

The Levels of Antioxidant Activity, Malondialdehyde and Nitric Oxide in Cows Naturally Infected with *Neospora caninum*

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Abstract: The aim of this study was to examine the effects of neosporosis on the oxidative stress cow naturally infected with *Neospora caninum*. Ten infected cow with *Neospora caninum* and ten healthy were included in this study. The neospora infection was diagnosed in the cows by using a commercially available competitive enzyme-linked immunosorbent assay kit. Compared with healthy controls, levels of malondialdehyde and serum concentration of nitric oxide metabolites higher in the infected group. Furthermore, total antioxidant activities were found lowered in the infected group compared to the control group. The results demonstrated that oxidative stress markers are greatly increased in cows naturally infected with *Neospora caninum*.

Key words: *Neospora caninum*, oxidative stress, cows, antioxidant, activity, Nitric oxide, Turkey

INTRODUCTION

Neospora caninum is an obligate intracellular protozoan parasite responsible for diseases in animals (Dubey *et al.*, 2007). Natural infections have been reported in dogs, cattle, sheep, goats, horses, deer and water buffalo (Dubey and Lindsay, 1996). The dog is the definitive host and the cattle are the intermediate host (Georgieva *et al.*, 2006). In cattle, *N. caninum* infections are associated with endemic and epidemic abortions, suggesting possible a point source exposure as well as neonatal mortality (Anderson *et al.*, 1997).

The mechanisms by which cellular defense kills microorganisms has been the subject of intense research. Numerous studies demonstrated that a variety of inflammatory cells are activated which induce or activate various oxidant-generating enzymes to kill intra-cellular and extra-cellular parasites (Kocyigit *et al.*, 2005). The induction and activation of these oxidant-generating enzymes in inflammatory cells are regulated by many pro-inflammatory cytokines including tumor necrosis factor, interleukins-1, IL-6 and others. These reactive species are produced primarily to attack invading microorganisms by nitration, oxidation and chlorination reactions

(Kocyigit *et al.*, 2005; Saran *et al.*, 1999). However, excess amounts of reactive oxygen and nitrogen species can cause injury to host cells (Kocyigit *et al.*, 2005). The aim of the present study was to evaluate the effect of *N. caninum* infection on oxidative status; therefore, Plasma total Anti Oxidant Activity (AOA) was measured Nitric Oxide (NOx) and Malon di aldehyde (MDA) levels in healthy as well as *N. caninum* infected cows.

MATERIALS AND METHODS

Chemicals: Vanadium (III) Chloride (VCl₃), NaCl, EDTA Na₂, NaOH, Thiobarbituric Acid (TBA), Trichloroacetic Acid (TCA), HCl, Sulfanilamide (SULF) and N-(1-naphthyl) Ethylenediamine Dihydrochloride (NEDD) were obtained from Merck (Darmstadt, Germany). Other chemicals used in the study were purchased from Sigma-Aldrich Chemical.

Subjects and parasitological examination: Ten naturally infected with *N. caninum* (infected group) and ten healthy dairy cows (control group), at 3-7 years of age were used in the study. The sera samples were conducted from Aksaray province in Turkey, November 2009. Ten

naturally infected with *N. caninum* cows, at 3-7 years of age were used in the study. The study group's animals were chosen from which have aborted. Entire control group animals were chosen from randomized healthy cows, from farms under the same management conditions. The study protocol adopted was in accordance with the Helsinki declaration as revised in 1989. *N. caninum* infection was diagnosed in cows by using a commercially available competitive enzyme-linked immunosorbent assay kit (VMRD, USA). The test was done following the instructions of manufacturer. The mean Optical Density (OD) at 630 nm was determined for all wells using a microplate reader (ELx 800 UV, Universal Microplate Reader, Bio-Tec Instrumens and Inc). The percent inhibition for each test sample was determined using the below mentioned formula:

$$\text{Inhibition (\%)} = 100 - \left[\frac{(\text{Sample OD})}{(\text{Mean negative control OD})} \times 100 \right]$$

The samples with values of $\geq 30\%$ inhibition were regarded as positive and those with the values $< 30\%$ inhibition were regarded as negative. Sample collection and biochemical estimation: Blood samples were taken from each animal, by puncture of the jugular vein into tubes. The blood was centrifuged at 3000 rpm for 10 min for serum separation. Serum samples were stored at -30°C for the analysis of MDA, AOA and -NOx.

