Veterinary Parasitology 266 (2019) 96–102

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

IDO, COX and iNOS have an important role in the proliferation of *Neospora caninum* in neuron/glia co-cultures

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ARTICLE INFO

Keywords:
Neospora caninum
Neuroinflammation
Indoleamine 2,3 dioxygenase
Cyclooxygenase 2

ABSTRACT

Central nervous system (CNS) is the main site for encystment of *Neospora caninum* in different animal species. In this tissue, glial cells (astrocytes and microglia) modulate responses to aggression in order to preserve homeostasis and neuronal function. Previous data showed that when primary cultures of glial cells are infected with *N. caninum*, they develop gliosis and the immune response is characterized by the release of TNF and IL-10, followed by the control of parasite proliferation. In order to elucidate this control, three enzymatic systems involved in parasite-versus-host interactions were observed on a model of neuron/glia co/cultures obtained from rat brains. Indoleamine 2,3-dioxygenase (IDO), induced nitric oxide synthase (iNOS) responsible for the catabolism of tryptophan and arginine, respectively, and cycloxigenase (COX) were studied comparing their modulation by respective inhibitors with the number of tachyzoites or the immune response measured by the release of IL-10 and TNF. Cells were treated with the inhibitors of iNOS (1.5 mM L-NAME), IDO (1 mM 1-methyl tryptophan), COX-1 (1 μM indomethacin) and COX-2 (1 μM nimesulide) before infection with tachyzoites of *N. caninum* (1:1 cell: parasite). After 72 h of infection, immunocytochemistry showed astrogliosis and a significant increase in the number and length of neurites, compared with uninfected co-cultures, while an increase of IL-10 and TNF was verified. *N. caninum* did not change iNOS activity, but the inhibition of the basal levels of this enzyme stimulated parasite proliferation. Additionally, a significant increase of about 40% was verified in the IDO activity, whose inhibition caused 1.2-fold increase in parasitic growth. For COX-2 activity, infection of cultures stimulated a significant increase in release of PGE_2 and its inhibition by nimesulide allowed the parasitic growth. These data indicate that iNOS, IDO and COX-2 control the proliferation of *N. caninum* in this *in vitro* model. On the other hand, the release of IL-10 by glia besides modulating the inflammation also allow the continuity of parasitism.

1. Introduction

The establishment of host-parasite relationship occurs through molecular interaction between them, due to the ability of the pathogen to proliferate inside the cell and due to the cell capacity to inhibit the pathogen growth (Buxton et al., 2002). Pathogen survival in the intracellular compartment is ensured by metabolic adaptations to which it is subjected, as well as its ability to regulate distinct mechanisms of immune responses to its favor (Innes et al., 2007; Adalid-Peralta, 2011). Many studies have been performed in an attempt to clarify the biochemical pathways established by some protozoa (Harris et al., 2014). Intracellular parasites can use energy substrates pre-synthesized by the host cell and activate enzymatic pathways that assist in their development and survival in the cellular microenvironment (Fairlamb, 1989).

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https://doi.org/10.1016/j.vetpar.2019.01.003
Received 23 May 2018; Received in revised form 3 January 2019; Accepted 5 January 2019

Neospora caninum is an obligate intracellular protozoa important to Veterinary Medicine once it infects various animal species, being encysted in the central nervous system and causing abortion in cows or provoking neuromuscular disorders in newborns (Dubey and Schares, 2011). Despite the advances made in recent years regarding the biology and interactions of *N. caninum* with its hosts, there is still the need to be clarified the mechanisms of parasite control and biochemical pathways established by this coccidian to evade the immune response triggered during its encysting in the central nervous system (Hemphill et al., 2004). This environment has been the focus of many studies in an attempt to clarify the neuropathogenesis, including those using cell lines and organotypic or primary cultures that have provided valuable information about cell invasion and the events that occur during parasite proliferation (Vonlaufen et al., 2002; Pinheiro et al., 2006a, b; Dubey et al., 2007). Our previous data showed that when primary cultures of glial cells are infected with *N. caninum*, they develop astrogliosis and the immune response is characterized by the release of TNF and IL-10, followed by the control of parasite proliferation (Pinheiro et al., 2010; Jesus et al., 2013). The effect of the inflammatory stimulus studied by Jesus et al (2013) with the treatment of primary cultures of mixed glial cells with IFN-y or TNF (50–300 IU/mL) reduced the number of *N. caninum* tachyzoites. Moreover, these authors also verified that treatment of cultures with antibodies for IL-10 reduced the number of tachyzoites by 52.8%. Considering that it is well known that interaction with neurons modulate the glial response including during parasite infection (Rozenfeld et al., 2005), the next step was to study the *N. caninum* infection in a model of glia/neurons co-culture (Jesus et al., 2014). In this model it was observed that the parasite infection inhibited neurite outgrowth, associated to IL-10 overexpression, corroborating with the finding that *N. caninum* induces inflammatory response with regulatory profile.

