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Short communication

## Astroglial cells in primary culture: A valid model to study *Neospora caninum* infection in the CNS

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### Abstract

The protozoan *Neospora caninum* has a veterinary importance because it causes abortion in cattle and neuromuscular alterations in dogs. We infected rat astrocytes, *in vitro*, with different concentrations of *N. caninum*. Astrocytes responded to infection by producing the pro-inflammatory cytokine TNF- $\alpha$  and the neurotoxic—free radical NO, 24 and 72 h post-infection. These data suggest that astrocytes, which are essential for brain function, are targets for the parasite and this represents a practical and valid model to study the effects of *N. caninum* on the CNS.

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**Keywords:** *Neospora caninum*; Astrocytes; Model; Infection; TNF- $\alpha$  and NO

### 1. Introduction

*Neospora caninum* is a protozoa of the phylum Apicomplexa that is responsible for abortion in cattle and neuromuscular disorders in dogs. This is an important parasite distributed worldwide and many studies have been conducted about neosporosis (Dubey, 1999, 2003). *N. caninum* has satisfactory growth and development in different kinds of cell

cultures, including primary cultures of bovine fetal brain which contain mainly neurons together with 10–15% of astroglia and about 20% un-identified cells (Yamane et al., 2000). Astrocytes represent one of the most important cell types in the mammalian CNS. These cells are involved in each stage of the brain development, in brain homeostasis control, in the synapse function, in the brain immune response and in most brain pathologies (for a review see Tardy, 1991; Norenberg, 1994; Pekny and Nilsson, 2005). We therefore propose rat brain astroglial primary cultures as an easy, practical and cheap *in vitro*

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model, to study the effects of a parasite infection in the CNS.

## 2. Material and methods

### 2.1. Astroglial primary cultures

Astrocytes were obtained from the cortex of newborn rats (<48 h) and mechanically dissociated through an 80  $\mu\text{m}$  filter and  $10^6$  cells were seeded onto culture dishes of 40 mm in diameter (TPP, Switzerland). The cultures were maintained in DMEM (Dulbecco's modified Eagle's medium, CULTILAB, Brazil) supplemented with 10% (v/v) fetal bovine serum (CULTILAB), 1 mM pyruvic acid (Sigma) and 2 mM glutamine (Sigma). Cultures were incubated at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> and the culture medium was changed three times a week (Costa et al., 2002). After 2–3 weeks of culture, cells covered all the surface of dishes and were considered confluent. At this time, cells were examined and counted by immunofluorescence microscopy using antibodies raised against glial fibrillary acidic protein (GFAP), a specific astroglial marker (described below). Each culture monolayer contained about 95% GFAP expressing cells. A group of astrocytes was infected after they became confluent ( $n = 12$ ) and cultured in parallel with non-infected controls ( $n = 12$ ). Three independent experiments were performed. Three plates from each group were used in each independent experiment for immunocytochemistry 6, 24, 48 and 72 h after infection. Cell culture medium was obtained 24 and 72 h post-infection and used for the assay of TNF- $\alpha$  and nitrite.

### 2.2. *N. caninum* culture

Tachyzoites of the NC-Bahia strain (Gondim et al., 2001) were maintained in Vero cells, with regular changes of the DMEM medium (supplemented with 10% fetal bovine serum) every 48 h.

### 2.3. Infection of astroglial cultures

Tachyzoites were purified by exclusion chromatography on a Sephadex G25 column. The parasites

were counted and cell:tachyzoite ratios of 0, 5:1, 1:1 and 2:1 were tested for 6, 24, 48 and 72 h after infection.

### 2.4. Immunocytochemistry

Cells were treated with ice-cold methanol for 20 min and blocked with 3% of BSA for 1 h at room temperature. They were stained with rabbit anti-GFAP IgG antibody (DAKO, Denmark) diluted 1:500 in PBS, and then incubated with tetramethyl-rhodamine isothiocyanate (TRITC) conjugated anti-IgG (Cappel, Durham, Canada) at 1:500. Nuclear dye Hoechst 33258 was used for detection of DNA fragmentation. Serum from a dog experimentally infected with *N. caninum* was diluted 1:50 (Pinheiro et al., 2005) and FITC-conjugated anti-dog IgG (BETHYL, USA) at 1:180 was added to detect the infection with the parasite. Normal dog serum and normal rabbit serum were used as negative controls.

### 2.5. TNF- $\alpha$

This cytokine was measured in the supernatant of control and infected cultures by using a commercial kit (Sandwich ELISA, Amersham, UK), according to manufacturer instructions.