Determination of malondialdehyde levels: MDA levels, an index of lipid peroxidation were measured by the double heating method of Draper and Hadley (1990). The method is based on spectrophotometric measurement of the purple color generated by the reaction of TBA with MDA. For this purpose, 2.5 mL of trichloroacetic acid solution (10%, w/v) was added to 0.5 mL serum in each centrifuge tube; the tubes were then placed in a boiling water bath for 15 min. After cooling to room temperature, the tubes were centrifuged at $1000 \times g$ for 10 min and 2 mL of each sample supernatant was transferred to a test tube containing 1 mL of TBA solution (0.67%, w/v). Each tube was then placed in a boiling water bath for 15 min. After cooling to room temperature, the absorbance was measured at 532 nm by using the Shimadzu UV 1601 spectrophotometer. The concentration of MDA was calculated based on the absorbance coefficient of the TBA-MDA complex ($\epsilon = 1.56 \times 10^5 \text{ cm M}^{-1}$).

Determination of plasma total antioxidant activity: The total AOA was determined using the method described by Koracevic *et al.* (2001). The assay measures the capacity of the serum to inhibit the production of TBA Reactive Substances (TBARS) from sodium benzoate under the influence of the oxygen-free radicals derived from Fenton's reaction. The reaction was measured

spectrophotometrically at 532 nm. The antioxidants from the added sample suppress the production of TBARS and the inhibition of color development is defined as AOA. A solution of 1 mmol L^{-1} uric acid was used as standard.

Estimation of serum nitric oxide levels: Nitric oxide decomposes rapidly in aerated solutions to form stable nitrite/nitrate products (NOx). Serum nitrite/nitrate concentration was measured by a modified method of Griess assay, described by Miranda *et al.* (2001). The principle of this assay is reduction of nitrate by vanadium combined with detection by the acidic Griess reaction. Briefly, samples were deproteinized prior to assay. The serum was added to 96% cold ethanol at 1:2 (v/v) and then vortexed for 5 min. After incubating for 30 min at 4°C , the mixture was centrifuged at $8000 \times g$ for 5 min and the supernatants were used for the Griess assay. Analysis was done in a microtiter plate. About 100 μL of filtrated plasma was mixed with 100 μL of VCl_3 and was rapidly followed by the addition of the Griess reagents which contain SULF 50 μL and NEDD 50 μL . The determination was performed at 37°C for 30 min. The absorbance was measured by a microplate reader (Multiskan Spectrum, Thermo Labsystems and Finland) at 540 nm. Nitrite/nitrate concentration was calculated using a NaNO_2 standard curve and expressed as $\mu\text{mol L}^{-1}$.

Statistical analysis: All data were presented separately as mean \pm SE for the MD and the healthy groups. The comparisons of parameters were performed with Student's t-test. Data were analyzed using the SPSS for Windows computing program (Version 10.0) and $p < 0.05$ were considered statistically significant (Sokal and Rohlf, 1969).

RESULTS AND DISCUSSION

The results of blood MDA, AOA and NOx levels in controls and *N. caninum* infected dogs are shown in Table 1. As shown in Table 1, the levels of MDA and plasma concentrations of NOx were found to be increased in infected group as compared with healthy controls ($p < 0.05$). Furthermore AOA levels were found to be lower in the infected group compared to the control group

Table 1: Levels of biochemical parameters in control and infected groups

Parameters	Infected group (n = 10)	Healthy control group (n = 10)
MDA (nmol mL^{-1})	6.59 \pm 0.16* (+31.8)	5.00 \pm 0.19*
AOA (mM)	1.91 \pm 0.03* (-31.78)	2.88 \pm 0.21*
NOx ($\mu\text{mol mL}^{-1}$)	18.80 \pm 1.34* (+92.8)	9.75 \pm 0.06*

Values are shown as \pm SE. *: $p < 0.05$; (+) % increase/stimulatory rate and (-) % decrease/inhibitory rate from control

($p < 0.05$). The different factors of variations were +31, 8% for the MDA concentrations, -31, 78% for the AOA, +92, 8% for the NOx concentrations.

N. caninum is an obligate intracellular pathogen (Hemphill, 1999). This suggests that cell-mediated immune responses are likely to play an important role in protective immunity. Early evidence of the importance of cell-mediated immune responses came from studies showing that treatment of cells *in vitro* with the cytokine Interferon Gamma ($IFN\gamma$) resulted in significant inhibition of parasite multiplication compared with untreated cells (Innes *et al.*, 1995). In addition, $IFN\gamma$ and Tumour Necrosis Factor alpha ($TNF\alpha$) inhibit multiplication of *N. caninum* tachyzoites within primary cultures of bovine brain cells (Yamane *et al.*, 2000). Several researchers have demonstrated antigen-specific cell proliferation and $IFN\gamma$ production in both naturally or experimentally infected cattle (Innes *et al.*, 1995).

