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ALEX BARBOSA DOS SANTOS

TESE DE DOUTORADO

RESPOSTA INFLAMATÓRIA EM CO-CULTURAS DE CÉLULAS GLIA/NEURÔNIO À *Neospora caninum*: POSSÍVEIS PAPÉIS DA INDOLAMINA 2,3 DIOXIGENASE E CICLOOXIGENASE

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Tese apresentada ao Programa de pós-graduação em Imunologia - Instituto de Ciências da Saúde da Universidade Federal da Bahia, como requisito para obtenção do grau de Doutor em Imunologia.

Orientadora: Profª. Drª. Maria de Fátima Dias Costa

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“Algo só é impossível até que alguém duvide e acabe provando o contrário.”

Albert Einstein (1879-1955).

RESUMO

O parasito *Neospora caninum* é um protozoário intracelular obrigatório que tem despertado especial interessse na Medicina Veterinária por causar desordens neuromuscular em cães e abortamentos em vacas gestantes. A resposta imune sistêmica contra o parasito é tipicamente do perfil Th1, com síntese de citocinas pró-inflamatórias, principalmente IFNγ, responsável pela redução da proliferação parasitária. Por outro lado, este perfil de resposta modifica-se durante o período gestacional, em que o balanço da resposta Th1 e Th2 aparentemente favorece a sobrevivência do concepto. Semelhante a isto, observa-se o mesmo padrão de resposta no sistema nervoso central (SNC), local de encistamento do parasito. Estudos anteriores apontaram que IDO (indolamina 2,3 dioxigenase) é modulada por IFNγ e que participa no controle da proliferação parasitária. Em estudos de neuroinflamação usando co-culturas de células gliais/neurônios infectadas por *N. caninum*, observou-se controle da proliferação parasitária por um mecanismo independente da enzima óxido nítrico sintase induzida por IFNγ. No interesse de esclarecer o mecanismo de controle parasitário neste modelo *in vitro*, a atividade da IDO e da ciclooxigenase 2 (COX-2) foram estudas. Co-culturas celulares glia/neurônios obtidas de ratos foram tratadas com o inibidor da IDO (1-metil triptofano/10^{-3}M/mL) e com inibidores da COX-1 (indometacina10^{-6} M/mL) e da COX-2 (nimesulida/10^{-6} M/mL) antes da infecção com taquizoítos de *N.caninum* (1:1 célula:parasito). Após 72 horas de infecção, as atividades enzimáticas foram avaliadas e seus fenótipos foram determinados usando anticorpos anti-βIII tubulin, OX-42 and GFAP para observar neurônios, microglia e astrócitos respectivamente. O perfil da resposta imunológica foi determinado por dosagem das citocinas IL-10, IFNγ e TNF pelo ensaio de ELISA. Notou-se duas vezes mais o aumento na atividade da enzima IDO em co-culuturas infectadas pela dosagem de cinurenina. Em culturas tratadas com o inibidor da IDO (1-MT) e infectadas com taquizoitos ocorreu aumento na proliferação parasitária de aproximadamente 40%, bem como aumento na atividade da IDO. Pertinente a atividade da COX-2, culturas infectadas produzem PGE_2, enquanto tratamento com nimesulida permite o crescimento parasitário e induz perda de aproximadamente 30% e 50% de astrócitos e microglia respectivamente, no entanto os neurônios foram preservados. A infecção por taquizoítos promove síntese de IL-10 e TNF, ainda na presença do inibidor da IDO, mas não ocorre liberação de IFNγ. Estes dados indicam que neste modelo experimental a atividade da IDO é ativada por um mecanismo independente IFNγ e que o controle parasitário pode ser mediado pelo efeito sinérgico de PGE_2 e TNF. Assim, a ativação da COX-2 parece ser um importante via de controle, ao passo que PGE_2 associada a IL-10 podem modular a inflamação e permitem a continuidade do parasitismo.

Palavaras chaves: *Neospora caninum*, neuroinflamação, indolamina 2,3 dioxigenase, ciclooxigenase 2, co-cultura glia/neurônio

ABSTRACT

Neospora caninum is an obligate intracellular protozoan that has been very studied by Veterinary Medicine because it causes neuromuscular disorders in dogs and abortion in cattle. The protective systemic immune response against this parasite is predominantly Th1 pattern, which there is proinflammatory cytokines production, mainly IFNγ, responsible for reduction of parasite burden. However, this response profile appears to be modified during pregnancy in chronically infected animals, in which a balance of production of Th1 and Th2 cytokines appears to favors fetal survival. The same was suggested occur in the central nervous system (CNS), local of parasite encystment. Previous data showed that IDO (indolamine 2,3 dioxygenase) is modulated by IFNγ in cell proliferation control. In studies of neuroinflammation using neuro-glia co-cultures infected by *Neospora caninum*, it was verified parasite control by a mechanism independent of type 2 nitric oxide synthetase (iNOS) induced by IFNγ. In order to clarify the mechanism of parasite control in this *in vitro* model, the activities of IDO and cyclooxygenase 2 (COX-2) were studied. Co-cultures of glia/neuron obtained from rat brains were treated with the inhibitor of IDO (1-methyl tryptohan/10^{-3}M/mL) and with inhibitors of COX-1 (indomethacin/10^{-6} M/mL) and COX-2 (nimesulide/10^{-6} M/mL) before infection with tachyzoites of *N.caninum* (1:1 cell:cell). After 72 hours enzymes activities were evaluated and cell phenotypes determinate using βIII tubulin, OX-42 and GFAP to observe neurons, microglia and astrocytes respectively. Immunological profile of response was determinate by ELISA tests of IL-10, IFNγ and TNF. It was verified that parasite infection in co-cultures increased twice IDO activity measured by kinurenin releasing. In cultures treated with IDO inhibitor 1-MT and infected with tachyzoites it was verified about 40% of parasite proliferation and an increasing of enzyme activity. Concerning to COX-2 activity, infected cultures stimulated the release of PGE_2, while nimesulide allowed the parasitic growth and a lost of 30 or 50% of astrocytes and microglia respectively, however was preserved neurons in cultures infected. Infection increases IL-10 and TNF even upon IDO inhibition but it does not release IFNγ. These data indicate that in this in vitro system IDO is activated by a mechanism independent of IFNy and parasite control could be mediated by synergistic effects of PGE_2 and TNF. In fact, COX-2 activation seems to be an important via in parasite control and PGE_2 associated to IL-10 besides to modulate the inflammation, allows continuity of parasitism.

Key words: *Neospora caninum*, neuroinflammation, indoleamine 2,3 dioxygenase, cyclooxygenase 2

LISTA DE ABREVIATURAS

BDNF - Fator neurotrófico derivado do cérebro

COX-1/2 - Ciclooxigenase tipo 1/2 (E.C. 1.14.99.1)

ELISA - Ensaio de imunoabsorbância ligada à enzima

GDNF - Fator neurotrófico derivado de células da glia

GFAP - Proteína ácida do gliofilamento

GM-CSF - Fator estimulante de colônia de granulócitos e macrófagos

GUSB- Enzima beta glicuronidase (E.C 3.2.1.31)

IDO 2- Indolamina 2,3 dioxigenase tipo 2 (E.C1.13.11.52)

IFI – Imunofluorescência indireta

IFN-α - Interferon alfa

IFN-β – Interferon beta

IFN-γ – Interferon gama

IgG – Imunoglobulina G

IgG2a – Imunoglobulina G do subtipo 2a

IL-1 – Interleucina 1

IL-10 – Interleucina 10

IL-12 – Interleucina 12

IL-4 – Interleucina 4

IL-6 – Interleucina 6

iNOS – Óxido nítrico sintase induzível (E.C 1.14.13.39)

LPS – Lipopolissacarideo

M-CSF – Fator estimulante de colônias de macrófagos

MIP-1 – Proteína de inflamação de macrófago 1

NAT – Teste de aglutinação para *Neospora*

NGF – Fator de crescimento neural

NK – Células *natural killer*

NO – Óxido nítrico

PGE_2- Prostaglandina E_2

PCR – Reação de polimerização em cadeia

RANTES – *Regulated upon Activation, Normal T-cell Expressed and Secreted*

ROS – Espécie reativa de oxigênio

SNC – Sistema nervoso central

T CD4+ – Linfocito T CD4+

TGF-β – Fator transformador de crescimento beta

Th1 – Resposta imune de células T auxiliares do tipo 1

Th2 – Resposta imune de células T auxiliares do tipo 2

TLR – Receptores *Toll-like*

TNF – Fator de necrose tumoral

UI/mL – Unidades internacionais por mililitro

SUMÁRIO

1. INTRODUÇÃO

O parasito *Neospora caninum* tem despertado especial interesse da Medicina Veterinária por causar relevantes prejuízos econômicos na indústria agropecuária mundial (REICHEL, et al., 2013). Este protozoário infecta várias espécies animais, com destaque para canídeos e bovinos, provocando desordens neuromusculares e, nesses últimos, abortamentos (DUBEY; SCHARES, 2011). Desde que foi identificado e descrito, (BJEKAS et al., 1984), estudos têm abordado a biologia e a interação do parasito com o hospedeiro, na tentativa de elucidar os mecanismos patogênicos da infecção e assim, promover seu controle epidemiológico e aperfeiçoar métodos de diagnóstico e tratamento.

O estudo da resposta imune celular durante a infecção por *N. caninum*, mostra que animais parasitados desenvolvem sistemicamente um padrão de resposta do tipo Th1 com a liberação de citocinas pró-inflamatórias. Estas contribuem para uma inibição da multiplicação dos taquizoítos ao passo que promovem uma maior lesão tecidual no sítio de infecção (KHAN et al., 1997; MARKS et al., 1998; BASZLER et al., 1999). Por outro lado, o perfil da resposta imune é passível de mudança para um padrão Th2 quando animais gestantes são infectados, havendo uma produção de citocinas anti-inflamatórias e regulatórias. Isto aponta para uma modulação da resposta, atuando como protetora, no interesse de garantir o sucesso da gestação (LONG; BASZLER, 2000; QUINN et al., 2004; KANO et al., 2005).

O tropismo deste protozoário pelo tecido nervoso e as consequentes manifestações clínicas, tem fomentado o interesse de pesquisadores em estudar a capacidade imunomodulatória deste sistema, frente à infecção parasitária. Modelos

experimentais utilizando culturas primárias de células de sistema nervoso, isoladas ou mistas (PINHEIRO et al., 2006; JESUS et al., 2013), assim como co-cultura neurônio-glia (JESUS et al., 2014) têm apontado promissores resultados. Células gliais infectadas com taquizoítos do *N. caninum*, mostraram-se reativas à infecção parasitária, com um perfil de resposta do tipo Th2, o que sugere modulação frente ao parasito, para proteger os neurônios dos efeitos nocivos de citocinas pró-inflamatórias (PINHEIRO et al., 2010).

A ativação de sistemas enzimáticos que contribuem para o controle da proliferação parasitária, bem como aqueles que regulam o sistema imune, para manutenção do tecido e preservação do parasito, têm sido o foco de alguns estudos (HUNT et al., 2006; SPEKKER et al., 2009; CARVALHO et al., 2010; MACHADO et al., 2011; NAHREVANIAN, 2006).

Sabe-se que o sistema nervoso central (SNC) responde a diferentes tipos de danos, por meio de uma complexa rede de integração, caracterizada pelo diálogo físico-químico entre as células que compõem este sistema ou ainda ou por células e mediadores da resposta imune sistêmica que afluem para este sítio. Assim, em condições de *stress*, as células deste tecido são passíveis de mudanças estruturais e do perfil imunológico que contribuem tanto para neurotoxicidade e/ou neuroproteção (BARRIENTOS et al., 2015).

O SNC é susceptível a infecção por diferentes tipos de parasitos, incluindo protozoários do gênero *Trypanossoma*, *Plasmodium* e *Toxplasma* provocando distúrbios neurológicos (FINSTERER; AUER, 2013). Sabe-se que os neurônios expressam a enzima óxido nítrico sintetase constitutiva (NOS-n), associada aos fenômenos de neurotransmissão, enquanto a glia expressa uma isoenzima tipo II (iNOS), induzida por processos inflamatórios (GHASEMI; FATEMI, 2014). Ainda, na

perspectiva de controle da proliferação parasitária, destaca-se o sistema enzimático indolamina 2,3 dioxigenase que oxida triptofano, consumindo este aminoácido essencial, além de produzir catabólitos potencialmente tóxicos para o SNC e para o parasito. Por outro lado, efeitos compensatórios são observados quando o tecido nervoso é agredido, visto que a cicloxigenase 2 (COX-2) quando ativada promove a liberação de prostanóides como PGE_2, que por sua vez, ao se ligar aos receptores EP2 promovem neuroproteção (JIANG; DINGLEDINE, 2013). Neste microambiente, o *N. caniunum* se encontra modulando as vias enzimáticas e mantendo uma relação parasito-hospedeiro estável.

2.0. REVISÃO DE LITERATURA

2.1. *Neospora caninum* e neosporose

Neospora caninum é um protozoário intracelular obrigatório que foi primeiramente relatado em cães, nos quais causa desordens neuromusculares (DUBEY et al., 2007; DUBEY; SCHARES, 2011). O protozoário *N. caninum* pertence ao filo Apicomplexa, família Sarcocystidae, subfamília Toxoplastinae (ELLIS et al., 1994). *N. caninum* é correlato ao protozoário *Toxoplasma gondii* e compartilha com estas características fenotípicas relacionadas à biologia, formação de cistos teciduais e excreção de oocistos. Apesar da similaridade filogenética com *T. gondii*, apresenta configuração antigênica diferenciada observada por técnicas moleculares e ultraestruturais (ELLIS et al., 1999; MUGRIDGE et al., 1999; REID, et al., 2012). As moléculas presentes na superfície de *N. caninum* participam diretamente nos

processos interativos com as células do hospedeiro e desencadeiam importantes eventos da imunopatogênese da infecção (ENGLISH, et al., 2015).

O parasito realiza replicação sexuada e assexuada em hospedeiros definitivos e intermediários respectivamente (McALLISTER et al., 1998; DUBEY, 1999, 2003), caracterizando-o como um protozoário heteroxênico. *N. caninum* tem como hospedeiros definitivos o cão (McALLISTER et al.,1998) e o coiote (GONDIM et al., 2004) e como hospedeiros intermediários, bovinos, bubalinos, equinos, caprinos, ovinos, cervos (DUBEY, 2003), raposas (SCHARES et al., 2001; NASCIMENTO, et al., 2015) e galinhas (COSTA et al., 2008). Animais como gatos, ratos, camundongos, coelhos, macacos, (DUBEY, 1999), porcos (JENSEN et al., 1998), pombos (McGUIRE et al., 1999), gerbis (GONDIM et al., 2001) e marsupiais (KING, et al., 2011) tornam-se hospedeiros intermediários quando experimentalmente infectados.

