UNIVERSIDADE PARANAENSE

CATCHIA HERMES-ULIANA

INFECÇÃO CRÔNICA CAUSADA PELO *Toxoplasma gondii* PROMOVE NEUROPLASTICIDADE MIENTÉRICA NO JEJUNO DE RATOS

UMUARAMA

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INFECÇÃO CRÔNICA CAUSADA PELO *Toxoplasma gondii* PROMOVE NEUROPLASTICIDADE MIENTÉRICA NO JEJUNO DE RATOS

Trabalho de conclusão de curso apresentado a banca examinadora do curso de mestrado em Ciência Animal da Universidade Paranaense – UNIPAR, como exigência parcial para obtenção do grau de Mestre em Ciência Animal, sob orientação da profa. Dra. Débora de Mello Gonçales Sant´Ana.

UMUARAMA

2010

TERMO DE APROVAÇÃO

CATCHIA HERMES-ULIANA

INFECÇÃO CRÔNICA CAUSADA PELO *Toxoplasma gondii* PROMOVE NEUROPLASTICIDADE MIENTÉRICA NO JEJUNO DE RATOS

Trabalho de conclusão aprovado como requisito parcial para obtenção do grau de Mestre em Ciência Animal, pela seguinte banca examinadora:

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Umuarama, 22 de abril de 2010

DEDICATÓRIA

Dedico este trabalho a meus pais por acreditarem em meus objetivos, depositando confiança e incentivo. Acima de tudo, por ser motivo maior do meu viver e pelo exemplo de vida a ser seguido.

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“Nunca abra mão de seus sonhos,
pois se eles morrem, a vida se torna
igual a um pássaro de asa quebrada:
não consegue voar”

(Langston Hughes)

UNIVERSIDADE PARANAENSE - UNIPAR

Mestrado em Ciência Animal

HERMES-ULIANA, C. **INFECÇÃO CRÔNICA CAUSADA PELO *Toxoplasma gondii* PROMOVE NEUROPLASTICIDADE MIENTÉRICA NO JEJUNO DE RATOS.** DISSERTAÇÃO (MESTRADO) MESTRADO EM CIÊNCIA ANIMAL. UNIVERSIDADE PARANAENSE, 2010, 69 f.

RESUMO

Objetivou-se investigar as alterações causadas pela infecção por *Toxoplasma gondii* em neurônios mientéricos do jejuno de ratos. Para tanto, utilizaram-se quinze ratos (*Rattus novergicus*) machos, adultos, da linhagem Wistar, com 90 dias de idade, provenientes do biotério da Universidade Paranaense - UNIPAR. Foram separados aleatoriamente em três grupos experimentais: controle (GC, n=5), infectado por 30 dias (G30, n=5) e infetado por 90 dias (G90, n=5). Os animais do GC receberam solução salina por via oral. Os ratos do G30 aos 150 dias de vida e os animais do G90 aos 90 dias de idade foram inoculados com 500 oocistos esporulados, de uma cepa genótipo III (M7741) de *T. gondii,* pela via oral. Aos 180 dias de idade, todos os animais foram anestesiados, submetidos à eutanásia e, por meio de laparotomia, o jejuno foi retirado, mensurado em seu comprimento e largura para cálculo da área. Colheu-se sangue dos animais anestesiados pela punção do plexo venoso retro-orbital, previamente a eutanásia, visando detecção de anticorpos séricos (IgG) anti-*T. gondii* pelo método de aglutinação direta. Depois de retirados, os segmentos intestinais foram lavados, e submetidos à técnica de Giemsa para avaliação da população neuronal total, e à técnica de NADPH-d para a subpopulação nitrérgica. Os segmentos foram dissecados, com a retirada da túnica mucosa e tela submucosa, restando preparados totais formados pela túnica muscular e serosa. Foi contado o número total de neurônios mientéricos de cada animal em 120 campos microscópicos com objetiva de 40x, distribuídos uniformemente por toda a circunferência intestinal. O