During the CNS inflammatory process caused by *Toxoplasma gondii*, a parasite correlated to *N. caninum* the activation of indoleamine 2,3 -dioxygenase (IDO) induced by IFNγ and TNF was observed (Suzuki, 2002; Carruthers and Suzuki, 2007), as well as the activation of induced nitric oxide synthase (iNOS) by IFNγ (Yarovinsky, 2014). IDO is responsible for tryptophan oxidative metabolism through the kinurenin pathway (KP), one of the major regulatory mechanisms of the immune response. Tryptophan degradation suppresses T-cell proliferation by dramatically depleting the supply of this essential amino acid (Moffett and Namboodiri, 2003), the same pathway observed in the control of the parasite growth (Pfefferkorn, 1984; Spekker et al., 2009). The iNOS catabolizes arginine producing nitric oxide that also controls the parasite growth due to its toxic activity (Rath et al., 2014). These two antiparasitic pathways regulate themselves: when activated, iNOS inhibits the activity of IDO and, on the other hand, when IDO is active, iNOS is suppressed (Stone and Darlington, 2002). This phenomenon is of interest, once the activation of a biological route by the pathogen indicates its ability to remain viable in the host tissue making a better environment for its growth and development and thus, ensuring tissue preservation. It was also observed that infection by *T. gondii* in monocytes and murine glial cell cultures (Lüder et al., 1998; Rozenfeld et al., 2003), as well as infection by *N. caninum* in rat glial cells (Jesus et al., 2013, 2014), induces the production of PGE_2 derived from the oxidative metabolism of arachidonic acid via activation of cyclooxygenase-2 (COX-2). This prostanoid is related to an immunoregulatory activity in the CNS by inhibiting NO production and its toxic effects (Levi et al., 1998; Zhang and Rivest, 2001). In order to elucidate some mechanisms associated to the proliferation of *N. caninum* in CNS, the three enzymatic systems IDO, iNOS and COX, involved in parasite-versus-host interactions, were studied in a model of neuron/glia co-cultures.

2. Material and methods

2.1. Culture of N. caninum

Neospora caninum tachyzoites (NC-Bahia strain) were maintained in VERO cells monolayer in RPMI 1640 medium (GIBCO BRL, USA) supplemented with 10% (v/v) fetal bovine serum (GIBCO BRL, USA), 100 IU/mL penicillin G and 100 g/mL streptomycin (CULTILAB, Brazil) as described by Jesus et al. (2014). To ensure that all parasites (intra and extracellular) were used, culture monolayers were scraped with their culture media and cells were ruptured by three passages through a 22-gauge needle. Tachyzoites were purified using a 5.0-μm filter (Millipore, Carrigtwohill, Ireland) and were counted before and after treatments using a Neubauer chamber, as described by Jesus et al. (2014).

2.2. Neuron/Glia co-cultures and N. caninum infection

Mixed glial cells cultures were obtained from brain cortices of newborn rats (< 48 h of age) by mechanical dissociation of the tissue, according to Jesus et al. (2014). The cultures were maintained in Dulbecco's modified Eagle's medium-F12 (DMEM-F12) supplemented with 10% (v/v) fetal bovine serum, 100 IU/mL penicillin G, 100 g/mL streptomycin, 2 mM L-glutamine, 0.011 g/L pyruvate, 3.6 g/L Hepes and 12 mM glucose, incubated at 37 °C in a humid atmosphere with 5% CO_2. All of these reagents were purchased from INVITROGEN (Brazil). These cultures were initially seeded onto 100 mm culture dishes (TPP, Switzerland) and after 14 days, the cells were re-seeded in 24-well (5×10^4) tissue culture plates for assays. In this time, timed pregnant rats were euthanized on the 17th or 18th gestational day, and embryos were used. Their cortex were dissected and dissociated in DMEM/F-12 as described above to obtain neurons (2.5×10^4/well), then plated on a glia monolayer. The cultures were maintained with regular DMEM/F-12 changed every 48 h for 7 days, *N. caninum* tachyzoites were purified and counted as described above, and cultures were infected with a ratio of 1:1 (cell: parasite).

2.3. Determination of iNOS activity

The culture medium of control neuron/glia co-cultures, infected and modulated by 1.5×10^{-3} M of N-nitro-L-arginine methyl ester (L-NAME) were assayed for nitrite levels 72 h after infection, which indirectly measure the NO production, using a colorimetric test based on the Griess reagent (1% sulfanilamide and 0.1% naphthyl-ethylenediamine dihydrochloride in 2.5% phosphoric acid in equal volumes). Equal volumes (50 μL) of culture medium of each culture and Griess reagent were mixed; the mixture was incubated for 15 min at room temperature and then the absorbance at 560 nm was measured using a microplate reader (Biotekinstruments, Inc., USA). Nitrite concentrations were calculated by comparison with a standard calibration curve of sodium nitrite ($NaNO_2$, 1.26–100 pg/mL) with DMEM-F12. Three independent experiments were performed.