N. caninum pode se disseminar entre os hospedeiros através de transmissão horizontal ou vertical. A primeira forma é aquela na qual o hospedeiro se infecta por ingestão de alimentos e fonte de água contaminada com oocistos ou através do consumo de tecidos contendo bradizoítos do protozoário. Já a transmissão vertical, ocorre quando fêmeas parasitadas, por via transplacentária, infectam sua prole e se constituem na principal forma de transmissão do parasito em rebanhos bovinos (HEMPHILL; VONLAUFEN; NAGULESWARAN, 2006; WILLIAMS et al., 2007). A principal forma de infecção em carnívoros se dá pelo consumo de carcaças de hospedeiros intermediários contendo cistos do protozoário ou restos fetais infectados com *N. caninum* (McALLISTER, 1998; DUBEY, 2003; GONDIM, 2006). Outras formas de infecção são apontadas pela literatura. A placentofagia por vacas sugere um mecanismo alternativo de transmissão horizontal da neosporose entre

bovinos (MODRY et al., 2001). Ainda neste sentido, a transmissão lactogênica tem sido demonstrada experimentalmente em bezerros recém-nascidos, alimentados com taquizoítos adicionados ao colostro, entretanto, tal via não foi observada naturalmente (DAVISON et al., 2001).

A neosporose em cães pode ser localizada ou generalizada e tem sido descrita como uma doença que causa dermatite (neosporose cutânea) (LAPERLÉ et al., 2001; ORDEIX et al., 2002), paralisia de membros, pneumonia, miosite, miocardite, pancreatite, hepatite e lesões no sistema nervoso central (SNC) (LINDSAY; DUBEY, 1990; McALLISTER et al., 1996).

Os principais sinais relatados, frequentemente em animais abaixo dos dois meses de idade, são neuromusculares e envolvem membros posteriores e/ou anteriores que podem estar flexionados ou hiperextendidos e o exame neurológico revela ataxia, diminuição do reflexo patelar e perda da propiocepção consciente. Além disso, defeitos congênitos, incluindo hidrocefalia e estreitamento da medula espinhal, podem também ser detectados (DUBEY; SCHARES, 2011). A doença pode ser fatal, principalmente se o diagnóstico e consequente início do tratamento forem tardios, podendo os animais sobreviventes permanecer com sequelas devido a lesões no sistema nervoso (GIRALDI; BRACARENSE; VIDOTTO, 2001).

Os sinais clínicos anteriormente descritos são compatíveis com os achados histopatológicos. Estes últimos são de ocorrência predominante no sistema nervoso central dos animais infectados e caracterizam-se por meningo-encefalite multifocal não supurativa, com ou sem áreas de malácia, presença de infiltrado perivascular, neovascularização, gliose, vasculite mononuclear e meningite (DUBEY et al., 1999; POLI et al., 1998).

O entendimento da fisiopatologia da neosporose tem possibilitado a otimização do diagnóstico e contribuído para o desenvolvimento de estratégias para prevenção e controle desta doença (DUBEY et al., 2003). Diferentes técnicas de diagnóstico têm sido utilizadas para identificar animais infectados por *N. caninum.* Os testes sorológicos se constituem importantes ferramentas para o diagnóstico da neosporose. Sendo possível detectar anticorpos anti-*N. caninum* através de ensaio de imunoabsorbância ligada à enzima (ELISA) (HAMIDINEJAT et al., 2015), teste de imunofluorescência indireta (IFAT) (PAIZ et al., 2015) e teste de aglutinação (NAT). Além destas ferramentas, o *western blotting* tem sido empregado para caracterizar antígenos de *N. caninum* e para identificar anticorpos específicos (SILVA et al., 2006; GHALMI et al., 2014; ALMERÍA; LÓPEZ-GATIUS, 2015).

2.2. Resposta imune durante infecção por *Neospora caninum*

A relação entre o sistema imunológico dos mamíferos com os seres apicomplexos está sujeita à composição e execução de funções baseadas na interação entre as moléculas do sistema imune do hospedeiro e as moléculas antigênicas do parasito. A intensa interação entre estes dois sistemas biológicos distintos promove o aperfeiçoamento da defesa do hospedeiro, ao passo que o invasor desenvolve sofisticados mecanismos de escape (BRAKE, 2002).

Ao detectar a atividade parasitária do *N. caninum,* o sistema imune do hospedeiro dispara seus mecanismos de resposta inata, os quais envolvem a liberação de quimiocinas que recrutam e ativam leucócitos e que também estão

envolvidas na regulação do processo inflamatório (TAUBERT et al., 2006 a, b). Adicionalmente, este protozoário é capaz de estimular uma resposta imunológica celular e humoral (NISHIKAWA et al., 2001a; INNES et al., 2005). Diversos estudos têm demonstrado o importante papel desempenhado pela resposta imune celular, durante a neosporose, devido à liberação de citocinas pró-inflamatórias por linfócitos T $CD4^+$ e T $CD8^+$ que asseguram a inibição da multiplicação parasitária (INNES *et al*., 2005). Ao passo que a resposta imunológica mediada por anticorpos, tem sido utilizada como importante ferramenta para estudos epidemiológicos e para o diagnóstico da neosporose (INNES et al., 2002).

A resposta imune inata consiste no recrutamento, para o local da infecção, de células polimorfonucleares, macrófagos e células *natural killer* (NK) (IWASAKI, MEDZHITOV, 2004). Neste contexto, ocorre síntese de interferon gama (IFN-γ) por células NK, que ativam neutrófilos e macrófagos, sendo que estes últimos estão envolvidos na produção de intermediários reativos do oxigênio (H_2O_2) e os intermediários reativos do nitrogênio como óxido nítrico (NO), os quais são capazes de destruir parasitos intracelulares controlando, assim, a replicação parasitária (DENKERS et al., 2004). Além disso, parasitos coccídios estimulam as células do sistema imune inato a sintetizarem interleucina-12 (IL-12), a qual induz a produção local de IFN-γ por linfócitos T e células NK (BRAKE, 2002). O IFN-γ secretado por linfócitos T ativa macrófagos e estes, quando ativados, atuam como primeira linha de defesa eliminando diretamente o parasito por fagocitose (NISHIKAWA et al., 2001).

A resposta celular ao *N. caninum* pode ser caracterizada pela indução de células T antígeno-específicas, mediada pela produção de citocinas, principalmente IL-12 e IFN-γ (KHAN et al., 1997). A importância dessas citocinas no

controle da infecção foi demonstrada quando os tratamentos com anticorpos utilizados para neutralizar suas respectivas atividades promoveram maior susceptibilidade para a infecção por *N. caninum* (KHAN et al., 1997; BASZLER et al., 1999).

O IFN-γ é também capaz de estimular uma resposta imunológica humoral (NISHIKAWA *et al*., 2001; INNES *et al*., 2005), sendo esta utilizada como importante ferramenta para estudos epidemiológicos e para o diagnóstico da neosporose (INNES et al., 2002). A resposta imune humoral do hospedeiro induzida pelo *N. caninum* é similar àquela observada com o *T. gondii*, na qual anticorpos específicos podem destruir taquizoítos na presença do complemento, dificultando a sua penetração nas células teciduais (MARKS et al., 1998).

O importante papel da resposta imune humoral foi demonstrado em infecção experimental com taquizoítos de *N caninum* em camundongos C57BL/6 *knockout* para linfócitos B, onde estes camundongos mostraram-se mais susceptíveis à infecção quando comparados com camundongos do tipo selvagem C57BL/6 (EPERON; BRONNIMANN; HEMPHILL, 1999). Além disso, Marez et al. (1999) observaram que bovinos infectados oralmente com oocistos de *N. caninum* desenvolviam uma forte resposta humoral com a produção de anticorpos IgG1 e IgG2.

O padrão da resposta imune humoral na produção de anticorpos está diretamente relacionado com o perfil de citocinas que são liberadas quando estimuladas pelo patógeno. Assim, citocinas do tipo Th1, tais como IL-12 e IFN-γ, favorecem a produção de anticorpos IgG2a. Por outro lado, citocinas do perfil Th2, tais como IL-4 e IL-10, estão associadas com a produção de anticorpos IgG1 (HEMPHILL et al., 2006). Apesar da necessidade de elucidar a homeostasia da

resposta Th1/Th2 na infecção por *N. caninum*, muitos estudos contribuem para a compreensão de que a atuação do linfócito TCD4$^+$ e, principalmente, da citocina IFN-γ são responsáveis por mecanismos de controle da proliferação parasitária. Neste sentido, pode-se afirmar que sistemicamente, uma resposta de padrão inflamatório estaria associada à resistência animal à infecção, bem como progressão para a fase crônica e assintomática da neosporose (KHAN et al., 1997; DUBEY et al., 1998; BASZLER et al., 999; INNES et al., 2005; WILLIAMS; TREES, 2006).

2.2.1. Resposta imune no sistema nervoso central durante infecção por *N. caninum*

O sistema nervoso central (SNC) é constituído por diversos tipos celulares, dentre os quais se destacam as células gliais. A glia, que compreende aproximadamente 90% do total de células que compõem o tecido nervoso, é subdividida em macroglia (astrócitos e oligodendrócitos) e em microglia. Os astrócitos representam as células mais numerosas da glia, onde contribuem para a homeostasia cerebral, garantindo a manutenção extracelular de potássio, regulando a liberação de neurotransmissores, participando na formação da barreira hemato-encefálica, liberando fatores de crescimento ou regulando a resposta imune no cérebro (GEE e KELLER, 2005, OWENS et al., 2005; FARINA et al., 2007; KETTENMANN; VERKHRATSKY, 2011). Já os oligodendrócitos, são encarregados do processo de mielinização das terminações neuronais. A micróglia, por sua vez, representa aproximadamente 20% do total de células da glia (VILHARDT, 2005) e deriva de precursores mielóides da medula óssea que migram para o SNC durante o desenvolvimento. Portanto, a micróglia são células imunes residentes no cérebro

que tem a função de detectar qualquer distúrbio fisiológico. Assim, quando os neurônios sofrem danos, provocados por diferentes agentes etiológicos, a micróglia se torna ativada mediante a liberação de ATP, neurotransmissores, fatores de crescimento, citocinas ou ainda a perda de moléculas inibidoras que são expostas pelos neurônios saudáveis (HANISCH; KETTENMANN, 2007). Além destas atribuições, a micróglia tem o papel de fagocitar debris celulares e patógenos invasores, o que lhes confere papel análogo aos macrófagos do sistema imune. Sabe-se que a micróglia expressa marcadores fenotípicos de macrófagos como CD11b. Por outro lado, quando o SNC não se encontra sobre *stress*, a micróglia em repouso expressa baixos níveis de moléculas MHC classe I e II em sua superfície (KAUR et al., 2010).

O papel fisiológico desempenhado por células gliais durante eventos de morte celular provocados por uma resposta inflamatória exacerbada tem sido demonstrado em vários estudos. Sabe-se que astrócitos e microglia se ativam sob condições patológicas resultando em comprometimento da função neuronal e no desenvolvimento ou agravamento de algumas doenças do SNC (BELANGER; MAGISTRETTI, 2009; ALLAMAN; BÉLANGER; MAGISTRETTI, 2011). Por outro lado, é necessário lembrar que eventos pós lesão tecidual apresentam uma via bi-direcional e refletem na homeostasia entre as células da glia ativadas e os neurônios (LIU et al., 2012; SHERIDAN; MURPHY, 2013).

Neste contexto, o papel desempenhado pelos neurônios está associado à indução do controle da resposta imune glial por vários neurotransmissores ou moduladores como NO, glutamato e fractalcina (LIU et al., 2006; LIU et al., 2011). Quando fisiologicamente ativos, neurônios geralmente apresentam um potencial supressivo da resposta glial, prevenindo ou limitando os danos ocasionados pela

resposta inflamatória (ITURRIA-MEDINA; EVANS, 2015). Apesar disto, o equilíbrio entre citocinas pró-inflamatórias e os elementos supressores derivados do SNC determina a capacidade apresentadora de antígenos da microglia e o resultado de reações inflamatórias no tecido cerebral (NEUMANN, 2001). Os neurônios, células gliais e células do sistema imunológico formam uma rede coordenada para manter a homeostase e restringir neuroinflamação no SNC. Esta rede integradora não só está envolvida na patogênese da neuroinflamação, mas principalmente desempenha um papel importante nas funções normais do cérebro (TIAN et al., 2012).

Dentre alguns estudos realizados para investigar o comportamento do sistema imune frente à infecção pelo *N. caninum* no sistema nervoso, cita-se aquele de Yamane et al. (2000). Os autores observaram que a adição de IFN-γ em co-culturas de células gliais e neurônios obtidas de cérebro bovino infectadas com taquizoítos foi responsável pela inibição do crescimento parasitário e que esta inibição podia ser mediada por receptores específicos de IFN-γ na superfície destas células. Esses autores observaram efeito semelhante para o TNF, ainda que com menor expressão. Estes dados são reafirmados por Vonlaufen et al. (2002), ao observarem que em culturas organotípicas obtidas do SNC de ratos pré-tratadas com IFN-γ e infectadas com taquizoítos de *N. caninum,* havia inibição da proliferação parasitária, indicando a importância desta citocina no controle da infecção.

A atividade de citocinas pró-inflamatórias também foi vista em culturas primárias de astrócitos obtidos de córtex cerebral de ratos e infectados com taquizoítos de *N. caninum*. Em períodos de 24 e 72 horas após infecção, estes secretaram níveis expressivos de TNF e de óxido nítrico (PINHEIRO et al., 2006). Posteriormente, PINHEIRO et al. (2010) utilizaram culturas mistas de células gliais

(astrócitos e micróglia) infectadas com *N. caninum*, onde notaram que havia produção de mediadores pró-inflamatórios como TNF e óxido nítrico.

Por outro lado, as células gliais produzem citocinas anti-inflamatórias para regular os efeitos deletérios causados por citocinas pró-inflamatórias. Um estudo utilizando culturas primárias de astrócitos de ratos tratadas com fator de crescimento transformador beta (TGF-β), IL-10 e IL- 6 mostrou que havia uma inibição da citocina pró-infamatória TNF, o que indica que citocinas anti-inflamatórias e/ou reguladoras são importantes para manter a homeostasia do SNC (BENENVISTE et al., 1995).

A secreção de IL-10 pelas células da micróglia pode ser parte dos mecanismos envolvidos na homeostase no SNC durante a infecção por *T. gondii* (ROZENFELD et al., 2003). Este efeito pode ser explicado pelo papel da IL-10 na redução do estresse oxidativo, promovendo uma regulação negativa na produção de NO por micróglia ativada por IFN-γ, e consequentemente na restauração do crescimento neuronal (GAZZINELLI et al., 1996; ROZENFELD et al*.,* 2003). Pinheiro et al. (2006 e 2010) mostraram que as células gliais de ratos quando infectadas por taquizoítos de *N. caninum* liberam IL-10, modulando a resposta inflamatória no interesse de preservar o tecido nervoso.

