local domestic species, which are well adapted to the climate of the area. The slaughterhouse was visited twice a week and blood were collected from mature bulls aged 24–48 months; estimated based on the dental combination (Cockrill, 1974). Since, the plasma levels of thyroid hormones may be altered also by other nutrition- and metabolism-related factors, such as selenium and/or iodine deficiency/supplementation (Wichtel et al. 1996; Awadeh et al. 1998), therefore it was tried to take samples from known farms in slaughterhouse (farms with acceptable management , fed with proper rations, with frequent veterinary care and etc.). The blood samples were collected from the jugular vein into sterile vacuum tubes. Sera were kept at –20 °C pending analysis.

Diagnosis of neosporosis by agglutination test and Elisa

In the present study two groups containing bulls with neosporosis and bulls without neosporosis were considered. To achieve bulls with neosporosis, *N. caninum* agglutination test (NAT) was performed in 96 round-bottom-well microplates according to the method previously described for toxoplasmosis (Desmonts and Remington, 1980). In brief, 50 µl of 0.2 M 2-mercaptoethanol in PBS was distributed in each well and sera were diluted two-fold up to 128, staring at 1:2. Tachyzoites of *N. caninum* NC-1 isolate 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 1 l; pH 8.7) and their concentration was adjusted at 2×10^4/µl. 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 clear-cut button-shaped deposit of parasite suspension at the bottom of the well was interpreted as a negative reaction, and a complete carpet of agglutinated organisms was considered positive. Each assay included two negative controls and one positive control. A serum sample obtained from a rabbit with an experimental *N. caninum* infection was selected as the positive control. Those samples with doubtful results were re-tested.

For definitive diagnosis of bulls infected with *N. caninum* and non- infected ones, positive and negative samples from NAT test were re-examined by an indirect non comparative ELISA. The sera were analyzed for detecting antibody to *N. caninum* by using the commercially ELISA kit (IDEXX, USA) according to the manufacture's instruction. OD of 0.15 was considered as cut-off based on the instruction of manufacture and, the ratio of sample for positive control was ≥0.2OD. Overall, 30 bulls infected with neosporosis and 15 non- infected bulls were selected for the present study.

Screening bulls for brucellosis

Since, brucellosis can affect the bulls reproduction therefore, all serums were tested for *Brucella* genus using slide agglutination by rose bengal test at cell concentrations and tube agglutination test (TAT) by 2-mercaptoethanol, using whole cell antigen (Razi Vaccine and Serum Research Institute) used for the presence of antibodies against *B. abortus* strain. The positive animals were deleted from the study.

Hormone analysis

For each sample, 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 T4 serum was gauged by competitive enzyme immunoassay utilizing T4 kit of Auto bio Diagnostic Co. with sensitivity of 0.2 (µgr/dl).

The testosterone level was assayed using the testosterone test kits (Monobind Inc., Lake Forest, USA) based on the enzyme-linked immunosorbent assay (ELISA) technique (Ekins, 1998).

Statistical analysis

Data generated from the study were subjected to one-way analysis of variance (ANOVA). Variant means were separated using the least significant difference (LSD) method. Significance was accepted at a probability level of less than 0.05.

RESULTS

The levels of testosterone and thyroid hormones in bulls with or without neosporosis are presented in Table1. Testosterone of bulls with neosporosis was decreased but the difference was not significant (p=0.06).Changes of serum T3 was not significant in bulls with or without neosporesis (p=0.12) but serum T4 of bulls with neosporosis was decreased significantly (p=0.04).