N. caninum-specific CD4+T cells derived from infected cattle were able to kill parasite infected autologous target cells (Staska *et al.*, 2003). Infection and inflammation activate a variety of inflammatory cells that induce and activate various oxidant-generating enzymes (Kocyigit *et al.*, 2005). The induction and activation of these enzymes in inflammatory cells are also regulated by many pro-inflammatory cytokines including tumor necrosis factor- α , interleukin-1 β , IL-6 and others. Enhanced production of such cytokines might increase cancer risk by inducing or activating enzymes involved in the production of inflammatory cytokines because these enzymes produce high concentrations of various free radicals and oxidants including the superoxide anion, nitric oxide, nitroxyl, nitrogen dioxide, hydrogen peroxide and hypochlorous acid (Kocyigit *et al.*, 2005; Beckman and Koppenol, 1996; Shoda *et al.*, 2000; Goff *et al.*, 2002). These oxidants react with each other to produce other reactive species that are far more reactive than the oxidants themselves (Keles *et al.*, 2010).

The impact by free radicals on lipids is known as Lipid Peroxidation (LP) (Fidan and Dundar, 2008). LP is a complicated radical chain reaction leading to the formation of various products including lipid hydroperoxide, conjugated dienes and malondialdehyde. Detection of lipid hydroperoxides and conjugated dienes and thiobarbituric acid-reactive substances such as MDA are often applied to the study of lipid peroxidation reactions. Since membrane phospholipids are major targets of oxidative damage, lipid peroxidation is often the first parameter analyzed for proving the involvement of free radical damage. Thus the presence of MDA is considered an indicator of free radical damage by membrane lipid peroxidation (Diplock, 1994; Katz *et al.*, 1996;

Enginar *et al.*, 2006). In a number of studies, it has been demonstrated that in the cells of hosts infected with different species of parasites, the amount of reactive oxygen radicals which cause LP are increased, thereby causing cell and tissue damage (Sarin *et al.*, 1993; Deger *et al.*, 2008). Saleh (2009) demonstrated that erythrocytic lipid peroxidation increased in cattle naturally infected with *Babesia bigemina*. Court *et al.* (2001) observed that around the time of peak parasitaemia, peripheral bovine monocytes and neutrophils engage in enhanced oxidative burst and production of oxidative radicals. Shiono *et al.* (2003) and Rezaei and Dalir-Naghadeh (2006) found marked increase of MDA levels in proportion to the decrease of PCV and the increase of parasitemia in bovine theileriosis. In this study, blood MDA levels were found to rise.

The antioxidant defense system includes small molecular antioxidants, antioxidant enzymes and metal chelating agents. The efforts of the endogenous antioxidant enzymes to remove the continuously generated free radicals that initially increased due to an induction but with later enzyme depletion resulted in oxidative cell damage (Vidyasagar *et al.*, 2004). The total AOA of body fluids suggests a simultaneous interaction between various antioxidants and is crucial for the maximum suppression of a free radical reaction in extracellular compartments (Cizova *et al.*, 2004). Such activity appears to indicate the antioxidant characteristics of only one antioxidant, whereas total antioxidant activity represents the aggregate antioxidant characteristics of all antioxidants found in the plasma. In addition to AOA, reduced GSH and its metabolizing enzymes constitute the major defense against ROS-induced cellular damage (Celik and Suzek, 2009). In the present study, the AOA levels in cows naturally infected with *N. caninum* were found significantly lowered compared to control groups. Therefore, it was observed that *N. caninum* infection causes a significant reduction in AOA. In this context, it is possible that the observed insufficiency in antioxidant activity was because the *N. caninum* directly modified antioxidant defenses.

Nitric oxide, an interesting free radical gas molecule is involved in numerous physiological and pathophysiological processes. The role of Nitric Oxide (NO) appears controversial because a tissue dysfunction or injury could occur after inhibition of NO. However, high production level of NO has been suggested as a cause of tissue injury (Bohloli *et al.*, 2007). Stimulation of tissue NO production is also associated with adverse events such as hypotension, inhibition of intermediary metabolism and the production of the potent oxidant peroxynitrite following radical-radical reaction with

superoxide (Rubbo *et al.*, 1994). The bio availability of NO is reduced due to the increased level of superoxide radical which transforms NO to peroxynitrite (Zourek *et al.*, 2008).

Dede *et al.* (2002) demonstrated that the concentration of nitrate increased significantly in goats infected with parasites. Kiral *et al.* (2005) found that in dogs infected with *H. canis* the concentrations of NO fell in comparison with healthy controls. In experimental leishmaniasis in rats, Bories *et al.* (1997) demonstrated that there was an increase in serum nitrate levels. The present study found the plasma NOx concentrations were significantly higher in cows naturally infected with *N. caninum*. According to the findings, MDA and plasma concentration of NOx were higher in cow naturally infected with *N. caninum* as compared with healthy controls. Furthermore was lowered in the infected group.

CONCLUSION

In this study, *N. caninum* induced oxidative stress in blood by reducing the activities of antioxidant enzymes and generation of free radicals in cows. In conclusion, oxidative stress markers are greatly increased in cows naturally infected with *N. caninum*.

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