Estudo recente com infecção *in vitro* por *N. caninum* em co-culturas glia-neurônio observou preservação neuronal e ausência de NO, sugerindo que fatores neurotróficos como Nerve growth factor (NGF), Brain-derived neurotrophic factor (BDNF), *Neurotrophin-3* (NT-3) e *Glial cell-derived neurotrophic factor* são responsáveis por inibir os efeitos deletérios ocasionados ao SNC (JESUS et al., 2014).

2.3. Ativação das vias enzimáticas da resposta imune durante infecção por protozoários

Em infecções por coccídios a resposta imune inata é disparada por antígenos presentes na superfície dos parasitos, conhecidos como padrões microbrianos associados à patógenos (PAMPs). Estes se ligam a receptores de reconhecimento de padrão da imunidade inata - receptores *Toll-like* - iniciando a resposta à infecção (A BRAKE, 2002). Além disso, foi demonstrado a participação de células *natural killer* (NK) durante a resposta inata, sendo estas ativadas por antígenos de superfície presentes nos taquizoítos (KLEVAR et al., 2007), produzindo INF-γ para inibir a multiplicação parasitária (BOYSEN et al., 2006). Esta citocina, quando liberada, é responsável por incitar três tipos de mecanismos antiparasitários, a saber: (i) mecanismo oxidativo, por meio da síntese de espécies reativas do oxigênio (JUN et al., 1993), (ii) mecanismo não oxidativo, com recrutamento de macrófagos para o sítio de infecção (ADAMS et al., 1990), e (iii) ativação da enzima 2,3 indolamina dioxigenase (IDO) (PFEFFERKORN et al., 1986).

A atividade da IDO (E.C.1.13.11.52) nas células da imunidade inata foi inicialmente associada com a defesa do hospedeiro contra patógenos tais como *Toxoplasma gondii*, *Chlamydia psitacci,* citomegalovírus (CMV) e na contenção do crescimento de células tumorais, por depletar triptofano e limitar a habilidade dos patógenos de sintetizar proteínas (TAYLOR et al., 1991; SEDLMAYR et al., 2002; UYTTENHOVE et al., 2003). A IDO age sobre o L-triptofano e o transforma em N-formil quinurenina, e a hidrólise deste composto por formidase libera quinurenina como catabólito, sendo este último utilizado comumente para mensurar a atividade da IDO (FRUMENTO et al., 2002; RAFICE et al., 2009). A degradação do triptofano

pela IDO ativada por IFN-γ pode limitar a proliferação de certos patógenos que são dependentes deste aminoácido para o seu crescimento (MACKENZIE et al., 2007). O composto 1-metil-triptofano (1MT) compete pela enzima IDO e inibe o seu efeito, consequentemente inibindo a formação dos catabólitos do triptofano como as quinureninas (YUASA et al., 2010). Além da ativação desta via por ação do IFNγ, outra forma também é observada. Braun et al. (2005), observaram que ativação da IDO 2,3 dioxigenase em células dendríticas ocorria em virtude do sinergismo entre PGE_2 e TNF. Posteriormente, Von Bergwelt-Baildon et al., 2006, observaram que a maturação de células dendríticas na presença de PGE_2 aumenta expressão de IDO 2,3 dioxigenase e consequentemente dispara a cascata de oxidação do triptofano. A função da IDO é catalisar a oxidação do TRP a N-formilquinurenina (NFK), posteriormente deformilada a quinurenina (QUIN).

Alguns estudos *in vitro* têm demonstrado atividade antiparasitária mediada por IFN-γ contra *T. gondii,* por meio da enzima IDO em células humanas (macrófagos, fibroblastos, células epiteliais e glioblastoma) (MURRAY et al., 1989; PFEFFERKORN et al., 1989; FUJIGAKI et al., 2001). Spekker et al (2009) mostraram que a citocina IFN-γ participa como indutor da síntese da enzima IDO, funcionado como um potente antiparasitário por catalisar a oxidação do triptofano. Estes resultados respaldam os achados de Carvalho et al. (2010), que observaram que em cultura de células epiteliais uterinas humanas (HeLa) e trofoblastos (BeWo) infectadas por taquizoitos de *N. caninum,* quando ativadas por IFN-γ e suplementadas com triptofano ou tratadas com metiltriptofano (inibidor competitivo específico da IDO), ocorria o aumento do crescimento parasitário, apontando a importância desta enzima no controle parasitário dependente de IFN-γ. Por outro lado, a atividade de IDO nas células é regulada por vários fatores bioquímicos tais

como a presença de óxido nítrico (NO) e a biossíntese de grupos heme. Citocinas como IL-6, IL-4, IL-13 e TGF-β são apontadas como supressoras de IDO (ORABONA et al*.,* 2005).

A citocina IFN-γ também está envolvida na ativação do sistema enzimático da óxido nítrico sintetase induzível (iNOS) ou do tipo II, culminando na destruição de *T. gondii* (ADAMS et al., 1990). A iNOS (E.C. 1.14.13.39) ou isoforma II não é expressa constitutivamente nos tecidos. Assim, quando induzida por citocinas do perfil pró-inflamatório, é capaz de produzir NO por longo tempo e isto caracteriza seu envolvimento em vários processos patológicos. Deste modo, o alto nível de NO liberado por células imunes efetoras, sejam elas do sistema imune sistêmico, ou por células residentes do tecido nervoso, além de ser tóxico para o patógeno, também é lesivo para o tecido adjacente, sendo este mecanismo responsável por amplificar o processo inflamatório (MARLETTA et al., 1988; JUN et al., 1993).

A reação química de formação do NO, parte-se da transformação da L-arginina em um intermediário, a NG-hidroxi-L-arginina com a presença de nicotinamida-adeninadinucleotídeo-fostato reduzido (NADPH) e Ca^{2+} sendo necessário mais adenosina difosfato reduzida (ADPH) e O_2 para a formação de L-citrulina e NO. A síntese enzimática de citrulina pode ser inibida por análogos da L-arginina tais como N^G-monometil-L-arginina (L-NMMA), N^G-nitro-Larginina (L-NNA) e N^G-nitro-L-arginina-metiléster (L-NAME). Estes inibidores têm grande importância na pesquisa dos prováveis efeitos do NO nos tecidos, uma vez que a substituição do substrato habitual (L-arginina) pelos análogos irá inibir a produção de NO e seus efeitos consequentes (REES et al., 1990).

Por outro lado, os coccídios também podem ativar rotas bioquímicas como a síntese de PGE_2, ou ainda inibir a iNOS estrategicamente no interesse de escapar

da resposta imune. A conversão de ácido araquidônico para PGE_2 é catalisada pela enzima cicloxigenase (COX) (E.C 1.14.99.1) que está presente sob duas isoformas. A COX-1 é constitutiva dos tecidos, enquanto que a COX-2 é altamente induzível em resposta a LPS ou a interleucina-1 (IL-1) (PERCIVAL et al., 1994; ARIAS-NEGRETE et al., 1995). Peng et al (2008) demonstraram que taquizoítos de *T. gondii* induziam a biossíntese de PGE_2 via COX-2 em macrófagos por meio da regulação de cálcio e da proteinacinase C (PKC). A infecção por *T. gondii* tanto *in vitro* como *in vivo* conduz a síntese de PGE_2 a partir do ácido araquidônico e esta molécula está envolvida na persistência e progressão da toxoplasmose (THARDIN et al., 1993; HENDERSON e CHI 1998). Este prostanóide também está envolvido em uma atividade imunomodulatória por inibir a resposta pró-inflamatória, proporcionando assim um mecanismo de escape do parasita, visto que inibe a ativação de macrófagos e a síntese de óxido nítrico (WILBORN et al., 1995). Células endoteliais de veia umbilical bovina (BUVEC) quando infectadas por taquizoítos de *N. caninum* expressaram quantidades aumentadas de RNAm das enzimas COX-2 e de iNOS após 6 e 72 horas de infecção, demonstrando que o processo lesivo provocado tanto pela infecção quanto pela produção de NO pode ser acompanhado pela síntese de prostanóides que modulam os efeitos inflamatórios da resposta imune inata (TAUBERT, et al., 2006).

3. JUSTIFICATIVA

A infecção por *N. caninum* constitui um importante problema na Medicina Veterinária, especialmente por induzir alterações neurológicas em cães e abortamento em bovinos, provocando elevadas perdas econômicas na pecuária mundial. Sua identificação e caracterização são relativamente recentes na literatura e muitos estudos têm sido desenvolvidos nos últimos anos, para o entendimento da patogênese da doença, bem como, dos fatores interferentes na relação parasito-hospedeiro.

A neuroinflamação decorrente de uma agressão ao SNC corresponde a uma complexa integração de resposta de suas células, podendo resultar em consequências favoráveis (neuroproteção) ou desfavoráveis (neurotoxicidade) àquele tecido. Uma resposta inflamatória bem-sucedida não somente elimina o agente inflamatório ou o patógeno invasor, como também promove cicatrização e angiogênese. Porém, a agressão inflamatória pode também progredir ou tender à cronicidade, seja por falência do tecido em combater o patógeno ou por necessidade de responder de forma limitada e circunscrita, o que no tecido nervoso preservaria funções, sobretudo neuronais. O equilíbrio entre a neuroproteção e a neurotoxicidade depende da interação - do “diálogo” - entre as células envolvidas na resposta. Em virtude dos conhecimentos incipientes no processo de neuroinflamação disparados pela presença do parasito *N. caninum* no tecido nervoso, faz-se necessário entender as rotas bioquímicas utilizadas pelas células da glia e neurônios para gerar uma resposta imune efetora.

Alguns estudos *in vitro* têm apontado a importância da ativação de algumas vias enzimáticas que participam no controle do crescimento parasitário e que

também podem participar na manutenção da viabilidade celular. Entre estas vias, destaca-se a atividade da indolamina 2,3 dioxigenase (IDO) que, ao ser ativada, controla o crescimento de *N. caninum* em co-culturas de glia/neurônio e conduz para manutenção da homeostasia parasito-hospedeiro. Além disto, ressalta-se o papel da da COX-2 ativada pelo parasito durante a infecção. Os prostanóides oriundos desta via, a exemplo da PGE_2, têm sido observados em modular os efeitos deletérios do agente agressor. Também é importante lembrar que estudos anteriores conduzidos *in house* apontaram a participação de óxido nítrico sintase induzível como via enzimática de eleição em cultivos primários de astrócitos e culturas mistas de astrócitos e micróglia.

As co-culturas de astrócitos, microglia e neurônios, revelam-se úteis em esclarecer o papel imunomodulador da glia frente à infecção por *N. caninum*, bem como sua capacidade de induzir neuroproteção ou neurotoxidade na dependência das características da resposta.

4.HIPOTESE

O controle do crescimento parasitário ocorre por ativação da indolamina 2,3 dioxigenase que oxida triptofano e limita o crescimento parasitário.

5. OBJETIVOS

5.1. Objetivo geral

Esclarecer possíveis mecanismos da resposta inflamatória de co-culturas glia-neurônio de cérebros de ratos através de sistemas enzimáticos ativados durante a infecção por *Neospora caninum* em co-cultura glia/neurônio.

5.2. Objetivos específicos

1. Investigar a atividade da óxido nitríco sintase induzível (iNOS) e da indolamina 2,3 dioxigenase (IDO) em co-culturas infectadas com taquizoítos de *N. caninum*, previamente moduladas com seus respectivos inibidores N-nitro-L-arginina-metil-éster (L-NAME) e 1-metil-triptofano (1-MT).

2. Investigar a produção de citocinas (INF-γ, TNF, IL-10 e TGF-β) em co-culturas infectadas com taquizoítos de *N. caninum* previamente moduladas com L-NAME e 1-MT.

3. Investigar a produção da PGE_2 em co-culturas infectadas com taquizoítos de *N. caninum*, tratadas e não tratadas com indometacina e nimesulida, inibidores da cicloxigenase Tipo-1 e tipo-2 (COX1-1 e COX-2).

4. Avaliar fenótipos celulares por meio de citometria em culturas de córtex cerebral de ratos recém-nascidos quando infectados por taquizoítos de *N. caninum*, tratadas e não tratadas com os antagonista de PGE_2**.**

5. Estabelecer protocolo de viabilidade celular por meio da avaliação da atividade da enzima beta glicuronidase.

4. RESULTADOS

MANUSCRIPT 1

POSSIBLE MECHANISM OF PARASITE CONTROL IN A NEUROINFLAMMATORY MODEL USING NEURON-GLIA CO-CULTURES

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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. When primary cultures of mixed glial cells obtained from newborn rats are infected with *N. caninum* they release nitric oxide (NO) and IL10. Co-cultures of glia/neurons pretreated with IFNγ before infection are able to control parasite growth and to preserve neuron viability, besides inhibition of NO release. Previous data showed that IDO (indolamine 2,3 dioxygenase) is modulated by IFNγ in cell proliferation control. In studies of neuroinflammation using neuro-glia co-cultures infected by *N. caninum*, it was verified parasite control by a mechanism independent of type 2 nitric oxide synthetase (iNOS) induced by IFNγ. In order of a better characterization of the immune response during neosporosis in central nervous tissue, it has been proposed the study of possible parasitic proliferation control mechanisms through the catabolism of the amino acids, such as arginine and tryptophan, and the participation of PGE_2 as a neuromodulator in the proposed model of murine glial/neuron co-cultures. Co-cultures of glia/neuron obtained from rat brains were treated with the inhibitor of iNOS (L-NAME/1.5 mM/mL), IDO (1-methyl tryptohan/10^{-3}M/mL) and with inhibitors of COX-1 (indomethacin/10^{-6} M/mL) and COX-2 (nimesulide/10^{-6} M/mL) before infection with tachyzoites of *N. caninum* (1:1 cell:cell). After 72 h infection enzymes activities were evaluated and cell phenotypes determined by flow cytometry. Immunological response profile was determined by ELISA to IL-10, IFNγ and TNF. Results: It was verified that parasite infection in co-cultures increased twice the IDO activity measured by kinurenin releasing. In cultures treated with IDO inhibitor 1-MT and infected with tachyzoites it was verified about

40% of parasite proliferation and an increasing of enzyme activity. Concerning to COX-2 activity, infected cultures stimulated the release of PGE_2, while nimesulide allowed the parasitic growth and a loss of 30 or 50% of astrocytes and microglia, respectively, however, neurons were preserved in infected cultures. Infection increased IL-10 and TNF even upon IDO inhibition but it does not release IFNγ. These data indicate that in this in vitro system IDO is activated by a mechanism independent of IFNγ and parasite control could be mediated by synergistic effects of PGE_2 and TNF. In fact, COX-2 activation seems to be an important via in parasite control and PGE_2 associated to IL-10 besides modulating the inflammation, also allows continuity of parasitism.