DISCUSSION

Tyroxine (T4) has been known as the predominant product of the thyroid gland for many years. Its production and liberation is governed by the hypothalamus/anterior pituitary axis. First, thyrotropin-releasing hormone (TRH), a neuropeptide produced in the paraventricular nucleus (PVN) of the hypothalamus, controls the release of thyroid-stimulating hormone (TSH) from the anterior pituitary. TSH acts on receptors on the thyroid to

promote synthesis and release of the thyroid hormones, mainly of T4, but also in a small quantity of 3,3',5-triiodothyronine (T3). T4 is changed to the active T3 and T3 is more potent than T4 (Yen, 2001). Thyroid hormones mainly influence the thermoregulation and homeostasis of energy and protein metabolism. Also, they involve in the metabolic response of animals to certain nutritional, environmental and/or disease-related challenges, as well as in regulation of certain ovarian functions. Furthermore, normal thyroid hormone levels play an important role in testicular development and its function (Achermann et al. 1999). Alteration in thyroid hormones (particular hypothyroidism) negatively affects gonadotropin secretion (like testosterone) and semen quality (Choksi et al. 2003; Wagner et al. 2008). Testosterone is the primary male sex hormone and an anabolic steroid. testosterone plays a key role in the development of male reproductive tissues such as the testis and prostate, as well as promoting secondary sexual characteristics such as increased muscle and bone mass, and the growth of body hair (Mooradian et al. 1987). In addition, testosterone is essential for health and well-being (Bassil et al. 2009), and for the prevention of osteoporosis. Insufficient levels of testosterone may lead to abnormalities including frailty and bone loss (Tuck and Francis, 2009). In the present study, serum T4 of bulls with neosporosis was decreased significantly but changes of serum T3 was not significant. Furthermore, testosterone of bulls with neosporosis was decreased but the difference was not significant. In association with *N. caninum* effect on thyroid and testosterone hormones five hypotheses can be raised.

In the first hypothesis central nervous system involvement is concerned. The hypothalamic–pituitary–adrenal axis (HPA axis) is a complex set of direct influences and feedback interactions among three endocrine glands: the hypothalamus, the pituitary gland and the adrenal glands (Otmishi et al. 2008). The mentioned axis has the key role on reproduction. The hypothalamus senses low circulating levels of thyroid hormone (T3 and Thyroxine T4) and responds by releasing thyrotropin-releasing hormone (TRH). The TRH stimulates the pituitary to produce thyroid-stimulating hormone (TSH). The TSH, in turn, stimulates the thyroid to produce thyroid hormones. On the other hand, gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus by GnRH-expressing neurons. The anterior portion of the pituitary gland produces luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and the gonads produce estrogen and testosterone. Due to the importance of hypothalamic–pituitary–adrenal axis in controlling hormones, any disorders in the mentioned axis can disturb the hormones levels. Nishimura et al. (2013) suggest that the cerebrum, especially amygdala, hippocampus, and hypothalamus, are the main areas that can be infected with *N. caninum*. Focal necrosis, glial activation and perivascular cuffing was the histopathological changes of cerebrum in their study. Based on their results and due to the injuries in cerebrum (hypothalamus) hypothalamic–pituitary–adrenal axis disorder can be proposed. Hypothalamic–pituitary axis dysfunction affects thyroid and sex steroid hormones which influence on male reproduction (Achermann et al. 1999, Choksi et al. 2003). Thyroid dysfunction (hypothyroxinaemia) as a result of thyrotropin-releasing hormone impairment (TRH), thyroid-stimulating hormone (TSH) and decreasing serum thyroxine (T4) levels reported in *T. gondii* infected mice by Stahl et al. (Stahl and Kaneda, 1998a; Stahl and Kaneda, 1998b).

Studies in Nylar female mice infected with *T. gondii*, exhibited hypogonadotrophic hypogonadism secondary to hypothalamic dysfunction. These mice infected with *T. gondii* Cornell strain, present atrophy in the thymus, ovaries, and uterus, cessation of cycling, anovulation, and decline of serum thyroxine (T4) levels (Stahl et al. 1985).

The second hypothesis about reduction of testosterone in bulls with neosporosis can be due to the direct effect of *N. caninum* and its antigens on testis function. Recently, the presence of *N. caninum* in the semen of naturally and experimental infected bulls have been described and it appears that certain cell types that are present in the cellular fraction of semen could harbor *N. caninum*. It seems that immune cells, such as mononuclear phagocytic cells, are responsible for protozoa transport in blood and semen, as circulating antibodies and complement could kill extra cellular parasites during reactivation

(Serrano- Martinez et al. 2007). Trafficking of leukocytes to disseminate intracellular parasites via a Trojan horse- type mechanism has been approved for *T. gondii* and *N. caninum* (Barragan et al. 2003). Abnormality of testis structure or function can lead to testosterone level changes.