número estimado de neurônios colinérgicos (NADPH-d negativos) foi determinado, reduzindo-se a média dos neurônios evidenciados pela marcação da NADPH-d, da população total de neurônios. Foi mensurada a área do corpo celular, do núcleo e calculado a área do citoplasma de 300 neurônios do plexo mientérico de cada animal para cada técnica. Todos os animais inoculados apresentaram anticorpos anti- *T. gondii*, enquanto os animais do GC tiveram resultado negativo. Os animais inoculados apresentaram maior ganho de peso corporal quando comparados ao GC. Todavia, não foram observadas alterações no comprimento, largura e área do jejuno. A análise quantitativa dos neurônios mientéricos evidenciados pela técnica de Giemsa e pela NADPH-d$^+$ (neurônios nitrérgicos), bem como, o número estimado de neurônios NADPH-d$^-$ (neurônios colinérgicos) não apresentaram diferença significativa. Os neurônios mientéricos da população total apresentaram atrofia nos animais do G30 e uma hipertrofia aos 90 dias (G90). A análise morfométrica dos neurônios NADPH-d$^+$ demonstrou atrofia tanto no G30 como no G90 quando comparados ao GC, contudo os neurônios do G90 demonstraram hipertrofia em relação ao G30. Verificou-se que após 30 dias de inoculação houve tendência de aumento da incidência de neurônios menores e redução de e neurônios maiores, tendência que se inverteu após 90 dias de inoculação. Conclui-se que a infecção por uma cepa genótipo III de *T. gondii* utilizado neste estudo não causou morte neuronal, no entanto observou-se atrofia em neurônios da população e da subpopulação nitrérgica durante 30 dias de infecção, e que esses neurônios tenderam a sofrer hipertrofia após 90 dias no jejuno de ratos.

Palavras-chave: intestino delgado, morfometria, plasticidade neuronal, sistema nervoso entérico, toxoplasmose.

UNIVERSIDADE PARANAENSE - UNIPAR

Mestrado em Ciência Animal

HERMES-ULIANA, C. **CHRONIC INFECTION WITH *Toxoplasma gondii* CAUSES MYENTERIC NEUROPLASTICITY OF THE JEJUNUM OF RATS.** DISSERTAÇÃO (MESTRADO) MESTRADO EM CIÊNCIA ANIMAL. UNIVERSIDADE PARANAENSE, 2010, 69 f.

ABSTRACT

The aim of this study was to investigate the changes caused by the infection with *Toxoplasma gondii* in the jejunum of rats. The experiment was conducted using 15, 90-day-old, Wistar rats (*Rattus novergicus*) from the UNIPAR – *Universidade Paranaense* vivarium. They were randomly divided into three groups: control (CG, n=5), infected for 30 days (G30, n=5), and infected for 90 days (G90, n=5). The CG animals received oral saline solution. Rats from the G30, at 150 days of age, and G90, at 90 days of age, were orally inoculated with 500 sporulated oocysts from a genotype III strain of *T. Gondii* (M7741). At 180 days of age, all animals were anesthetised, euthanized, and had their jejunum removed through laparotomy to measure its length and height for the calculation of its area. Blood from the anaesthetised animals was collected by orbital plexus puncture previously to the euthanasia. The serum was examined for the presence of (IgG) anti-*T. gondii* by testing direct agglutination. After removal, the intestinal segments were washed and underwent Giemsa banding analysis for the evaluation of the total population, and NADPH-d histochemistry was applied for the nitrergic subpopulation. They were then dissected, had the tunica mucosa and tela submucosa removed, resulting in the whole mounts formed by the muscle tunic and the