2.4. Determination of IDO activity

Cells from neuron/glia co-cultures were treatedduring 1 h with 1 mM of tryptophan (TRP) or 1.5 mM of 1-methyl-tryptophan (1-MT), an IDO inhibitor (Sigma-Aldrich, Germany), diluted in culture medium, then infected with *N. caninum* in at rate of 1:1 (cell: parasite) for 72 h. The enzyme activity was assessed through the concentration of N-formyl-kynurenine in samples of 160 μL of the media of culture cells.

After the addition of 10 µL 30% trichloroacetic acid, each tube was incubated at 50 °C for 30 min to hydrolyze the N-formyl-kynurenine to kynurenine. After centrifugation for 10 min at 600 × g, 100 µL of the supernatant was transferred to 96-well flat-bottom plates, 100 µL 1.2% (wt/vol) 4 (dimethylamino) benzaldehyde (Ehrlich Reagent; Sigma-Aldrich, Germany) in glacial acetic acid was added and plates were incubated for 10 min at room temperature. The optical density was determined at 492 nm with a microplate reader (BIOTEK INSTRUMENTS, Inc., USA). Data were expressed as percentage of optical densities for triplicate cultures. The concentration of kynurenine was calculated using a standard curve for L-kynurenine sulfate (Sigma-Aldrich, Germany).

2.5. Determination of COX activity

Neuron/glia co-cultures were treated for 1 h with the inhibitor of COX-1 indomethacin (1 µM, Sigma-Aldrich, Germany) or with the inhibitor of COX-2 nimesulide (1 µM, Sigma-Aldrich, Germany) both diluted in culture medium, and then infected with *N. caninum* tachyzoites in a rate of 1:1 (cell: parasite) for 72 h. The concentrations of PGE_2 were measured using a commercially-available ELISA (CAYMAN CHEMICAL Co., USA).

2.6. Cytokine assays

Culture media of control and infected cultures, treated or not with the enzymes inhibitors, were collected 72 h after experiments and centrifuged at 3500 × *g* for 5 min. The samples were stored at -20 °C until the assay. IL-10 and TNF were measured by Sandwich ELISA Kit obtained from R&D (USA) and Amersham (UK), respectively, according to the instructions of manufacturers. Results were expressed as pg/mL of cytokines.

2.7. Immunocytochemistry

Neuron/glia co-cultures obtained from the cerebral cortex of neonatal (24 h) and embryonic rats (18 days) were prepared on glass cover slips pre-treated with poly-D-ornithine (10 µg/mL) and infected with *N. caninum* tachyzoites, as described. Cells were fixed, 48 h after infection, for 15 min in ice cold methanol and washed with PBS three times at 5-minute intervals. After that, cells were permeabilized with Triton X-100 (0.5%) in PBS for 15 min and coverslips were again washed with PBS three times at 5-minute intervals. Subsequently, non-specific binding sites were blocked by incubation with 5% FBS-PBS for 30 min and incubated with the primary antibody β-Tubulin III (1: 200, Santa Cruz Biotechnology®) or anti-GFAP (1:300, Invitrogen™) diluted in 1% FBS-PBS. The coverslips were incubated in the wet chamber for 12 h at 4 °C. After the incubation period, the material was washed with PBS and incubated with the secondary antibodies goat anti-mouse IgG Alexa fluor® 594 (1:500, Molecular Probes Eugene, Oregon, USA) or goat anti-rabbit IgG Alexa fluor® 488 (1:500, Molecular Probes Eugene, Oregon, USA). Control immunostaining was performed by incubating cultures with only secondary antibodies. The nuclear chromatin was stained with DAPI (Molecular Probes, Eugene, Oregon, USA) at a concentration of 5 µg/mL, for 10 min, at room temperature. The excess of reagents was removed and the slides were mounted with the anti-fading agent N-propylgallate (Sigma-Aldrich). Then, the cells were observed and photographed under fluorescence microscopy (Olympus AX70) and images were captured with Image-Pro Plus software (Media Cybernetics). Photomicrographs were analyzed with Image J 1.33 u (Wayne Rasband, National Institute of Health, USA). One random field from each experimental condition was represented.

2.8. Quantification of tachyzoites

For the quantification of tachyzoites in cultures, the number of tachyzoites was counted in each culture 72 h after infection. To ensure that all parasites (intra- and extracellular) were counted, culture monolayers were scraped with their culture media and centrifuged at 1.500 × g at 37 °C for 10 min and the tachyzoites, present in the pellet, were purified with a filter unit (5-µm Millipore) and quantified using Neubauer Chamber, as described previously (Jesus et al., 2014) Three independent experiments were performed in triplicate by two independent investigators in a blind assay. The results are expressed as the mean of tachyzoite percentages compared with the untreated control cultures (considered as 100%).