KEY WORDS: *N. caninum*, neuroinflammation, indoleamine 2,3 dioxygenase, cyclooxygenase 2

INTRODUCTION

Establishment of host-parasite relationship occurs through molecular interaction between them, the pathogen's ability to proliferate inside the cell and 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, 2007 et al., ADALID-PERALTA 2011). Many studies have been performed in attempt to clarify the biochemical pathways established by some protozoa (HARRIS; MITCHELL; MORRIS, 2014). Intracellular protozoan 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). Despite the advances made in recent years regarding the biology and interactions of the parasite *Neospora caninum* with its hosts, it is still necessary to clarify the parasite control mechanisms and biochemical pathways established by this coccidia to evade the immune response triggered during its encysting in central nervous system. *N. caninum*, an obligate intracellular protozoa, is of great importance to Veterinary Medicine by infecting various animal species, and especially for causing abortion in cows and provoking neuromuscular disorders in newborns (DUBEY; SCHARES, 2011). *N. caninum* has tropism for the central nervous system (CNS) (HEMPHILL et al., 2004) and this environment has been the focus of many studies in an attempt to clarify the neuropathogenesis, including those using cell lines and organotypic cultures that have provided valuable information about cell invasion and the events that occur during parasite proliferation (VONLAUFEN et al., 2002; PINHEIRO et al., 2006; DUBEY, SCHARES; ORTEGA-MORA, 2007).

During the CNS inflammatory process caused by *Toxoplasma gondii*, a *N. caninum* correlate parasite, the activation of indoleamine 2,3 -dioxygenase (IDO) induced by IFNγ and TNF was observed (SUZUKI, 2002; CARRUTHERS; SUZUKI, 2007), furthermore the activation of induced nitric oxide synthase (iNOS) by IFNγ (YAROVINSKY, 2014). IDO is responsible for tryptophan oxidative metabolism and has an important function controlling the parasite growth by depleting this essential amino acid of the microenvironment (PFEFFERKORN, 1984; SPEKKER et al., 2009). The iNOS catabolizes arginine with nitric oxide production, being capable of controlling parasite growth by this enzyme's toxic activity (RATH et al., 2014). These antiparasitic pathways regulate themselves. On the one hand, iNOS when activated inhibits the operation of IDO, and on the other hand, when IDO is activated iNOS is

suppressed (STONE; DARGLINGTON, 2002). This phenomenon is very interesting, since the activation of a biological route by the pathogen, to the detriment of the other, indicates its ability to remain viable in the host tissue to make it a less hostile environment for its growth and development and thus ensuring tissue preservation. It was also observed that infection by *T. gondii* in monocytes and murines glial cell cultures (LÜDER et al., 1998; ROZENFELD et al., 2003), as well as infection by *N. caninum* in mice glial cells (JESUS et al., 2013, 2014), induce the production of PGE_2 prostanoid, 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 central nervous system by inhibiting NO and its toxic effects (LEVI; MINGHETTI; ALOISI, 1998; ZHANG; RIVEST, 2001). In behalf of a better characterization of the immune response during neosporosis in central nervous tissue, it has been proposed the study of possible parasitic proliferation control mechanisms through the catabolism of the amino acids, such as arginine and tryptophan, and the participation of PGE_2 as a neuromodulator in the proposed model of murine glial/neuron co-cultures.

MATERIAL AND METHODS

Culture of N. caninum

Neospora caninum tachyzoites of the NC-Ba 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). The VERO cells were washed with phosphate

buffered saline (PBS) and then mechanically disrupted to obtain the parasites. Soon after, the tachyzoites were purified using a 5.0 μm filter (Millipore, Carrigtwohill, Ireland) as described by Pinheiro et al (2010).

Neuron/Glia co-cultures

Mixed glial cells (astrocytes and microglia) were first obtained from brain cortexes of newborn rats (<48 hours of age) by mechanical dissociation of the tissue. 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% CO2. 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, they were re-seeded (5 X 104) in 24-well tissue culture plates for assays. In this time, timed pregnancy rats were sacrificed on the 17^{th} or 18^{th} gestational day, and embryos were removed by caesarian section. Cortex dissection cells were dissociated in DMEM/F-12 as described above. Neurons (2.5 X 10^4/well) were then plated on astrocyte/microglia monolayer and the cultures were maintained with regular DMEM/F-12 changed every 48 hours to 7 days, when the experiments were performed.

Determination of iNOS activity

The supernatants from co-cultures neuron/glia were assayed for nitrite levels, which reflect the NO production, using a colorimetric test based on the Griess method. The co-cultures were stimulated with L-Nitroarginine methyl ester (L-NAME) (1.5 mM/mL) during 1 hour and infected with N. caninum in a rate 1:1 cell parasite for 72 h. The activity of iNOS correlates directly with the concentration of nitrite in supernatants of tissue culture cells, and thus measurement of the nitrite concentration can be used to determine iNOS activity. Triplicate 50 μL aliquots of the culture medium were mixed with an equal volume of a 1:1 (v/v) solution of 1.0% sulfanilamide and 0.1% naphthyl-ethylenediamine dihydrochloride in 2.5% phosphoric acid. After 10 min of incubation at room temperature, the absorbance was measured at 560 nm using a microtiter plate reader (BIOTEK INSTRUMENTS, Inc., USA). The nitrite concentrations were calculated by comparison with a standard calibration curve of sodium nitrite ($NaNO_2$: 1.26–100 Mmol/L) with DMEM as the baseline control. Data were expressed as percentage of optical densities for triplicate cultures. Three independent experiments were performed in triplicate wells for each analysis.

Determination of IDO activity

Neuron/Glia co-cultures were pretreated for 24 hours with 300 IU/mL of recombinant rat IFNγ (R&D Systems, USA) and thereafter cells were stimulated with tryptophan (TRP) 1 mM/mL and 1-metyl-tryptophan (1 MT) 1.5 mM/mL (Sigma-Aldrich, USA) for

1 hour diluted in culture medium, then infected with N. caninum in a rate 1:1 cell parasite for 72 hours. The activity of IDO correlates directly with the concentration of N-formyl-kynurenine in supernatants of tissue culture cells, and thus measurement of the kynurenine concentration can be used to determine IDO activity. Approximately about 160 μL of the culture supernatant was removed and transferred to microtubes. After addition of 10μL 30% trichloroacetic acid to each tube, the supernatant were incubated at 50°C for 30 min to hydrolyze the N-formyl-kynurenine to kynurenine. After centrifugation for 10 min at 600g, 100μL of supernatant was transferred to 96-well flat-bottom plates, and 100μL 1.2% (wt/vol) 4(dimethylamino) benzaldehyde (Ehrlich reagent; Sigma-Aldrich, Deisenhofen, Germany) in glacial acetic acid was added. After incubation 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, Deisenhofen, Germany).

Determination of COX activity

Neuron/Glia co-cultures were pretreated for 24 hours with 100 IU/mL of recombinant rat IFNγ (R&D Systems, USA) and after cells were stimulated with inhibitor of COX-1 and COX-2 (indomethacin/nimesulide -10^{-6} M/mL) (Sigma-Aldrich, USA) for 1 hour diluted in culture medium, then infected with N. caninum in a rate 1:1 cell parasite for 72 hours. The concentrations of PGE_2 were measured using a commercially-available ELISA (CAYMAN CHEMICAL Co., USA).

Flow Cytometry

For flow cytometry, cells were resuspended in flow cytometry buffer, consisting of HBSS (Hank's Balanced Salt Solution- GIBCO), pH 7.2, containing 1.55 g/L glucose and 0.1% of bovine serum albumin (BSA; Sigma-Aldrich). Cells were counted and diluted to a density of 10^6 cells per milliliter of buffer; all analyses were performed with 100 μL aliquots containing 10^5 cells. Cells were stained with anti-β tubulin III Alexa 647 (1:200), anti-GFAP-Alexa 488 (1:50), rat anti-CD11b/c-PE (1:200) and incubed for 45 min on ice. B-Tubulin III, CD11b and GFAP antibodies were purchased from BD Biosciences.

RESULTS

iNOS is important to the CNS homeostasis during infection by *N. caninum*

N. caninum infection in Neuron/Glia co-cultures did not stimulate nitrite synthesis (Figure 1), however iNOS inhibition by L-NAME enhanced the parasitic proliferation (Figure 2), indicating that it is important to have basal concentrations of nitric oxide to maintain the homeostasis in the CNS. Modulation with IFNγ 100 IU/mL was not able to activate iNOS and did not interfere in the parasitic proliferation control.

Immune response profile during infection by *N. caninum* after iNOS inhibition.

The immune response was investigated by dosing IFNγ, TNF and IL-10. In Glia/Neuron co-cultures there was no participation of IFNγ in the inflammatory process caused by *N. caninum* after 72 hours of infection (Figure 3). However it was observed that TNF was present at increased levels, in approximately 1.5-fold, compared to control (Figure 4). On the other hand, infection by *N. caninum* stimulated the synthesis of IL-10 only in cultures that weren't previously modulated by IFNγ (100 UI/mL), indicating that the pretreatment with this cytokine opposes to IL-10, a typically regulatory cytokine. When under IFNγ stimulus, an associative effect of the infection with iNOS inhibition was observed, increasing the levels of this cytokine in approximately 2-fold compared to its control group (Figure 5).

IDO controls *N. caninum* growth in Glia/Neuron co-cultures by an IFNγ independent mechanism

The activity of indoleamine 2,3 -dioxygenase was measured by the dosage kynurenine found in the supernatant of the culture medium. Therefore, higher concentrations of this product (generated by tryptophan oxidation) represent a greater activity of IDO. The concentration of IFNγ used, 100 UI/mL, aimed to investigate the involvement of IDO in an iNOS independent way, since this concentration is not capable of inducing iNOS activation in this experimental model.

In control cultures, it was observed that IFNγ modulation induces the activity of IDO, with a 3-fold increase, when compared to cultures without previous exogenous IFNγ treatment. Moreover, in cultures with no IFNγ premodulation but infected with tachyzoites, an increase of approximately 50% of kynurenine when compared to the control was observed, demonstrating that the parasite`s presence induced IDO activity. Reduction in the activity of IDO in IFNγ pre-stimulated cultures, treated with the inhibitors TRP and 1MT, and infected by N. caninum (Figura 6) was also noted. This indicates that IDO, in this experimental model, was a metabolic route used by these cells to control parasitism. This idea was supported by observing that in cultures without IFNγ modulation but treated with inhibitors (TRP and 1MT), and respectively infected, showed basal levels of kynurenine production when compared to control.

Once IDO's activity was demonstrated, the parasite controlling capacity of these cells was assessed. The Figure 7 illustrate that there was an increase in the tachyzoite number when the cultures were treated with IDO inhibitors without IFNγ stimulus. The blocking effect of 1MT and TRP indicates that IDO is a potent antiparasitic target able to control the proliferation of *N. caninum* in this model. Futhermore, it was noted that exogenous IFNγ was capable of controlling tachyzoits growth, since IDO inhibition in this condition did not result in an increase in the parasite proliferation.

Taking into consideration that there was a participation of IDO in the control of parasite proliferation, but this activation was IFNγ independent (given the fact that the parasite proliferation growth has occurred only in those cultures with IDO activity blocked in the absence of IFNγ), it was evaluated the immune profile of these cells in an attempt to discriminate which cytokines participates in the metabolic pathway

regulation. Thus, IFNγ, TNF and IL-10 were measured from the supernatants of these cultures. As shown in figure 8, there was IFN-γ release only in those culture which has received the exogenous cytokine, pointing that this inflammatory mediator did not participate in this model.

However, regarding the investigation of TNF presence, it was observed that in both cultures, those which were previously stimulated by IFNγ and those that were unmodulated, the synthesis of TNF was performed, indicating that infection as well as the association tachyzoite/IDO blocker do not interfere with this cytokine expression (Figure 9).

IL-10 synthesis profile, as expected, reflected the balance of the immune response under a proinflammatory stimulus. It could be verified that IL-10 was produced in the cultures that were infected by *N caninum*, and in those infected and blocked for IDO, in both IFNγ unstimulated and stimulated groups (Figure 10).

COX_2 ensures cellular homeostasis during infection of Neuron/Glia co-cultures by *N. caninum*

Cyclooxygenase-2 activity was measured by synthesis of PGE_2 in the supernatants from cultures that were under different stimuli. It was noted that in cultures infected by *N. caninum*, PGE_2 synthesis were increased around 2-fold when comparing to control. This data repeated itself in the infected and IFNγ treated cells (Figure 11). Knowing the importance of this prostanoid by participating in the CNS immunomodulation and contributing to the cell integrity maintenance in this tissue,

the culture cells were counted by immunophenotyping. Figure 12 indicates reduction in the number of astrocytes in approximately 30% when infected and under the effect of a COX_2 blocker, nimesulide. The same treatment was also capable of reducing, of approximately 33% in the number of microglia (Figure 13), but did not refer to losses in neuron number. On the other hand, there was a synergist effect of infection and IFNγ (100 IU/mL), since there was a reduction of approximately 40% in the neurons number (Figure 14).

DISCUSSION

The immune response triggered in the CNS during the acute phase of infection by obligate intracellular parasites, such as *T. gondii*, is carried out by a pattern of proinflammatory cytokines, measured mainly by IFNγ (MORDUE et al., 2001; BLANCHARCD; DUNAY; SCHLÜTER, 2015). This cytokine has been identified in the literature as responsible for activating biochemical pathways such as the enzymatic activity of iNOS (SILVA et al., 2009; DINCEL; ATMACA, 2015) and IDO (DÄUBENER et al., 2001; FUJIGAKI et al., 2002, 2003). Distinct patterns in the immune response were found in CNS cells infected by *N. caninum* tachyzoites, using murine models. The infection of isolated cells from the CNS, following the example of primary astrocyte cultures infected by *N. caninum,* indicated that the immune response is measured by IL-10, without the participation of IFNγ (PINHEIRO et al., 2006 a,b) by TNF and by iNOS activation (PINHEIRO et al., 2006a). In another study, using glial cells culture (astrocyte and microglia) it was observed that the infection by *N. caninum* induced the synthesis of NO and TNF, therefore, these mediators were

presented as responsible for the control of parasitic proliferation, whereas IL-10 synthesis was observed maintaining the host/parasite relationship stable (PINE et al., 2010). Using a similar experimental model, Jesus et al. (2013) observed that the control of parasitic growth was independent of iNOS and that these cultures showed PGE_2 synthesis, contributing to the idea of a possible role that glial cells may have in preserving neuron. Subsequently, it was observed that the infection of co-cultures (glia/neurons) by *N. caninum* was controlled by exogenous IFNγ, indicating the importance of this cytokine in parasite control (JESUS et al., 2014). Thus, a clarification of the IFNγ cytokine's involvement in controlling the parasitism and which biochemical pathway is activated during the infection by *N. caninum* is necessary.