serosa. The total number of myenteric neurons from each animal within 120 microscopic fields uniformly distributed on the intestinal circumference was counted. The estimated number of cholinergic neurons (NADPH-d negative) was determined by decreasing the average of neurons evidenced by the NADPH-d staining of the total population of neurons. The area of the body cell and nucleus was measured and the area of the cytoplasm of 300 neurons in the myenteric plexus of each animal per technique was calculated. All animals presented anti-*T. gondii* antibodies, whereas the CG animals presented negative results. Infected animals had higher body mass gain when compared to the CG. No alterations with respect to length, width and area of the jejunum were observed. The quantitative analyses of the myenteric neurons evidenced with Giemsa staining and NADPH-d$^+$ histochemistry, as well as the estimated number of NADPH-d$^-$ (cholinergic neurons) presented no significant difference. The myenteric neurons of the total population presented atrophy in the G30 animals and a recovery resulting in hypertrophy at 90 days (G90). Morphometric analysis of the NADPH-d$^+$ neurons showed atrophy for both the G30 and G90 when compared to the CG; however, neurons from the G90 showed hypertrophy in relation to the G30. It was observed that at 30 days, the infection tended to increase the incidence of smaller neurons and decrease of larger neurons – this tendency was inverted at 90 days of infection. We concluded that the infection with a genotype III of *T. gondii* used in this study caused no neuronal death, however, we observed atrophy in neurons from the nitrergic population and subpopulation at 30 days of infection, and that those neurons tend to hypertrophy at 90 days in the jejunum of rats.

Keywords: enteric nervous system, morphometry, neural plasticity, small intestine, toxoplasmosis.

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Infecção crônica causada pelo *Toxoplasma gondii* promove neuroplasticidade mientérica no jejuno de ratos

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Resumo: O *Toxoplasma gondii* é o agente etiológico da toxoplasmose, que comumente causa diarreia em uma variedade de espécies, esse achado juntamente à afinidade do parasito pelo tecido nervoso, são indícios que a infecção toxoplásmica pode afetar os neurônios mientéricos. Objetivou-se avaliar as alterações causadas pelo *Toxoplasma gondii* (genótipo III) sobre neurônios mientéricos do jejuno de ratos. Distribuíram-se quinze ratos em três grupos: controle (GC), inoculado por 30 dias (G30) e inoculado por 90 dias (G90). Os ratos do G30 e G90 foram inoculados com 500 oocistos de uma cepa genótipo III (M7741) de *T. gondii,* pela via oral. Aos 180 dias de idade todos os animais foram anestesiados e mortos. Preparados totais foram marcados pelas técnicas de Giemsa e NADPH-diaforase. Observou-se manutenção na largura, comprimento, área e na densidade neuronal, contudo houve atrofia neuronal no G30 e tendência a hipertrofia no G90.

Palavras-chave: intestino delgado, morfometria, plasticidade neuronal, sistema nervoso entérico, toxoplasmose.

1. INTRODUÇÃO

O *Toxoplasma gondii* (*T. gondii*) é um parasito intracelular obrigatório, agente etiológico da toxoplasmose, zoonose de distribuição mindial (Tenter et al., 2000). Em animais infectados por esse parasito, a diarreia é um dos sinais clínicos (Dubey et al., 1979), outro achado comum é a presença de cistos teciduais encefálicos, que em imunodeprimidos pode gerar alterações funcionais e comportamentais (Ascenzi et al., 2005). A prevalência de diarreia entre animais infectados e a afinidade do parasito pelo tecido nervoso sugerem que o sistema nervoso entérico (SNE) também possa ser um alvo do *T. gondii*.

Os corpos celulares dos neurônios do SNE organizam-se nos plexos, submucoso e mientérico (Furness, 2006). Estudos recentes demonstram que a infecção causada pelo *T. gondii* promove alterações morfométricas nos neurônios mientéricos de ratos (Sugauara, et al., 2008, 2009; Soares et al., 2009; Pereira et al., 2010) e de suínos (Odorizzi et al., 2010). No entanto, em ratos foi avaliada a população total e a subpopulação NADH-diaforase positivivo dos neurônios mientéricos, não havendo estudos com as duas subpopulações neuronais mientéricas predominantes: os neurônios nitrérgicos e os colinérgicos.