2.9. Statistical analysis

The distribution of the data defined the choice of central tendency and dispersion measures, being considered normal when approved in the test (D'Agostino and Pearson test) and Skewness parameters with interval between -1 and +1 and kurtosis in the interval between - 1.5 and + 1.5. The comparisons between the experimental groups and the corresponding controls were performed with GraphPad Prism 6 for Mac OS X (GraphPad Software, Inc.) using a two-way ANOVA, except to parasite number evaluation that one-way ANOVA followed by a Tukey post-test was performed. Results were expressed by means ± standard deviations (SD). The confidence interval of the test was defined as 95% of statistical significance $p < 0.05$.

3. Results

3.1. Effect of N. caninum infection upon morphology of neuron-glia co-cultures

The astrocyte morphology and reactivity in neuron/glia co-cultures after *N. caninum* thacyzoites infection were analyzed through immunocytochemistry for GFAP (Fig. 1). Polygonal astrocytes were observed in control cultures (Fig. 1A–B); however, the proportion of reactive GFAP-positive astrocytes was increased in infected cultures characterizing astrogliosis (Fig. 1C–E). Moreover, the integrity of neurons and the neuritis length were analyzed through immunocytochemistry for the structural marker β-tubulin III (β-TubIII). It was observed that infection by *N. caninum*, besides astrogliosis, also induced an increase ($p < 0.05$) in the number of β-$TubIII^+$ cells and an increase ($p < 0.05$) in the number and length of neurites, compared with uninfected co-cultures, suggesting neuritogenesis and/or neuroprotection (Fig. 1A–F).

3.2. Role of iNOS, IDO and COX_2

In order to better characterize the proliferation of *N. caninum* of CNS cells, the involvement of the enzymatic systems iNOS, IDO and Cox were investigated.

The infection of co-cultures by *N. caninum* did not induce an increase in the basal levels of the enzymatic activity of iNOS, measured by the release of nitrite (Fig. 2A). However, when iNOS was inhibited, we observed a parasite proliferation of 37% (Fig. 2B). To verify if this proliferation induced by the inhibition of iNOS could change the release of TNF and IL-10, these cytokines were measured but the results were similar to those without inhibition (Fig. 2C–D).

The activity of IDO was measured by the dosage of kynurenine found in the culture medium. Cultures infected with tachyzoites of *N. caninum* showed an increase ($p < 0.05$) of 50% of kynurenine levels when compared with the control uninfected cultures (Fig. 3A). Infected cultures treated with 1-MT, the IDO inhibitor or with TRP presented basal levels of kynurenine production when compared with control.

Once IDO activity was demonstrated, the capacity of these cells in controlling parasite proliferation was assessed. The Fig. 3(B) illustrates that there was an increase ($p < 0.05$) increase in the number of tachyzoites, not only in the presence of the substrate TRP, but also when