Similarly to what had been observed by our group (JESUS et al., 2014), co-cultures infected by *N. caninum* did not show increased levels of nitrite, even in those infected and modulated by IFNγ (100 UI/mL). This possibly indicates the parasite's ability to inhibit the activity of iNOS for tissue preservation and consequently remain viable in the microenvironment. This is reinforced by a study conducted by Rozenfeld et al. (2005) that showed a reduction of nitrite levels in co-cultures infected with *T. gondii* and previously treated with exogenous IFNγ. In another study, it was observed that the immune response triggered by *T. gondii*, and mediated by PGE_2 and IL-10, was responsible for the reduction of nitric oxide synthesis, supporting the concept that *T. gondii* reduces inflammation for neuronal preservation (ROZENFELD et al., 2003). However, it became clear that the maintenance of baseline levels of nitric oxide is important for the homeostasis of the microenvironment, since the depletion of the nitrite inhibitor of iNOS (L-NAME) resulted in increased parasitic proliferation. Nitric oxide is an important neurotransmitter, being observed in the activation of glial cells (CALABRESE et al., 2007; BROWN; NEHER, 2010) and its complete depletion

implies a limited local immune response, with consequent glial inactivity, explaining the parasitic growth in the presence of L-NAME. Knowing that the enzymatic pathway of iNOS does not represent any gain in antiparasitic defense in this experimental model, a new route that could control parasitism was sought.

Some studies pointed to the down-regulation of iNOS via IDO (THOMAS et al., 1994; ALBERATI-GIANI et al., 1997). Thus, we investigated the involvement of IDO in our model by means of the inhibitory effect of 1 MT and tryptophan supplementation combined or not with the synergistic effect of IFNγ. It was observed that previous stimulus by IFNγ was capable of inducing IDO activity, and its effect were reversed in the presence of infection associated inhibitors. This data corroborate those observed by Spekker et al. (2009), which working with bovine endothelial cells infected with *N. caninum* observed IDO activity in the presence of IFNγ. In addition, we observed that the infection induced IDO activity without prior IFNγ stimulation. It was also noted that the parasitic growth occurred in cultures that were inhibited by 1 MT and supplemented with tryptophan. This event confirms that IDO is a potent antiparasitic (HESELER et al., 2008; MURAKAMI et al., 2012) and that this model contributes to CNS homeostasis. However, the parasite control that occurred in the presence of IFNγ with IDO's pathway inhibited, reinforces the argument that iNOS becomes active in the absence of IDO (LÓPEZ et al., 2006; WANG et al., 2010). Our data suggest an IDO activity independent from IFNγ activation, which is intriguing, since cytokine dosages did not reveal any participation of this inflammatory mediator. Moreover, we observed the participation of TNF and of IL-10 in all cultures that were infected and treated with 1MT. The absence of IFNγ and nitric oxide corresponded to a gain in tissue preservation, as it reduced the deleterious effects of these inflammatory mediators on the microenvironment (GRESA-ARRIBAS et al., 2012;

JESUS et al., 2013). Given the above, it is clear that activation of IDO's classical pathway does not happen in this model, given the fact that the cell culture did not present IFNγ synthesis when subjected to infection. This indicates that perhaps there is an alternative mechanism able to activate IDO. Studies have demonstrated the involvement of the synergistic effect of PGE_2 prostanoid with the TNF cytokine, in triggering the pathway of tryptophan's oxidative metabolism (BRAUN et al., 2005; VON BERGWELT-BAILDON et al., 2006). In this sense, we investigated the involvement of PGE_2 by blocking COX through the use of selective COX-1 inhibitor, indomethacin, and selective COX-2 inhibitor, nimesulide. We have observed that infection induced the release of PGE_2 and that parasitic growth occured by the inhibition of COX-1 and COX-2, thus confirming the idea that this prostanoid also participates in the control of parasite growth. Furthermore, it is believed that down-regulation of iNOS is also associated with PGE_2 presence in cultures. Some studies have demonstrated the inhibitory effect of PGE_2 on prostanoid 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 et al., 2003). In the interest of showing the protective effect of COX in this experimental model, a immunophenotyping was performed for quantitative detection of infected cells modulated by COX inhibitors with or without prior stimulation of IFNγ. It was identified that when selectively inhibited with COX2, there was a reduction in the number of astrocytes and microglia infected with the parasite; however, there was no reduction in the number of neurons in these same conditions. This probably occurs because they are immune competent cells resident to the CNS, and responsible for neuronal preservation (BÉLANGER e MAGISTRETTI, 2009; GIMSA et al., 2013; SHINOZAK et al., 2014). Furthermore, synergism between IL-10 and

PGE_2 can contribute to homeostasis of the microenvironment by reversing pro-inflammatory conditions, given the fact that it has been observed that the interaction between PGE_2 and both EP4 and EP2 receptors promotes anti-inflammatory effects and neuroprotectection (ECHEVERRIA et al., 2005; SHI et al., 2010). The data set suggest a few interpretations such as: (1) the control of parasitic growth was maintained by enzymatic activity from indolamine 2,3 dioxygenase; (2) the endogenous cytokine IFNγ in this experimental model did not participate in the activation of indoleamine 2,3 dioxygenase; (3) PGE_2 can work synergistically with TNF and alternatively activate the pathway of indolamine 2,3 dioxygenase; (4) the regulatory effects of IL-10 and PGE2 were able to modulate inflammatory processes and maintaining homeostasis of the microenvironment; (5) the enzyme cyclooxygenase 2 participated in the control of parasite proliferation by PGE_2 synthesis. Further studies are needed to clarify some questions such as: (1) Do the products generated from the tryptophan metabolism during infection by *N. caninum*, like the kynurenic acid released by astrocytes, participate in neuroprotection mechanisms in this model? (2) Can constituent molecules of the parasite induce neuropreservation? The answers to these questions will help to understand how the parasite/host relationship is maintained in this system and how the immunoregulatory mechanisms are targeted for the benefit of the parasite and/or tissue.

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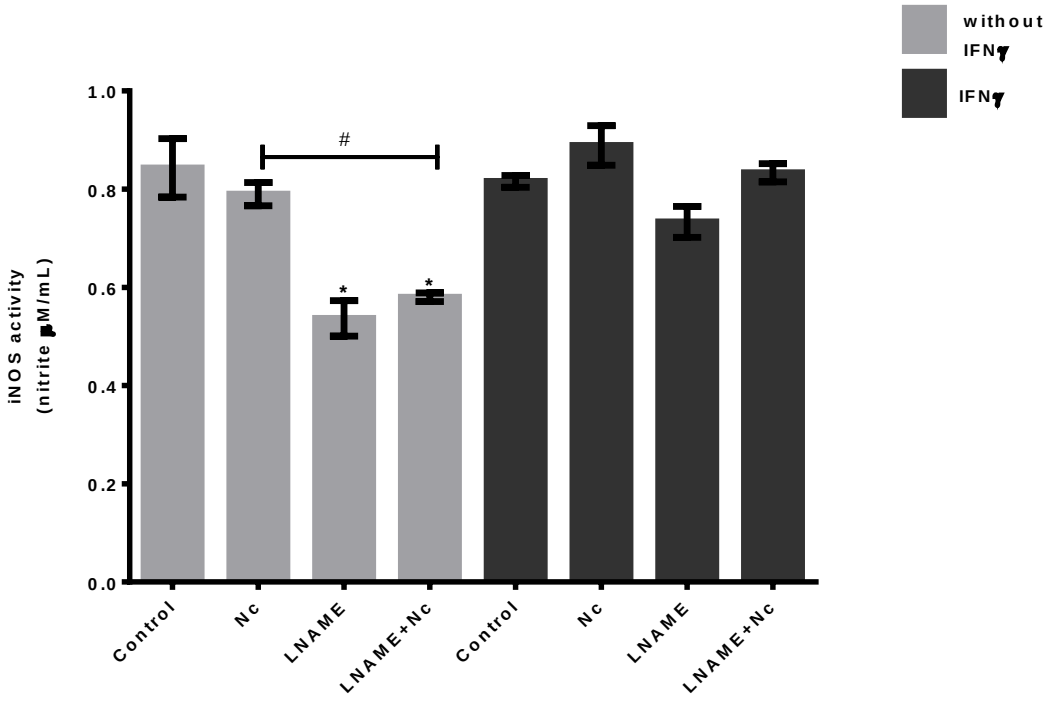

Figure 1. Dosage of nitrite in Neuron/Glia co-cultures obtained from the cerebral cortices of neonatal (24 h) and embryos rats (18 days) with and without IFNγ modulation (100 IU/mL/24h). L-NAME (1.5 mM/mL) was added to the cell's medium for 1 h and then the cells were infected by *N. caninum* in a proportion of 1:1 parasites per cell per 72h. (*) $P < 0.05$ (comparison between control group and treated groups without IFNγ). (#) $P < 0.05$ (comparison between infected group and group treated with L–NAME).

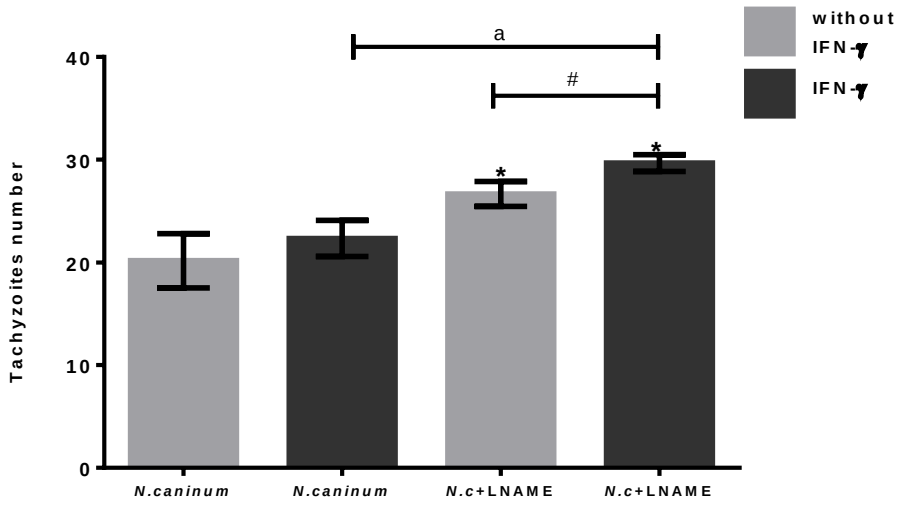

Figure 2. Count of *N. caninum* tachyzoites in glia/neuron co-cultures obtained from the cerebral cortex of neonatal (24 h) and embryos rats (18 days) with and without IFNγ modulation (100 IU/mL/24h). L-NAME (1.5 mM/mL) was added to the cell's medium for 1 h and then the cells were infected by *N. caninum* in a proportion of 1:1 parasites per cell per 72h. (*) P < 0.05 (comparison between control group and the other groups). (#) P <0.05 (difference between groups with and without modulation by IFNγ). (a) P <0.05 (difference between groups modulated by IFNγ).

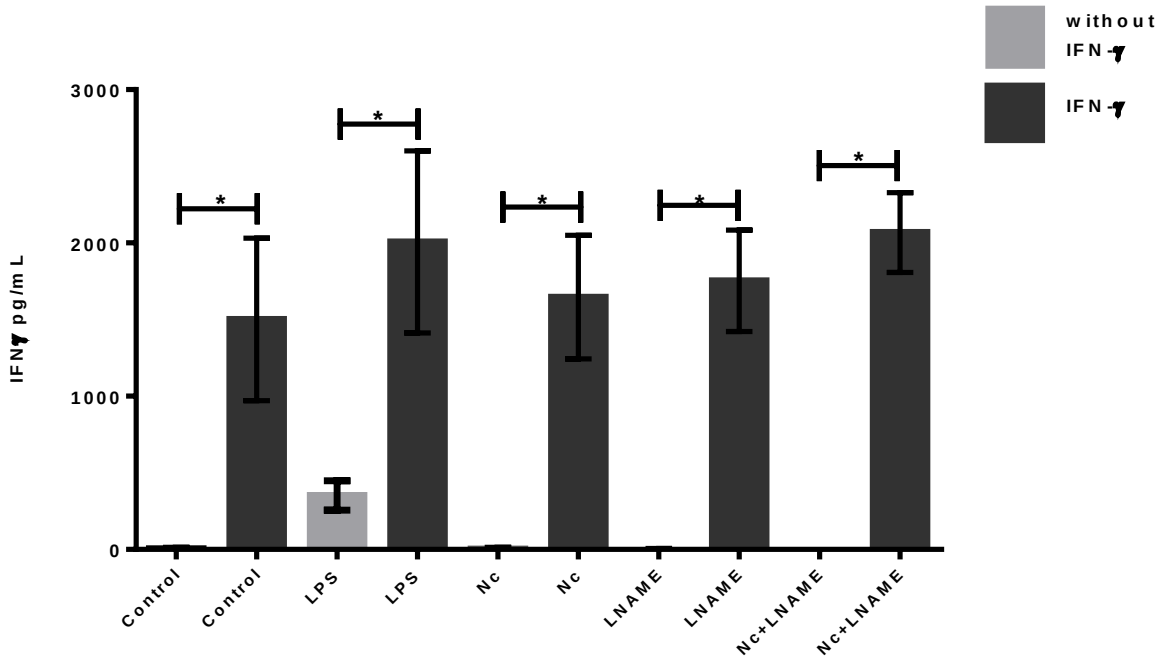

Figure 3. Dosage of IFNγ in the supernatants of glia/neuron co-cultures obtained from the cerebral cortex of neonatal (24 h) and embryos rats (18 days) with and without IFNγ modulation 100 IU/mL/ 24h). L-NAME (1.5 mM/mL) was added to the cell's medium for 1 h and then the cells were infected by *N. caninum* in a proportion of 1:1 parasites per cell per 72h. (*) P <0.05 (difference between groups with and without modulation by IFNγ).

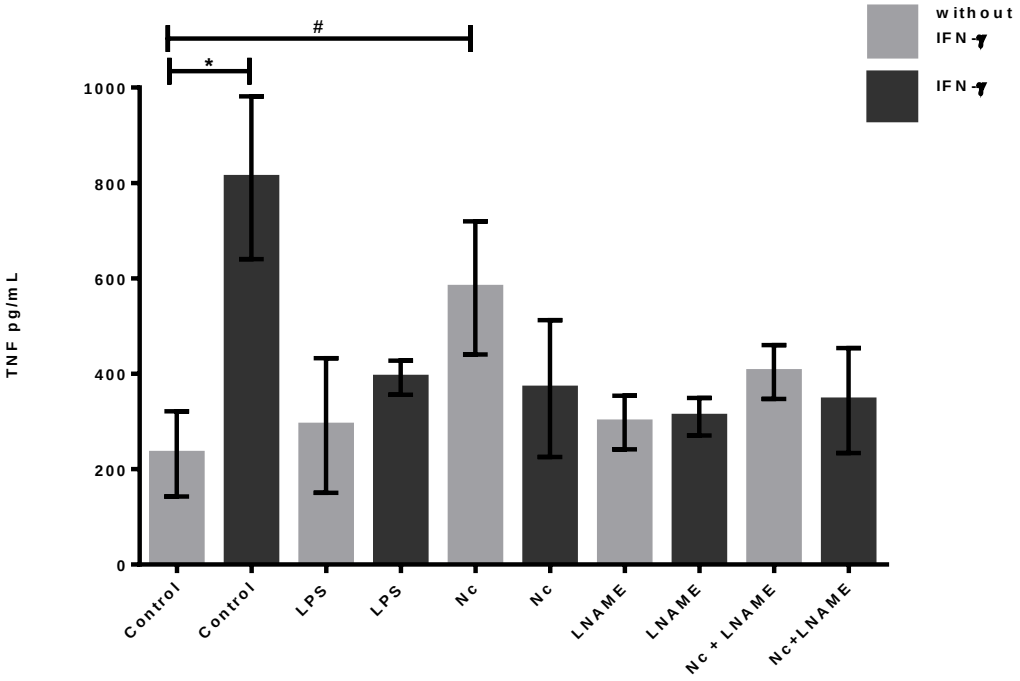

Figure 4. Dosage of TNF in the supernatants of glia/neuron co-cultures obtained from the cerebral cortex of neonatal (24h) and embryos rats (18 days) with and without IFNγ modulation (100 IU/mL/24h). L-NAME (1.5 mM/mL) was added to the cell's medium for 1h and then the cells were infected by *N. caninum* in a proportion of 1:1 parasites per cell per 72h. (*) P <0.05 (difference between groups with and without modulation by IFNγ). (#) P <0.05 (difference between groups non-modulated by IFNγ).