The next hypothesis about testosterone and thyroid hormones imbalance can be related to the interrelationships between thyroid dysfunction and hypogonadism in bulls with neosporosis. Several studies have confirmed that thyroid hormone deficiency affects all tissues of the body, including multiple endocrine changes that alter growth hormone, corticotrophin, glucocorticoids, and gonadal function. Primary hypothyroidism is associated with hypogonadotropic hypogonadism, which is reversible with thyroid hormone replacement therapy (Meikle, 2004). It seems that neosporosis may affect this relationship.

High concentrations of testosterone are known to have immunosuppressive effects (Roberts et al. 2009). Therefore, the results of the present study, namely the decreased concentration of testosterone in *Neospora* infected bulls, make the immunosuppression based explanation of the association between *Neospora* infection and testosterone concentration unlikely. Therefore, the forth hypothesis is raised about the adaptive response of infected animals to *N. caninum*. In fact, it could be speculated that the decrease of testosterone concentration could be an adaptive response of infected animals to *Neospora*- induced immunosuppression. By decreasing the concentration of testosterone, the infected bulls could partly compensate the chronic neosporosis associated down regulated cellular immunity. Such compensation might increase the probability of the survival of infected bulls after contact with various pathogens in their natural environment.

Despite the low oxygen tensions that characterize the testicular micro-environment, this tissue remains vulnerable to oxidative stress due to the abundance of highly unsaturated fatty acids (particularly 20:4 and 22:6) and the presence of potential reactive oxygen species (ROS)-generating systems. ROS generation can be from the mitochondria and a variety of enzymes including the xanthine- and NADPH-oxidases (Banfi et al. 2001; Kumagai et al. 2002), and the cytochrome P450s (Zangar et al. 2004). These enzymes specialize in the professional generation of ROS or produce these toxic metabolites as an inadvertent consequence of their biochemical activity. In order to address this risk, the testes have developed a sophisticated array of antioxidant systems comprising both enzymatic and non-enzymatic constituents. Concerning the enzymatic constituents of this defense system, the induction of oxidative stress in the testes precipitates a response characterized by the NFκB mediated induction of mRNA species for superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) activities (Kaur et al., 2006). Recent investigations indicate that parasitic infections with high tolerance of the host are the result of defense mechanisms which include enhanced generation of reactive oxygen species (ROS) (Boczon et al., 1996; Sanchez-Campos et al., 1999). Therefore, occurrence of oxidative stress in testis of bulls with neosporosis can be the fifth hypothesis. Presence of *N. caninum* in testis and its antigens may trigger oxidative stress. In our previous study there was no significant differences in SOD activity and MDA levels in testis of bulls with neosporosis but GPX activity was significantly elevated in infected bulls and this finding indicated oxidant/antioxidant imbalances in testis of bulls with neosporosis (Bahrami et al., unpublished).

In conclusion, it seems that during the neosporosis alterations of thyroid hormones and testosterone can occur that can affect several behavioral, physiologic and immunological parameters for a long time. Albeit several influencing factors including parasite strain, doses, and routes of parasite inocula, as well as host variation in susceptibility to infection may directly affect the course of infection and hormones alterations. It's our future plan to investigate experimental neosporosis effects on male and female reproductive parameters and hormonal alterations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 1: Mean ± standard deviation of Testosterone, T3 and T4 levels (ng/ml) in non-infected and *N. caninum* infected bulls.

Groups	Hormones (ng/ml)		
	Testosterone	T3	T4
Infected	3.1±0.8[a]	3.31±0.1[a]	7.42±1.9[a]
Non- infected	4.6±1.4[a]	3.42±0.2[a]	9.61±1.7[b]

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

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