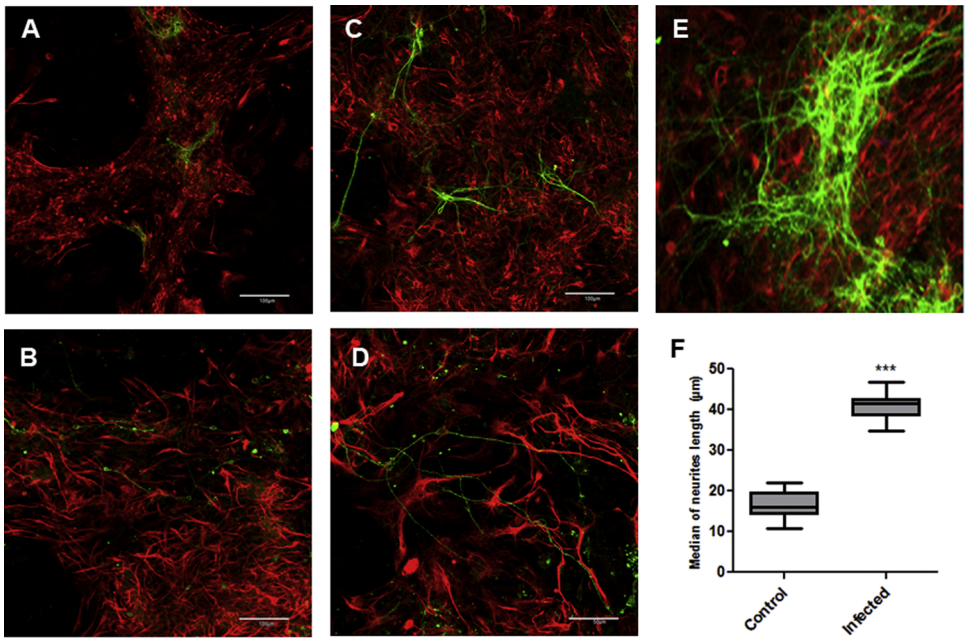

Fig. 1. Presence of neurons and astrocytes in neuron/glia co-cultured infected or not with *Neospora caninum* tachyzoites analyzed through immunocytochemistry for GFAP and β-tubulin III, 48 h after *N. caninum* infection. (A–B) Astrocytes (red) and neurons (green) in control cultures; Obj. × 20 and Obj. × 40, respectively scale bar 100 µm. (C – D) Reactive astrocytes (red) and neurons (green) in infected cultures; Obj. × 20 scale bar 100 µm and Obj. × 40 scale bar 50 µm, respectively. (E) Reactive astrocytes (red) and neurons (green) in infected cultures; Obj. × 60. (F) Quantification of neurites lengths 72 h after *N. caninum* infection. Results from three independent experiments expressed as mean ± SD; (*) $p < 0.05$ (compared with control not infected cultures) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

the cultures were treated with the IDO inhibitor 1-MT, indicating that in the present experimental model this catabolic route is used to control the parasite proliferation. It was confirmed that the release of IL-10 and TNF in the cultures infected with *N. caninum* was increased ($p < 0.05$) and, additionally, it was shown that the inhibition of IDO did not change this profile in infected cultures (Fig. 3C–D).

We also investigated whether cyclooxygenase-2 activity could have an important role in the control of *N. caninum* proliferation in CNS cells. For this, we measured its activity according to the levels of PGE_2 in the media of neuron/glia co-cultures infected or not with *N. caninum* tachyzoites. It was observed that in co-cultures infected the synthesis of PGE_2 increased ($p < 0.05$) around 2-fold when compared with control cultures. Pre-treatment of cultures with COX inihibitors indometacin and nimesulide, as expected, reduced PGE_2 synthesis (Fig. 4A). Moreover, PGE_2 inhibition by nimesulide, a COX_2 inhibitor, enhanced ($p < 0.05$) the parasite proliferation (Fig. 4B).

4. Discussion

Many evidences show the modulator role of glial cells during the immune response to intra cellular parasites in the CNS. This response protects nervous functions but, also favors parasite survival in this tissue. The acute phase of *T. gondii* infection is carried out by a pattern of proinflammatory cytokines, measured mainly by IFNγ (Mordue et al., 2001; Blanchard et al., 2015). This cytokine has been identified as responsible for activating biochemical pathways such as the enzymatic activity of iNOS (Silva et al., 2009; Dincel and Atmaca, 2015) and IDO (Däubener et al., 2001; Fujigaki, 2002; Fujigaki et al., 2003). Concerning *N. caninum,* distinct patterns of immune response were found in CNS cells. Primary cultures of astrocytes respond to *N. caninum* infection releasing IL-10, TNF and NO (Pinheiro et al., 2006a, 2006b) and the same response was verified when mixed glial cells (astrocytes and microglia) were also infected with this parasite (Pinheiro et al., 2007; 2010).

In the present study, reactive astrogliosis was observed after *N. caninum* infection associated with neurite outgrowth in neuron/glia co-cultures. Moreover, similar to what has been observed previously (Jesus et al., 2014), the immune profile of co-cultures infected with this parasite was characterized by TNF and IL-10 but not by IFN-γ nor by NO release. The ability of the parasites to inhibit the activity of iNOS contributes to the preservation of the tissue and, therefore, to keep it viable in this environment. Our results showed a recovery in the TNF and IL-10 synthesis by the parasite when iNOS was inhibited. Rozenfeld et al. (2003) observed that the immune response triggered by *T. gondii* mediated by PGE_2 and IL-10 was responsible for the reduction of the nitric oxide synthesis, supporting the concept that *T. gondii* reduces inflammation for neuronal preservation. Nitric oxide is an important neurotransmitter and the maintenance of its baseline levels is important for the tissue homeostasis, being observed during the activation of glial cells (Calabrese et al., 2007; Brown and Neher, 2010; Fujigaki et al., 2017). The complete depletion of NO implies a limited local immune response, with consequent glial inactivity. When co-cultures were treated with 1.5 mM of L-NAME, an increase of about 35% of parasite proliferation was verified. Since the enzymatic pathway of iNOS was not increased in our model, probably by the *N. caninum* action, another metabolic pathway that could control parasitism was supposed. Some studies pointed to the down regulation of iNOS via IDO (Thomas et al., 1994; Alberati-Giani et al., 1997) and thus, we investigated its involvement in our model through the detection of kynurenine, observing an increase of 28% in the activity of IDO by *N. caninum* infection. Once TNF and the neutralization of IL-10 showed to contain parasitic proliferation in glial cells (Jesus et al., 2013), it was verified whether parasite growth favored by IDO inhibition would alter the profile of these cytokines. However, the inhibition of this enzyme by 1-MT did not affect the release of neither TNF nor IL-10.