### 2.6. NO evaluation

The amount of nitrite (NO<sub>2</sub><sup>-</sup>) formed in the cell culture supernatant was used as a marker for NO production according to Griess (Won et al., 2004). Briefly, duplicate aliquots of culture medium (50  $\mu\text{L}$ ) were mixed with an equal volume of a 1:1 (v/v) mixture of 1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-(1-naphthyl) ethylenediamine. Absorbance was read at 490 nm using an Elx 800 Universal microplate reader (Biotek instruments, Inc., USA). Quantitative standards were generated by serial diluting sodium nitrite into culture medium.

### 2.7. Statistical analysis

The results were expressed as mean  $\pm$  S.D. and the comparisons between two groups were done using Student's *t*-test.

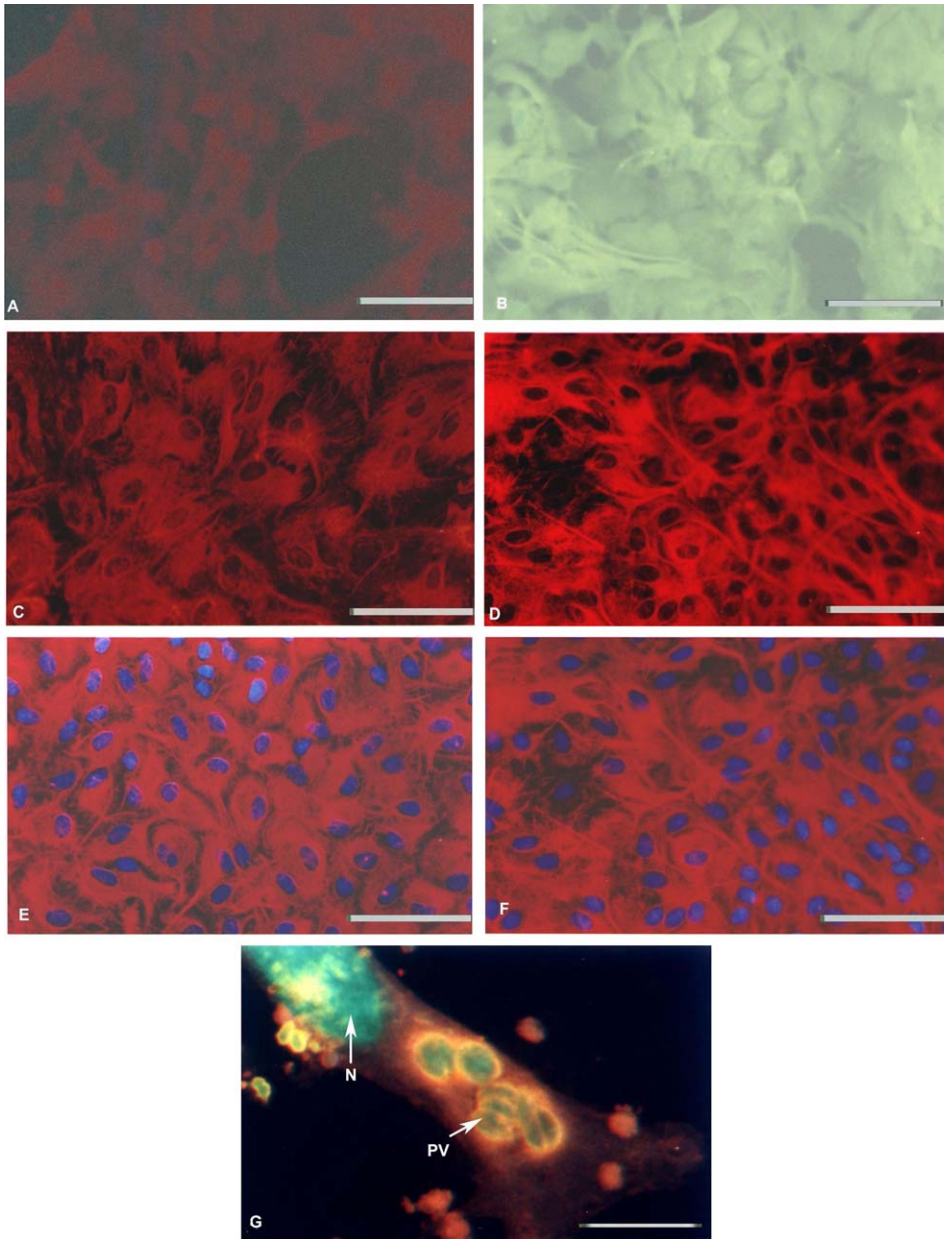


Fig. 1. Immunocytochemistry of non-infected astrocytes and infected astrocytes with *Neospora caninum*. (A) Non-infected astrocytes were incubated with normal rabbit serum, and then incubated with TRITC conjugated anti-IgG. (B) Infected astrocytes with *Neospora caninum* were incubated with non-infected dog serum, and then incubated with FITC conjugated anti-IgG. (C) Non-infected astrocytes incubated with rabbit anti-GFAP IgG antibody, and then stained with TRITC conjugated anti-IgG. (D) Infected astrocytes incubated with rabbit anti-GFAP antibody, and then stained with TRITC conjugated anti-IgG. Cell nuclei stained with Hoechst 33258 in non-infected (E) and infected cells cultures (F) shows a regular nuclear morphology. (G) Infected astrocytes incubated with *N. caninum* positive dog serum, and then stained with FITC conjugated anti-IgG. PV, parasitophorous vacuoles; N, nuclei. Bars: A–F, 50  $\mu$ m; G, 10  $\mu$ m.

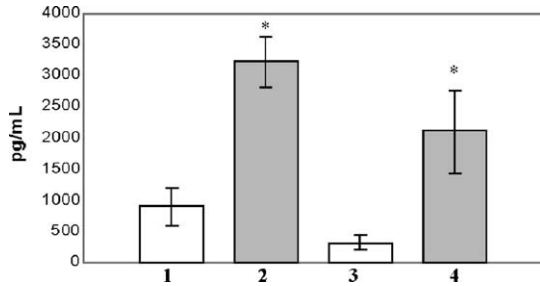


Fig. 2. Evaluation of TNF- $\alpha$  level, 24 h (2) and 72 h (4) post-infection and in the control conditions (1 and 3). Results are expressed as mean  $\pm$  S.D. “\*\*” represent a significant difference between infected and control culture ( $p < 0.001$ ).