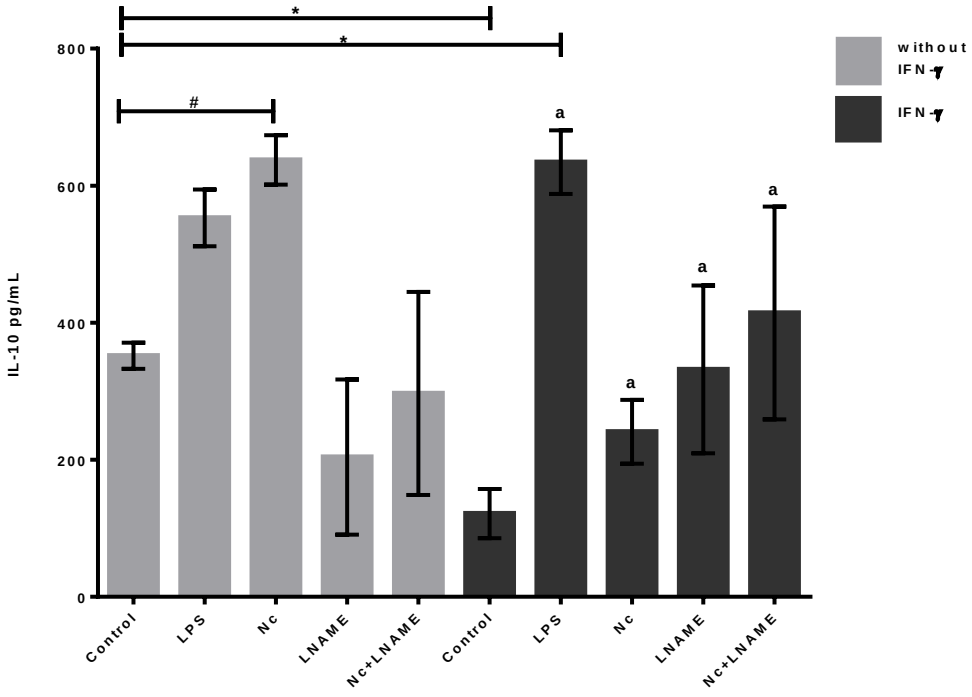

Figure 5. Dosage of IL-10 in the supernatants of glia/neuron co-cultures obtained from the cerebral cortex of neonatal (24h) and embryos rats (18 days) with and without IFNγ modulation (100 IU/mL/24h). L-NAME (1.5 mM/mL) was added to the cell's medium for 1h and then the cells were infected by *N. caninum* in a proportion of 1:1 parasites per cell per 72h. (*) P <0.05 (difference between groups with and without modulation by IFNγ). (#) P <0.05 (difference between groups non-modulated by IFNγ). (a) P <0.05 (difference between groups modulated by IFNγ)

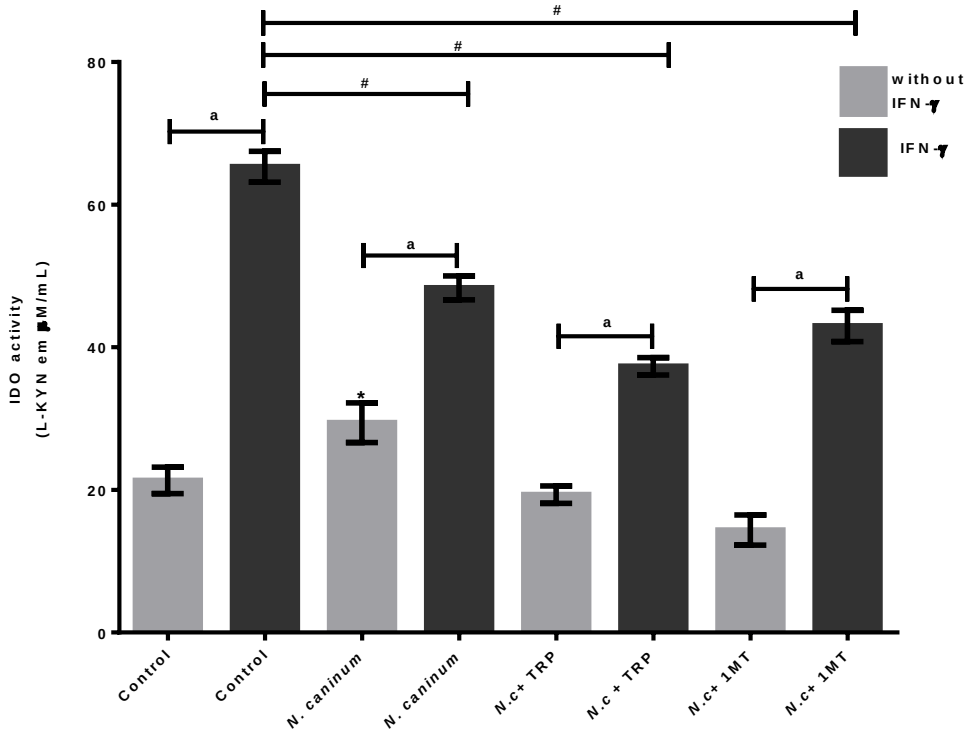

Figure 6. Dosage of kynurenine in the supernatants of glia/neuron co-cultures obtained from the cerebral cortex of neonatal (24 h) and embryos rats (18 days) with and without IFNγ modulation (100 IU/mL/24h). TRP (1 mM/mL) and 1-MT (1.5 mM/mL) were added to the cell's medium for 1 h and then the cells were infected by *N. caninum* in a proportion of 1:1 parasites per cell per 72 h. (*) P <0.05 (comparison between groups non-modulated by IFNγ). (#) P <0.05 (comparison between groups modulated by IFNγ). (a) P <0.05 (comparison between groups with and without modulation by IFNγ).

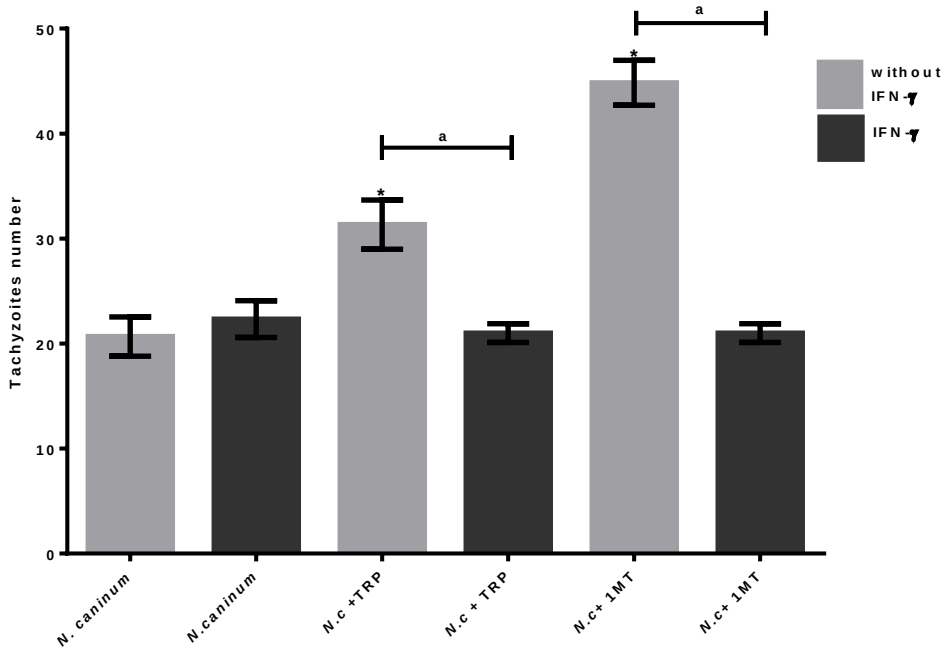

Figure 7. Count of *N. caninum* tachyzoites in glia/neuron co-cultures obtained from the cerebral cortex of neonatal (24 h) and embryos rats (18 days) with and without IFNγ modulation (100 IU/mL/24h). TRP (1 mM/mL) and 1-MT (1.5 mM/mL) were added to the cell's medium for 1h and then the cells were infected by *N. caninum* in a proportion of 1:1 parasites per cell per 72h. (*) $P < 0.05$ (comparison between control group and the other groups). (a) $P < 0.05$ (difference between groups with and without modulation by IFNγ).

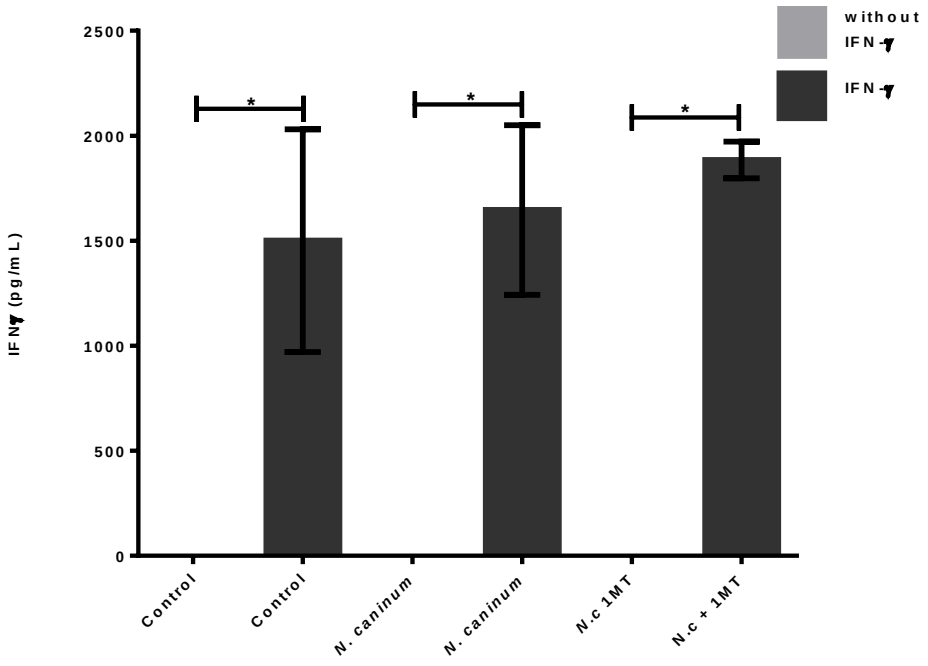

Figure 8. Dosage of IFNγ in glia/neuron co-cultures obtained from the cerebral cortex of neonatal (24 h) and embryos rats (18 days) with and without IFNγ modulation (100 IU/mL/24h). 1-MT (1.5 mM/mL) was added to the cell's medium for 1 h and then the cells were infected by *N. caninum* in a proportion of 1:1 parasites per cell per 72h. (*) P <0.05 (difference between groups with and without modulation by IFNγ).

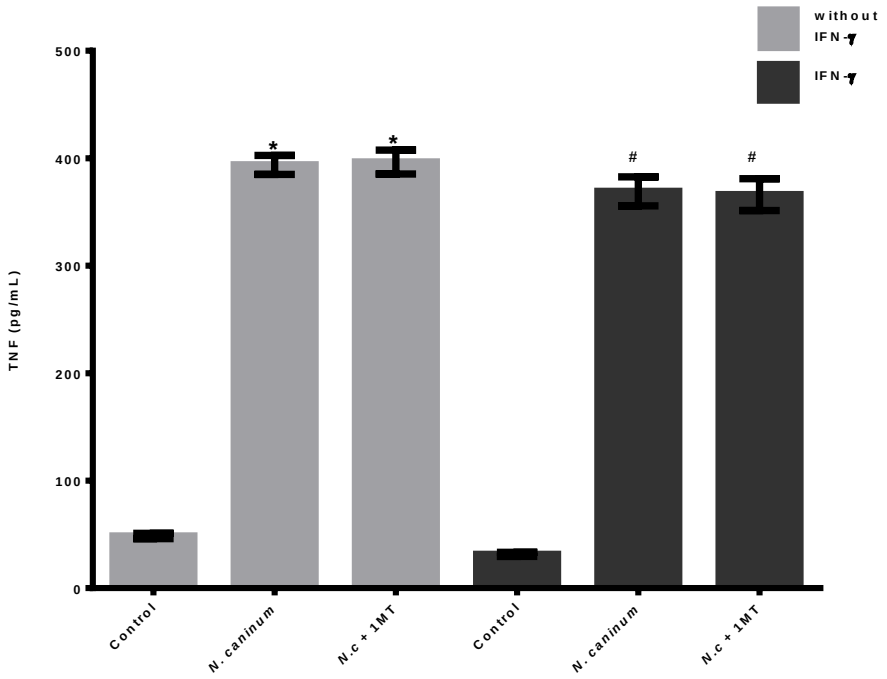

Figure 9. Dosage of TNF in glia/neuron co-cultures obtained from the cerebral cortex of neonatal (24 h) and embryos rats (18 days) with and without IFNγ modulation (100 IU/mL/24h). 1-MT (1.5 mM/mL) was added to the cell's medium for 1 h and then the cells were infected by *N. caninum* in a proportion of 1:1 parasites per cell per 72h. (*) P <0.05 (difference between groups non-modulated by IFNγ). (#) P <0.05 (difference between groups modulated by IFNγ).