The activation of IDO by *N. caninum* was observed by Spekker et al. (2009) in bovine endothelial cells in the presence of IFNγ. Meanwhile, we observed that the infection induced IDO activity without IFNγ stimulation (data not shown). It was also noted that IDO was responsible for parasite control, since its inhibition by 1-MT increased tachyzoite proliferation. The tryptophan pathways triggered by IDO is described as a potent antiparasitic mechanism (Heseler et al., 2008; Murakami et al., 2012) and this enzyme, which activation contributes to CNS homeostasis, is expressed by brain cells including microglia and astrocytes (Guillemin et al., 2001). Our data pointed to an alternative mechanism able to activate IDO that can be also induced, to a lesser extent, by TNF (Pemberton et al., 1997; Suzuki, 2002). This proinflammatory mediator was detected in other murine models of CNS infection by *N. caninum* (Pinheiro et al., 2006a; Jesus et al., 2013, 2014) and we observed the release of TNF and IL-10 in all cultures that were infected and treated with 1-MT. IDO and iNOS were described as retromodulators themselves (López et al., 2006). Additionally, the lack of IFNγ and nitric

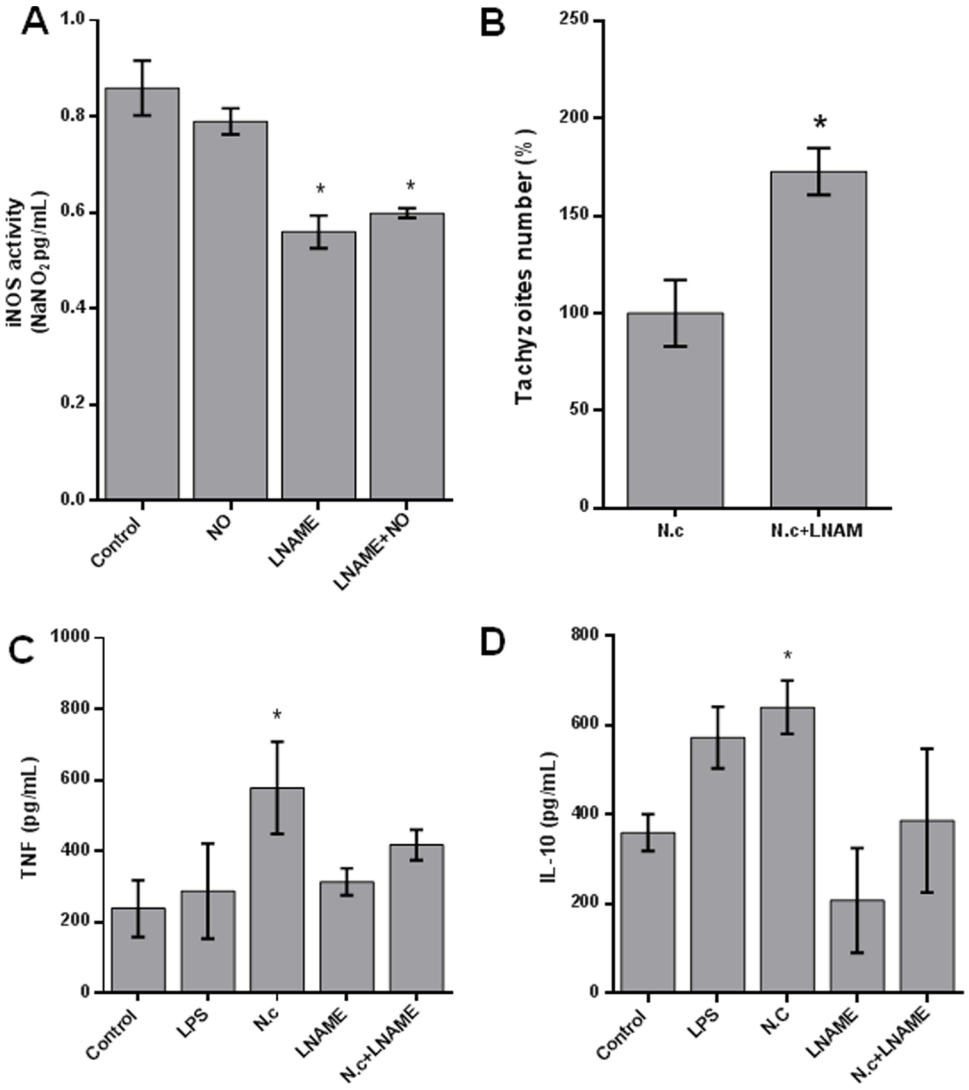

Fig. 2. Characterization of the involvement of the enzymatic system iNOS in the parasitism of *N. caninum* neuron/glia co-cultures. L-NAME (1.5 mM) was added to the culture medium for 1 h and then the cells were infected by *N. caninum* (1:1 parasite/cell) 72 h after treatments. (A) iNOS activity, as measured by nitrite levels. (B) Number of tachyzoites in cultures expressed as the percentual related to control, not infected cultures, considered as 100%. (C–D) TNF and IL-10 cytokines in the culture medium. Results from three independent experiments expressed as means ± SD; (*) $P < 0.05$ (compared with control not infected cultures).