### 3. Results and discussion

We have shown that cultured astrocytes are targets for *N. caninum* and astrocytes respond to the infection with a high reactivity, illustrated by the gliofilament reorganization in the first 24 h of infection (Fig. 1C–F). The presence of parasites inside the cells was confirmed by immunocytochemistry (Fig. 1G), and no cross reactivity was observed (Fig. 1A and B). The best effector/target ratio for the parasite was 1:1, with good astrocyte viability in the monolayer allowing visualization of parasites in parasitophorous vacuoles inside the infected cells. Using immunocytochemistry there was no visible difference in the parasites number, on the cell monolayer, at 48 and 72 h post-infection.

*N. caninum* infected astrocytes released TNF- $\alpha$  into the culture medium 24 h post-infection, suggesting a high pro-inflammatory response of infected cells to the parasite (Fig. 2). The nitric oxide (NO) level in

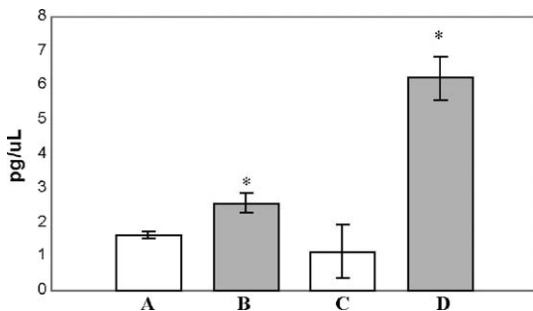


Fig. 3. Production of NO in astrocytes at 24 h (B) and 72 h (D) post-infection and in their controls (A: 24 h and C: 72 h). Results are expressed as mean  $\pm$  S.D. “\*\*” represent a significant difference between infected and control culture ( $p < 0.001$ ).

the medium also increased at 24 h post-infection and continued to increase during the course of the infection (Fig. 3), illustrating another potential pro-inflammatory effect and oxidative events in the infected cells (Gibson et al., 2005). NO is a physiological messenger in the CNS and a marker for oxidative stress. NO can be toxic to neurons and may contribute to the neurodegenerative process associated with gliosis. Its implication in other important functions has been recently reported (Moro et al., 2005). In conclusion, primary cultures of the rat brain cortex predominantly contain astrocytes and these cells are susceptible to *N. caninum* infection. Infected cells have the capacity to release highly pro-inflammatory and toxic agents in the cell culture environment suggesting that this represents a valid *in vitro* model to analyze the effects of *N. caninum* infection on the CNS.

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