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Nitric oxide production increases during *Toxoplasma gondii* encephalitis in mice

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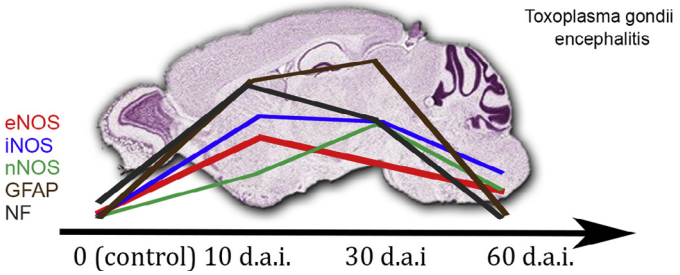
HIGHLIGHTS

- NO has protective and neuropathological effects in the CNS in *Toxoplasma gondii* infection.
- NF expression might be a useful prognostic indicator and monitoring in Toxoplasmic encephalitis pathogenesis.
- Expression of NF provides insight into the severity of toxoplasmosis.

GRAPHICAL ABSTRACT



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ABSTRACT

Toxoplasma gondii is an intracellular parasite with the potential of causing severe encephalitis among immunocompromised human and animals. The aim of this experimental study was to investigate the immunomodulatory and immunopathological role of nitric oxide (NO) in central nervous systems and to identify any correlation between toxoplasmosis neuropathology and investigate the consequences of the cellular responses protect against *T. gondii*. Mice were infected with ME49 strain *T. gondii* and levels of endothelial, neuronal and inducible nitric oxide synthase (eNOS, nNOS, iNOS), glial fibrillary acidic protein (GFAP) and neurofilament (NF) were examined in brain tissues by immunohistochemistry, during the development and establishment of a chronic infection at 10 30 and 60 days post infection. Results of the study revealed that the levels of eNOS ($p < 0.05$), nNOS ($p < 0.05$), iNOS ($p < 0.005$), GFAP ($p < 0.005$) and NF ($p < 0.005$) were remarkably higher in *T. gondii*-infected mice than in uninfected control. The most prominent finding from our study was 10 and 30 days after inoculation data indicating that increased levels of NO not only a potential neuroprotective role for immunoregulatory and immunopathological but also might be a molecular trigger of bradyzoite development. Furthermore, this findings were shown that high expressed NO origin was not only inducible nitric oxide synthase but also endothelial and neuronal. We demonstrated that activation of astrocytes and microglia/macrophages is a significant event in toxoplasma encephalitis (TE). The results also clearly indicated that increased levels of NO might contribute to neuropathology related with TE. Furthermore, expression of NF might gives an idea of the progress and critical for diagnostic significance of this disease.

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1. Introduction

Toxoplasma gondii is an obligate intracellular protozoon parasite that infects all warm-blooded animals, including humans (Hill et al., 2005; Dubey, 2009). The reactivation mechanism in tissue cysts, which are found in the central nervous system (CNS) of humans and other animals chronically infected with *T. gondii*, has been studied in recent years. Chronic toxoplasmosis has a subclinical course in healthy individuals, but tissue cyst reactivation can cause toxoplasma encephalitis (TE), a severe and sometimes fatal condition, in immunosuppressed individuals (Kasper and Boothroyd, 1992; Bhopale, 2003).

The pathogenesis of tissue cyst reactivation remains unclear. A decrease in inducible nitric oxide synthase (iNOS) expression in chronic toxoplasma infections might be associated with reactivation in cerebral immunity (Schlüter et al., 1999; Gazzinelli et al., 1993; Khan et al., 1997). Moreover, many studies have shown that nitric oxide (NO) triggers the conversion between tachyzoite to bradyzoite forms and has parasiticidal effects. In addition to its immunoregulatory functions (Sibley et al., 1991; Candolfi et al., 1994; Bohne et al., 1994; Hayashi et al., 1996; Ibrahim et al., 2009), NO has immunopathological effects (Khan et al., 1997; Hayashi et al., 1996; Scharton-Kersten et al., 1997; Liesenfeld et al., 1999).

At physiological levels, NO has a neuroprotective effect in the CNS, as is in other tissues. Above physiological levels, NO, like other free radicals, has a neurotoxic effect (Calabrese et al., 2007), leading to neuronal degeneration *in vitro* (Dawson et al., 1991; Boje and Aroa, 1992). In addition, NO produced by microglial cells and macrophages that infiltrate the CNS inhibits *T. gondii* replication and plays a vital role in controlling the progression of the infection (Khan et al., 1997; Schlüter et al., 1999; Scharton-Kersten et al., 1997; Liew and Cox, 1991; Langermans et al., 1992; Peterson et al., 1995).