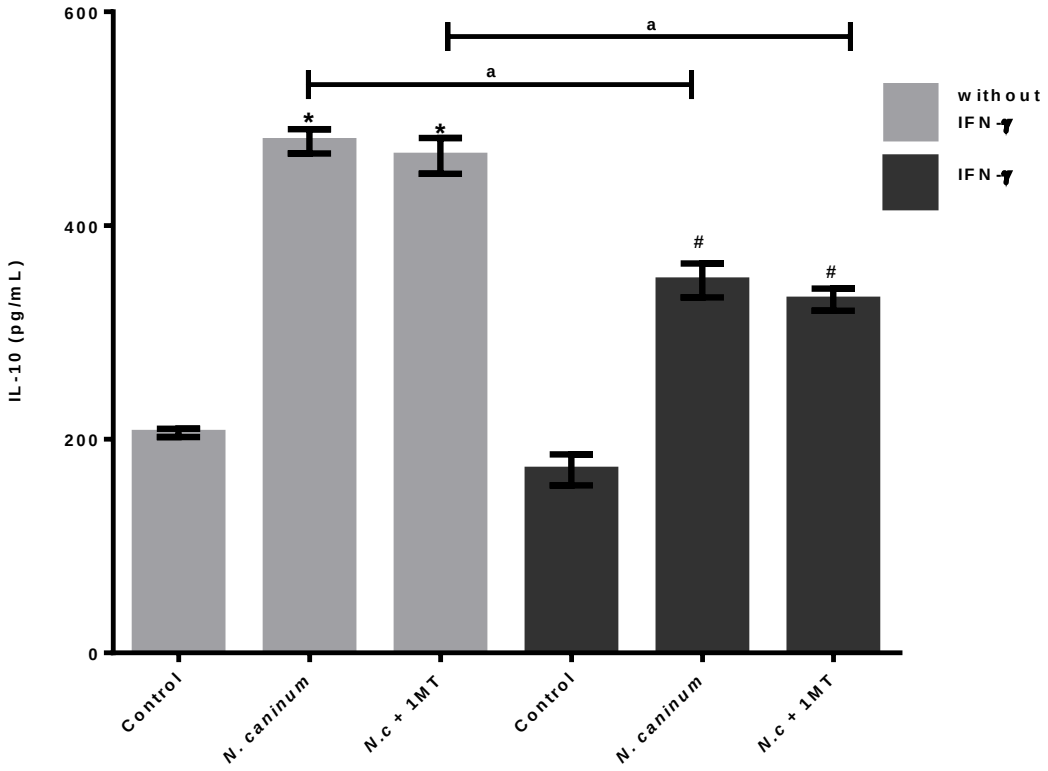

Figure 10. Dosage of IL-10 in glia/neuron co-cultures obtained from the cerebral cortex of neonatal (24h) and embryos rats (18 days) with and without IFNγ modulation (100 IU/mL/24h). 1-MT (1.5 mM/mL) was added to the cell's medium for 1 h and then the cells were infected by *N. caninum* in a proportion of 1:1 parasites per cell per 72 h. (*) $p<0.05$ (difference between groups non-modulated by IFNγ). (#) $p<0.05$ (difference between groups modulated by IFNγ). (a) $p<0.05$ (difference between groups with and without modulation by IFNγ).

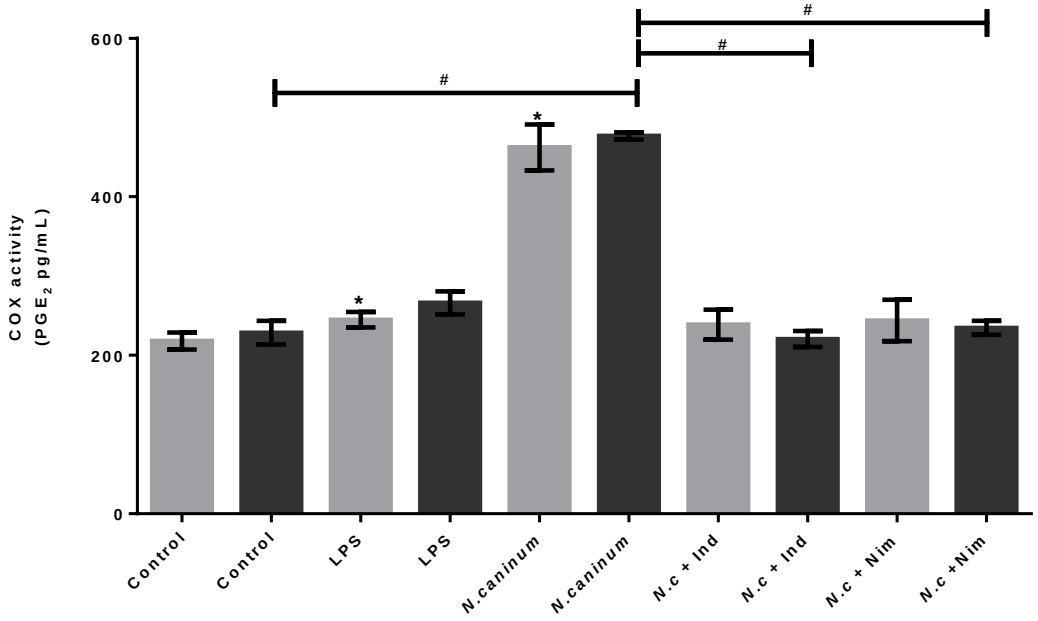

Figure 11. Dosage of PGE_2 in glia/neuron co-cultures obtained from the cerebral cortex of neonatal (24 h) and embryos rats (18 days) with and without IFNγ modulation (100 IU/mL/24h). Indometacin and nimesulide (10^{-6} M/mL) were added to the cell's medium for 1 h and then the cells were infected by *N. caninum* in a proportion of 1:1 parasites per cell per 72 h. (*) $p < 0.05$ (comparison between control group and the other groups). (*) $p < 0.05$ (difference between groups non-modulated by IFNγ). (#) $p < 0.05$ (difference between groups with and without modulation by IFNγ).

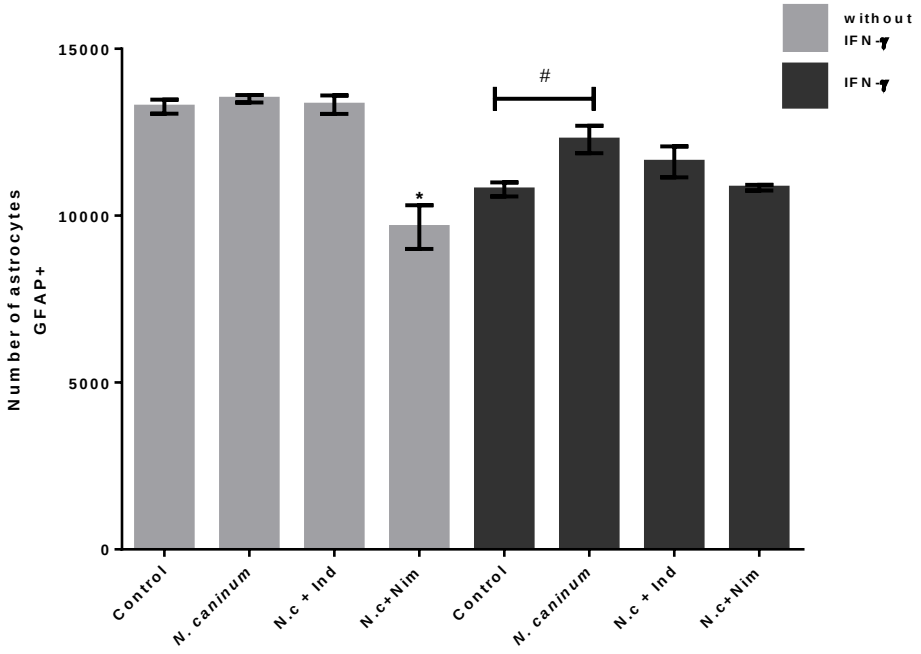

Figure 12. Flow cytometry - GFAP positive cells in glia/neuron co-cultures obtained from the cerebral cortex of neonatal (24h) and embryos rats (18 days) with and without IFNγ modulation (100 IU/mL/24h). After cells were infected with *N. caninum* tachyzoites (ratio cell:parasite 1:1) for 72 h. (*) $p<0.05$ represents a significant statistical difference when compared to control cultures. (#) $p<0.05$ represents a significant statistical difference when compared to infected cultures.

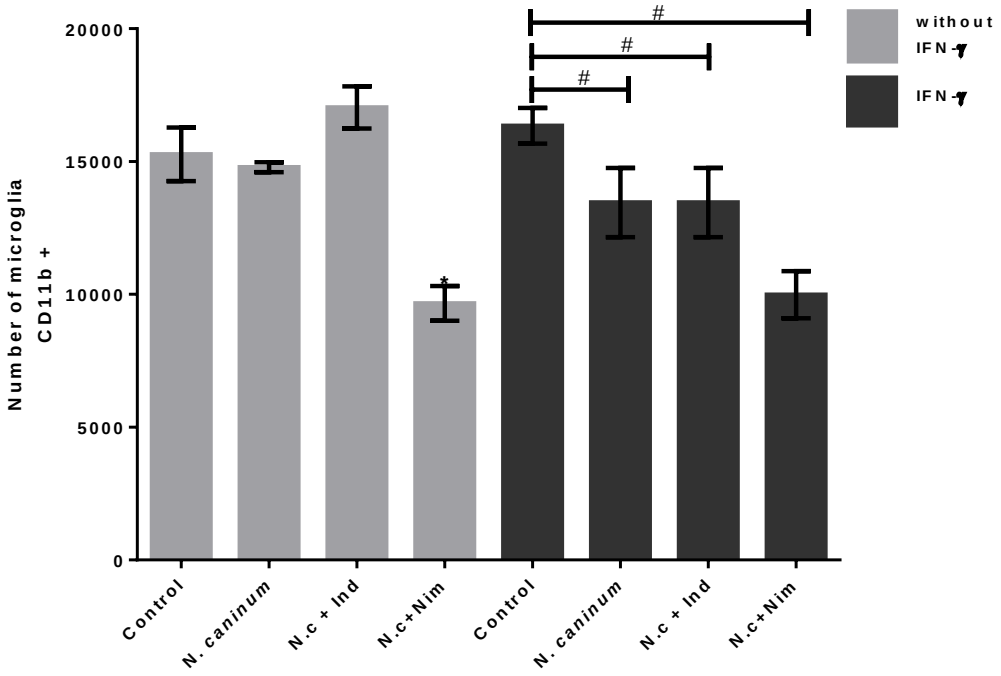

Figure 13. Flow cytometry – CD11b positive cells in glia/neuron co-cultures obtained from the cerebral cortex of neonatal (24h) and embryos rats (18 days) with and without IFNγ modulation (100 IU/mL/24h). After cells were infected with *N. caninum tachyzoites* (ratio cell:parasite 1:1) for 72 h. (*) $p<0.05$ represents a significant statistical difference when compared to control cultures. (#) $p<0.05$ represents a significant statistical difference when compared to infected cultures.

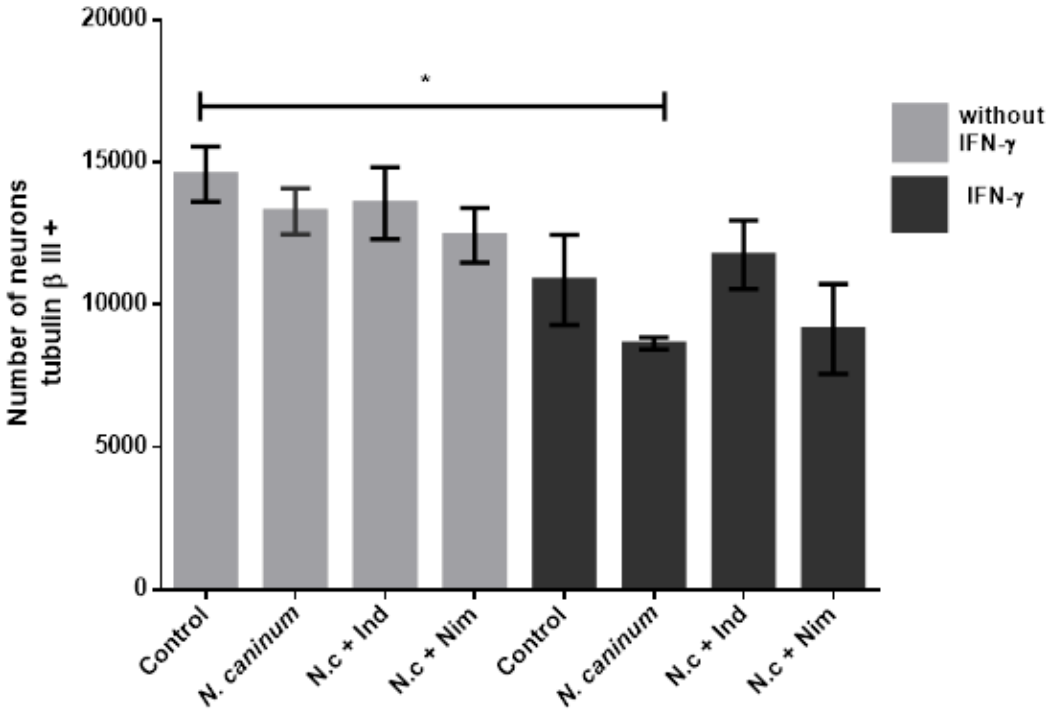

Figure 14. Flow cytometry – βIII tubulin positive cells in glia/neuron co-cultures obtained from the cerebral cortex of neonatal (24 h) and embryos rats (18 days) with and without IFNγ modulation (100 IU/mL/24h). After cells were infected with *N. caninum* tachyzoites (ratio cell:parasite 1:1) for 72 h. (*) $p<0.05$ represents a significant statistical difference when compared to control cultures.

Manuscrito 2:

IS BETA-GLUCURONIDASE A GOOD MARKER FOR NEUROGLIA VIABILITY?

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ABSTRACT

The enzyme beta-glucoronidase (EC 3.2.1.31) is a lysosomal enzyme catalyzing the decomposition of beta-D-glucoronides compounds arising as a result of the combination of beta-D-glucoronic acid and a number of compounds both exo- and endogenous, containing hydroxylic, carboxylic, amine, imine or thiol groups. The beta-glucoronidase is a sensitive indicator signalling cell damage. Thus, objective of this study was to evaluate the enzyme beta-glucuronidase as a possible biomarker for cell damage induced by infection with the parasite *N. caninum* in co-cultures of neuron-glial cells from rats. Neuron/Glia co-cultures were treated with 100 IU/mL of recombinant rat IFN or 1.0 mg/mL of lipopolysaccharide diluted in culture medium.