oxide corresponded to a gain in tissue preservation, by the reduction of the deleterious effects of these inflammatory mediators on the microenvironment, as it was described (Gresa-Arribas et al., 2012; Jesus et al., 2013).

After the initial step of tryptophan pathway catalyzed by IDO, the kynurenine has two distinct branches: one of them responsible for the production of quinolinic acid (QUIN) and the other branch regulated by kynurenine aminotransferase (KAT), which produces kynurenic acid (KYNA). QUIN is described as an agonist of NMDA receptors while KYNA is its antagonist (Fugigaki et al., 2017). In CNS cells, QUIN is usually released by the activation of microglia (Alberati-Giani et al., 1996; Heyes et al., 1996) while astrocytes predominantly express the isoenzyme KAT-II to produce KYNA (Guillemin et al., 1999). *N. caninum* infection could contribute to the preservation of the tissue by the activation of the KAT branch prior to the one related to neurotoxicity during the glia activation. Studies have demonstrated the synergistic effect of PGE_2 with TNF in triggering the pathway of oxidative metabolism of tryptophan (Braun, 2005; Von Bergwelt-Baildon, 2006). In this sense, we investigated the involvement of PGE_2 by blocking COX through the use of the selective inhibitors indometacin (COX-1) and nimesulide (COX-2). We observed that infection with *N. caninum* induced the release of PGE_2 and also that parasitic proliferation occurred by the inhibition of both isoenzymes COX-1 and COX-2, confirming that this prostanoid also participates in this parasite control. A down regulation of iNOS could also be associated with the presence of PGE_2 in infected cultures. Some studies have demonstrated the inhibitory effect of PGE_2 in the synthesis of nitric oxide to prevent the deleterious effects of an exacerbated inflammatory process (Minghetti et al., 1997; D'Acquisto et al., 1998; Kobayashi et al., 2001; Boje, 2003). According to our data, a synergistic effect between IL-10 and PGE_2 is likely to contribute to the homeostasis of the microenvironment, by reversing pro-inflammatory conditions. It has been observed that the interaction between PGE_2 and both its receptors, EP4 and EP2, promotes anti-inflammatory effects and neuroprotection (Echeverria et al., 2005; Shi et al., 2010).

Altogether, the data suggest some interpretations such as: (1) the parasitic growth was controlled by enzymatic activity of IDO and COX-2 while basal level of iNOS could contribute in this control; (2) the regulatory effects of IL-10 and PGE2 are able to modulate inflammatory processes and maintain the homeostasis of the microenvironment.