There are many studies that demonstrate eNOS and nNOS expressions increase as well as iNOS expressions due to viral, bacterial and prion diseases, and these expressions contribute to pathogenesis of these diseases. High induces iNOS and especially eNOS expression were seen in systemic lipopolysaccharide treatment (Iwase et al., 2000), acute phase of experimental bacterial meningitis (Winkler et al., 2001) and neurons of cattle with rabies infections (Shin et al., 2004). In addition to eNOS was detected significant up-regulation of prion disease animal models (Park et al., 2011). nNOS expressions play an important role in host immunity against neurotropic viral infections in neurons (Komatsu et al., 1996). nNOS expression increased significantly in human influenza (Fatemi et al., 1998, 2000) and border disease virus infections (Dincel and Kul, 2015a). Moreover, both nNOS, iNOS and eNOS expressions significantly increased in Border Disease virus infections and related to apoptosis and neurodegeneration in this disease (Dincel and Kul, 2015b). In addition to nNOS play an important role in the systemic inflammatory responses (Duma et al., 2011) and signals mediated by the enzyme nNOS play a key role in elimination of *Giardia lamblia* infections (Li et al., 2006).

In the present study, the expression of NO synthases, glial fibrillary acidic protein (GFAP), and neurofilament (NF) was assessed immunohistochemically in mice with chronic *T. gondii* infection at 10, 30, and 60 days post-infection. The protective and neuropathological effects of NO were investigated at 10 days post-infection, at the transition from the acute period to the chronic period, and during potential reactivation periods. The severity of degeneration was also investigated.

2. Materials and methods

2.1. Ethics statement

This study was performed in strict accordance with the recommendations of the National Centre for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs). The protocol was approved by the Committee on the Ethics of Animal Experiments at Kirikkale University (Permit Number: 11/02-11/-02.11.2011).

2.2. Animal infection model

All animals (Swiss albino mice; age 12–16 weeks; weight 22–25 g; female) were obtained from Saki Yenilli Experimental Animal Production Laboratory (Ankara, Turkey) and were continually monitored for survival, clinical condition, and weight until the time of tissue collection. The low-virulence ME-49 strain of *T. gondii* was used for infection as previously described by Atmaca et al. (Atmaca et al., 2014). Tissue cysts of *T. gondii* were harvested from the brains of mice that had been intraperitoneally inoculated with 20 cysts. For experimental infection, mice were intraperitoneally inoculated with 15–20 ME-49 cysts suspended in 0.25 mL of sterile physiologic saline. Typical symptoms of acute toxoplasmosis, including lethargy, ruffled fur, or hunched posture, were not observed after infection with tissue cysts. Animals were anesthetized with 20 mg/kg of ketamine and 5 mg/kg of xilazine. No animals died before euthanasia processes. They were then perfused transcardially with saline solution followed by formalin solution using a peristaltic pump. In groups of six, Swiss albino mice were sacrificed at 10, 30, and 60 days after inoculation. Six healthy mice, sacrificed at the beginning of the study, were used as controls in immunohistochemical analysis.

2.3. Necropsy and histopathology

Mice were anesthetized with pentobarbital (10 mg/kg) and sacrificed by cervical dislocation. The brains were quickly removed. The squash smear technique was used to identify *T. gondii* tissue cysts in the sacrificed animals. Briefly, randomly selected five small pieces taken from fresh brain tissue spread on the placed onto poly-L-lysine-coated glass slides. Sections were fixed in 10% neutral buffered formaldehyde for 1 min. Rehydrated through graded series of alcohol for 1 min, Mayer's hematoxylin was applied for 30 s and washed distilled water. Thereafter, dehydrated through graded series of alcohol for 1 min and clear in xylene for 2 min and mounted on glass slides. Unstained and H&E-stained tissues were analyzed. Sections were fixed in 10% neutral buffered formaldehyde for 48 h and washed under tap water overnight. Following routine tissue preparation procedures, tissue samples were dehydrated through graded series of alcohol and xylene and embedded in paraffin blocks. Paraffin serial sections were cut at a thickness of 4–5 μm and mounted on glass slides. Brains were sectioned to 5-μm thickness, stained with H&E, and examined under a light microscope (Olympus BX51; Olympus, Japan).

2.4. Antibodies

Commercial anti-mouse antibodies against eNOS (Thermo Scientific, USA), iNOS (Thermo Scientific), nNOS (1:100; Santa Cruz Biotechnology, USA), NF (Thermo Scientific), and GFAP (1:100; Thermo Scientific) were used in the present study.