The control conditions were obtained by fresh medium addiction. Twenty-four hours after treatment, neuron/glia co-culture were infected with tachyzoites of *N. caninum* (host:parasite ratio of 1:1). Under the experimental conditions cultures, untreated/infected and treated/infected showed no increase in enzyme activity. This result must be also considered in abstract to sustain the conclusions In this experimental model, using tachyzoites of *N. caninum* as injuring agent, the dosage of the enzyme beta-glucoronidase in this scenario appears as a new tool for analysis of cell viability. **Keywords**: Beta-glucorinidase, *Neospora caninum*, neuron-glial cells

INTRODUCTION

The enzyme beta-glicuronidase (GUSB) (E.C 3.2.1.31) is expressed in a variety of tissues (SPERKER et al., 1997; ZHU et al., 2000; NAZ et al., 2013) and catalises the hydrolisis of residues of the beta-glucuronic acid in the non-reducing end of glycosaminoglycans (chondroitin sulfate, heparan sulfate, dermatan and keratan sulfate and hyaluronic acid) (GEHRMANN et al., 1994). The increase of the lisossomal activity of this enzyme point towards the presence of several diseases, such as, tissue inflammation (MORO; BERNARD; GONANO, 1975; GOLDLUST; RICH, 1981) acquired immunodeficiency syndrome (AIDS) (SAHA et al., 1991), tuberculosis (JASWAL et al., 1993; SELVARAJ et al., 1997), cirrhosis (GEORGE, 2008; YAMAGUCHI et al., 2013), neoplasms (ANTUNES et al., 2012; XIE et al., 2014). Besides, it can be used as an indicator for cellular damage (Horie et al., 1971; FINCH et al., 1987). Some studies point to an increased release of beta glucuronidase from activated microglia during the process of neuro inflammation

caused by viruses (MACKENZIE; WILSON; DENNIS, 1968; BOWEN et al., 1974; ANTUNES et al., 2012), or in demyelinating diseases such as multiple sclerosis (MCMARTIN; HORROCKS; KOESTNER, 1972; CUZNER et al., 1976), and Alzheimer's disease models (SUZUKI et al., 1988; MCGEER et al., 1989). Glial cells when infected with *Neospora caninum*, obligate intracellular coccidian parasite, respond to this stimulus by changing their morphology and releasing potentially harmful inflammatory mediators for this microenvironment (PINHEIRO et al., 2010). *N. caninum* infection in primary cultures of astrocytes induces loss of cell viability measured by lactate dehydrogenase (EC 1.1.1.27) activity, according to Pinheiro et al. (2006). Jesus et al. (2013) reported that, in neuron-glia co-cultures infected by *N. caninum*, there was no decrease in cell viability according to trypan blue and MTT assays. The objective of this study was to evaluate the enzyme beta-glucuronidase as a possible biomarker for cell damage induced by infection with the parasite *N. caninum* in co-cultures of neuron-glial cells from rats.

MATERIAL AND METHODS

Culture of *Neospora caninum*

N. caninum tachyzoites of the NC-Ba 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). To obtain the parasites, the VERO cells were first, washed with phosphate buffered saline (PBS) and then mechanically disrupted. Soon

after, the tachyzoites were purified using a 5.0 μm filter (Millipore, Carrigtwohill, Ireland) as described by Pinheiro *et al* (2010).

Neuron/Glia co-cultures

Mixed glial cells (astrocytes and microglia) were first obtained from brain cortexes of newborn rats (<48 hours of age) by mechanical dissociation of the tissue. 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 CULTILAB (Brazil).

These cultures were initially seeded onto 100 mm culture dishes (TPP, Switzerland) and after 14 days, they were re-seeded (5 X 10^4) in 24-well tissue culture plates for assays. In this time, timed pregnancy rats were sacrificed on the 17^{th} or 18^{th} gestational day, and embryos were removed by caesarian section. Cortex dissection cells were dissociated in DMEM/F-12 as described above. Neurons (2.5 X 10^4/well) were then plated on astrocyte/microglia monolayer and the cultures were maintained with regular DMEM/F-12 changed every 48 hours to 7 days, when the experiments were performed.

Neuron/Glia co-culture infection and treatment

Neuron/Glia co-cultures were treated with 100 IU/mL of recombinant rat IFN-γ (R&D Systems, USA); 1.0 mg/mL of lipopolysaccharide (LPS) (Sigma-Aldrich, USA); 4

mM/mL of digitonin (Sigma-Aldrich, USA) diluted in culture medium. The control conditions were obtained by fresh medium addiction. Twenty-four hours after treatment, neuron/glia co-culture were infected with tachyzoites of *N. caninum* (host:parasite ratio of 1:1). Analysis were performed 72 hours post-infection as determined in previous studies.

Cell viability assay

MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] conversion method was performed to evaluate the cellular oxidative metabolism. The assay is based in the ability of active mitochondrial dehydrogenases to convert MTT in purple formazan crystals. Briefly, cells under different culture conditions were incubated with MTT at a final concentration of 1.0 mg/mL for 2 hours. Thereafter, cells were lysed with 20% (w/v) sodium dodecyl sulfate (SDS), 50% (v/v) dimethyl formamide (DMF) (pH 4.7), plates were kept overnight at 37°C in order to dissolve formazan crystals and optical density was quantified at 580nm (Hansen *et al.*, 1989). Three independent experiments were carried out with eight replicate wells for each analysis. Results are shown as viability percentage compared to untreated/uninfected control cultures, considered as 100%.

Enzymatic assay of Beta-glucuronidase (E.C 3.2.1.31)

Beta-glucuronidase (GUSB, EC 3.2.1.31) catalyzes the hydrolysis of *b*-linked D-glucopyranosiduronic acids (*b*-glucuronides) to glucopyranosiduronic acid (glucuronic acid) and aglycone: Phenophtalein-Glucuronide + H2O $\rightarrow$ D-Glucopyranosiduronic Acid + Phenophtalein. Each determination is run in duplicate with a single control. The volume of 70 μL of acetate buffer 100 mM at pH 3,8 are pipetted into test-tubes, and 70 μL of sodium phenolphthalein glucuronide 1.2 mM is added to the two experimental tubes but not the control. The tubes are placed in a water bath at 37ºC and allowed to come to temperature, 10 μL of enzyme solution is added to each tube at timed intervals, and the contents mixed by whirling. The tubes are stoppered and allowed to incubate for an exact period of time, usually 30 minutes. At the end of this time, 500 μL of glycine buffer 200 uM are added to each tube, including the control, and then 100 μL of the substrate is added to the control tube. The phenolphthalein calibration curve is prepared in the same buffer mixture which the experimental tubes finally contain. Colorimeter tubes are prepared to contain 70 ul of acetate buffer 100 mM at pH 3.8, 500 ul of glycine buffer 200 mM and 70 μL of phenolphthalein solution of varying dilutions. The phenolphthalein dilutions are prepared by diluting the ethanol 95% (v/v), just prior to use. Readings are made against a water blank with a 540 nm filter.

RESULTADOS

MTT assay

The test of reduction of tetrazolium salts by mitochondrial metabolism analysis was required to confirm that the various treatments described above induced changes in cell physiology. Results from MTT assay were expressed as a percentage considering the control as 100%. It was found that there was no reduction in the formation of formazan crystals in cultures infected with tachyzoites of *N. caninum* ($p<0,05$) when compared with the control culture. This fact is also observed in cultures that were previously treated with exogenous cytokine IFNγ 100UI/mL as shown in figure 01.

Beta glucuronidase enzyme activity

This test assessed whether *N. caninum* infection induced cell death by the dosage of free phenolphthalein generated from the hydrolysis of phenolphthalein glucuronide substrate under the action of the enzyme GUSB. Under the experimental conditions cultures, untreated/infected and treated/infected showed no increase in enzyme activity. However, in LPS-untreated/treated cultures with IFNγ, and in digitonin cultures untreated/treated with IFNγ showed a reduction of the viability of cells as seen in figure 02.

DISCUSSION

The beta glucuronidase is an enzyme that has been widely observed in different pathological processes and has been implicated as a biomarker of cell death (FINCH et al., 1987). Injuries in the cell endomembrane system caused by different agents (chemical, physical or environmental pathogens) induce the activation of phospholipase A2, phospholipase C and diacylglycerol lipase that are responsible for the release of arachidonic acid from the cell membrane and subsequent formation of inflammatory prostanoids, via cascade of cyclooxygenase 2 (XU et al., 2013). Some studies have pointed out that the increased release of the GUSB enzyme is directly related to high levels of arachidonic acid and other unsaturated fatty acids derived from damaged cell membrane (CHEAH, 1981; BEAUMIER; FAUCHER; NACCACHE, 1987; PACKHAM et al., 1995). Thus, increased levels of GUSB in cultures treated with digitonin cell permeabilizing and inflammatory inductor LPS (not treated / treated with IFNγ), corresponding to loss of cell viability, since this enzyme is not found compartmentalized in the lysosomes and appears active in the extracellular space. This is corroborated by the MTT assay that showed that impairment in mitochondrial oxidative metabolism when cultures were under action of digitonin and LPS. On the other hand, the cultures that were infected untreated/treated with IFNγ did not show loss of viability for any of the three tests applied. Carvalho et al. (2010) working with human uterine cervical cells (HeLa) and trophoblastic (BeWo) observed that infection with *N. caninum* tachyzoites induced loss of cell viability, measured by the colorimetric MTT and LDH. In a study performed with human brain microvascular endothelial cells (HBMEC) infected with tachyzoites of *N. caninum*, the MTT assay

showed no significant difference in the rate of cell proliferation when compared with control cultures in the first 24 hours of infection, suggesting that *N . caninum* is able to invade and replicate within HBMEC without causing substantial cell damage (ELSHEIKHA et al., 2013). Recently, Jesus *et al*., 2013 reported the synergistic effect of the infection of *N. caninum* and IFNγ in co-cultures glia/neuron. Probably neurotrophic factors such as Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and Glial cell-derived neurotrophic factor (GDNF) acts as immunomodulatory molecules from the central nervous system and can therefore be responsible for the preservation of these cells when exposed to an injuring agent (BARBACID, 1995; WANG et al., 1997; SUZUMURA et al., 2006). Moreover, it is known that some intracellular pathogens can modulate the microenvironment in their favor and consequently escape the immune response. Thus, it is ensured the viability of the host cell and the permanence of the protozoa at the site of infection (LALIBERTÉ; CARRUTHERS, 2008). In view of the different responses observed in various experimental models for the study of inflammatory processes, using tachyzoites of *N. caninum* as injuring agent, the dosage of the enzyme GUSB in this scenario appears as a new tool for analysis of cell viability.

CONCLUSION

The beta-glucuronidase enzyme has its highest activity during pathological processes. Thus, this enzyme appears as a new biomarker for cellular lesions induced by infection with the parasite *N. caninum* in co-cultures of neuron-glial cells from neonatal rat cortex.

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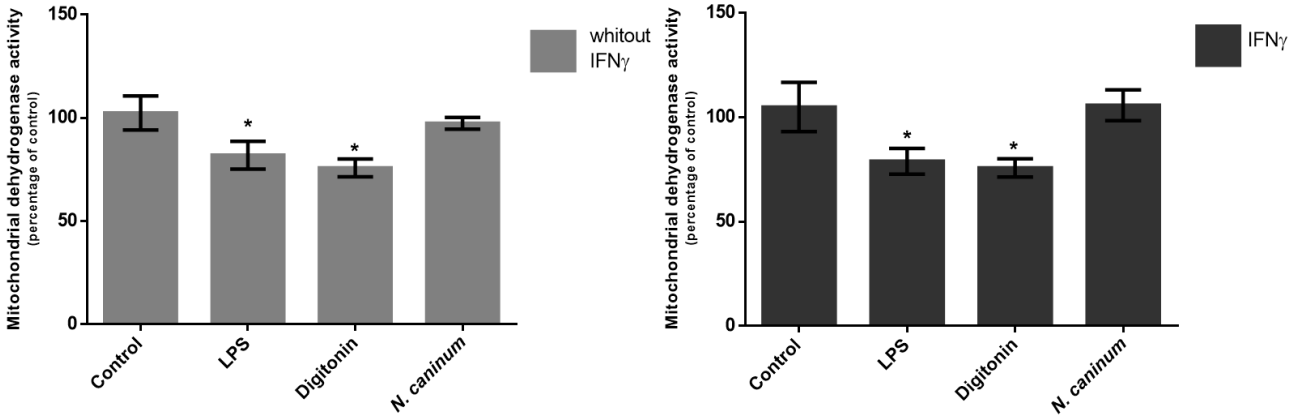

Figure 1. MTT test in neurons/glial co-cultures taken from the cerebral cortex of newborns rats (24h) and fetal (18 days) modulated by IFNγ (100 IU/mL) and without modulation by IFNγ in a period of 24h . Cells were stimulated with LPS (1 mg/mL) for 1 hour, digitonin (4mM/mL) and infected with *N. caninum* in a rate 1:1 cell parasite for 72h. p< 0,05.

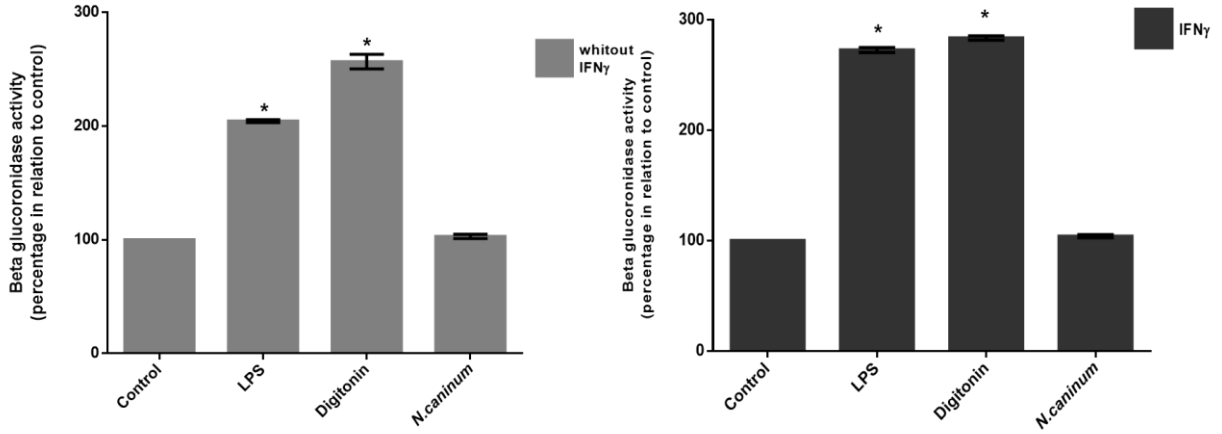

Figure 2. Beta-glucuronidase activity in neurons/glial co-cultures taken from the cerebral cortex of newborns rats (24h) and fetal (18 days) modulated by IFNγ (100 IU/mL) and without modulation by IFNγ in a period of 24h. Cells were stimulated with LPS (1 mg/mL), digitonin (4mM/mL) and infected with *N. caninum* in a rate 1:1 cell parasite for 72h. p< 0,05.

CONSIDERAÇÕES FINAIS

A resposta imune deflagrada por células glia/neurônio durante a infecção por *Neospora caninum* é independente de IFNγ.

A resposta resultante da interação entre o conjunto de células glia/neurônio e *Neospora caninum* durante a infecção é capaz de desencadear mecanismos de controle de proliferação parasitária, mediada pela indução da enzima indoalmina 2,3 dioxigenase associada ao possível efeito sinérgico da citcocina TNF e do prostanóide PGE_2.

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