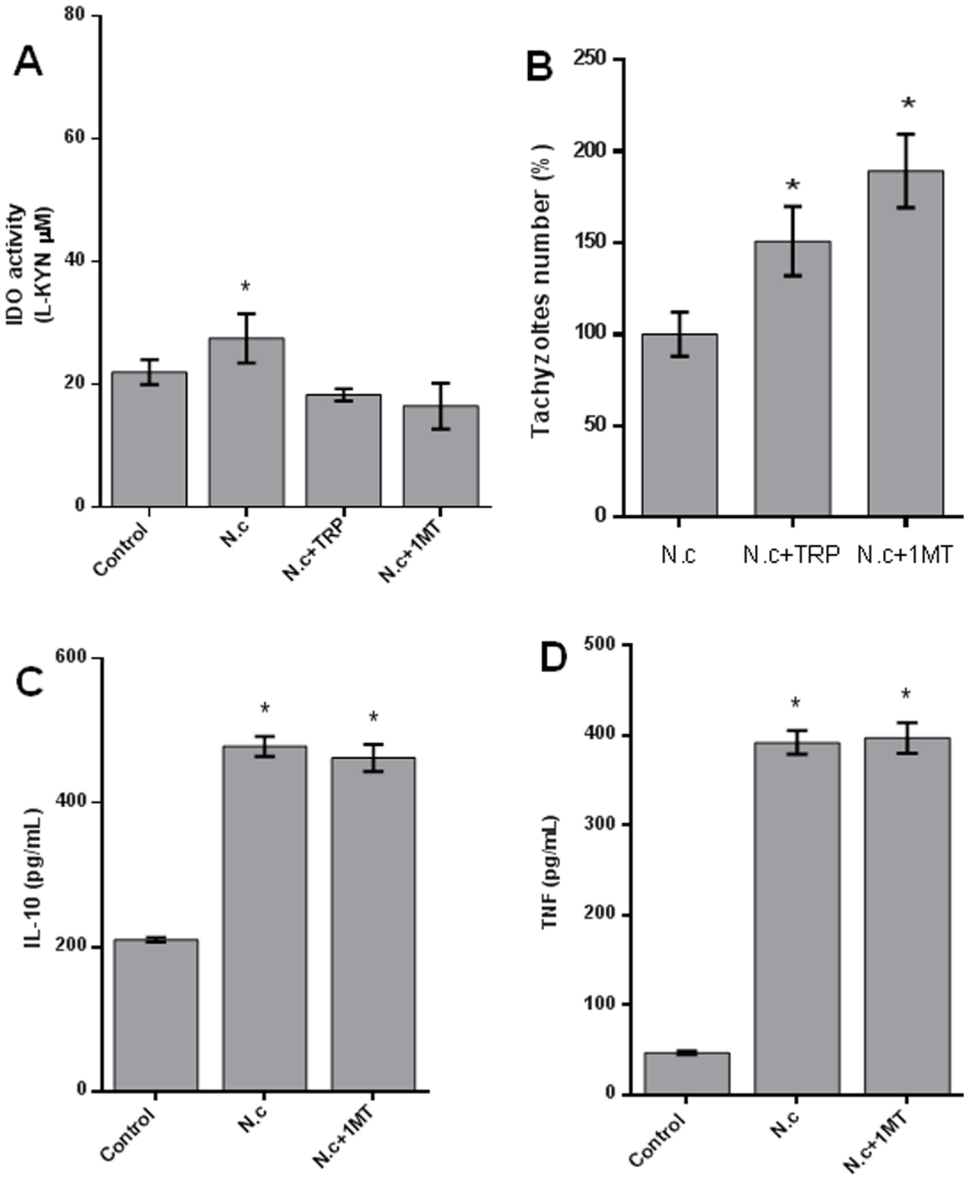

Fig. 3. Characterization of the involvement of the enzymatic system IDO in the parasitism of *N. caninum* neuron/glia co-cultures. L-TRP (1 mM) and 1-MT (1.5 mM) were added to the culture medium for 1 h and then the cells were infected by *N. caninum* (1:1 parasite/cell) and analyzed 72 h after treatments. (A) IDO activity, as measured by L-kirunine levels. (B) Number of tachyzoites in cultures expressed as the percentual related to control, not infected cultures, considered as 100%. (C–D) Levels of TNF and IL-10 cytokines in the culture medium. Results from three independent experiments expressed as means ± SD; (*) $P < 0.05$ (compared with control not infected cultures).

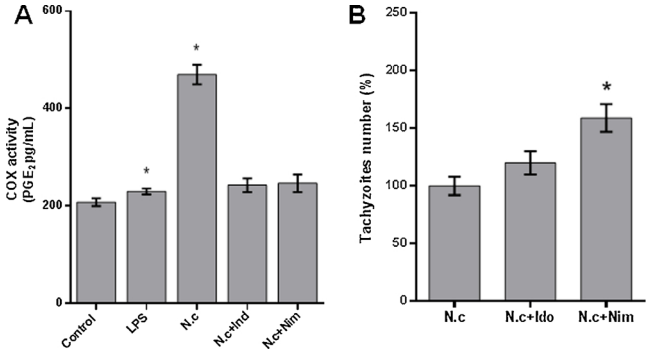

Fig. 4. Characterization of the involvement of the enzymatic system COX in the parasitism of *N. caninum* neuron/glia co-cultures. Indometacin (Ind, 1 µM) or nimesulide (Nim, 1 µM) was added to the culture medium for 1 h and then the cells were infected by *N. caninum* (1:1 parasite/cell) and analyzed 72 h after treatments. (A) COX activity, as measured by PGE2 levels. (B) Number of tachyzoites in cultures expressed as the percentual related to control, not infected cultures, considered as 100%. Results from three independent experiments expressed as means ± SD; (*) $P < 0.05$ (compared with control not infected cultures).

Further studies are needed to clarify some questions specially related to the protection of the tissue attributed to tryptophan catabolites in CNS cells by *N. caninum*.

Acknowledgements

We gratefully acknowledge the research support provided by Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil (CNPq), Função de Aperfeiçoamento de Pessoal (CAPES), Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB) and Programa de Pós-graduação em Imunologia da Universidade Federal da Bahia. We also thank Abilio Borghi for the grammar review of the manuscript. The authors thank the Program for Technological Development in Tools for Health-PDTIS-FIOCRUZ for use of Confocal Microscopy Service-CPqGM facilities.

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