O *T. gondii* possui três formas de vida infectante: taquizoíto, bradizoíto e esporozoíto. Os esporozoítos estão localizados no interior de oocistos, que permanecem livres no ambiente e por isso, representam a forma infectante mais comum (Dubey et al., 1998; Tenter, 2009). O único trabalho, que avaliou a infecção causada pela inoculação de oocistos por via oral em ratos analisou a subpopulação mientérica de neurônios NADH-dp (Pereira et al., 2010). Nos demais estudos preliminares com

roedores foram avaliados os efeitos da infecção causada por taquizoítos (Sugauara et al., 2008; Soares, et al., 2009) e bradizoítos (Sugauara et al., 2009), não tendo sido analisada a infecção por oocistos desse parasito.

Em virtude da lacuna existente quanto a estudos das demais subpopulações neuronais mientéricas de ratos infectados, objetivou-se avaliar as possíveis alterações causadas durante uma infecção crônica por oocistos, de uma cepa genótipo III (M7741) de *T. gondii,* sobre os neurônios mientéricos da população neuronal total, NADPH-d positivos e NADPH-d negativos do jejuno de ratos.

2. MATERIAIS E MÉTODOS

Todo o procedimento experimental foi aprovado previamente pelo Comitê de Ética em Pesquisa Envolvendo Experimentação Animal (CEPEEA) da Universidade Paranaense (UNIPAR, Umuarama, Paraná, Brasil; Protocolo: 12361/2008), que está de acordo com a Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL). Foram seguidas também as orientações presentes no NRC *Guide for the Care and Use of Laboratory Animals*.

2.1 Delineamento e grupos experimentais

Foram utilizados quinze ratos (*Rattus novergicus*) machos, adultos, da linhagem Wistar, com 90 dias de idade e 286,0±39,2g de peso corporal, provenientes do biotério da Universidade Paranaense - UNIPAR. Foram separados aleatoriamente em três grupos

experimentais: controle (GC, n=5), inoculado por 30 dias (G30, n=5) e inoculado por 90 dias (G90, n=5). Permaneceram em uma sala climatizada (temperatura entre 22 a 24°C) com ciclo claro/escuro de 12 horas com água (clorada) e ração comercial para roedores (Nuvilab®, Nuvital, Colombo, Paraná, Brasil) disponíveis *ad libitum.* Alojados em caixas de polipropileno tampadas com grade zincada, com depressão central para deposição da ração e para garrafa de água. Os animais do GC receberam solução salina por via oral. Os ratos do G30 aos 150 dias de vida e os animais do G90 aos 90 dias de idade foram inoculados com 500 oocistos esporulados de uma cepa genótipo III (M7741) de *T. gondii,* pela via oral.

2.2 Inóculo

Foram utilizados oocistos da cepa M7741 de *Toxoplasma gondii* (genótipo III) provenientes do Laboratório de Medicina Veterinária Preventiva e Saúde Pública da UNIPAR, originalmente isolados por Jacobs et al. (1960) de diafragma ovino. Os oocistos foram obtidos pela inoculação de gatos domésticos (*Felis cattus*), pela via oral com cistos teciduais provenientes de camundongos (*Mus musculus*), previamente infectados com o parasito. A partir do quinto dia após a inoculação, as fezes dos gatos foram recolhidas e submetidas ao método de Sheatter (Sloss et al., 1999) para concentração dos oocistos, que foram contados em câmara de Newbauer e esporulados em solução de ácido sulfúrico a 2%. Para a inoculação dos ratos, 500 oocistos esporulados foram ressuspendidos em 1 mL de solução salina fosfatada (pH 7,4), para facilitar a administração do inóculo pela via oral.

2.3 Coleta das amostras

Aos 180 dias de idade todos os animais foram anestesiados com maleato de acepromazina 2% (1,26 mL/kg) (Acepram®, Univet, São Paulo, SP, Brasil) + cloridrato de cetamina 10% (1,26 mL/kg) (Ketalar®, Pfizer, Santo Amaro, SP, Brasil) + cloridrato de xilazina 2,5% (0,42 mL/kg) (Xilasin®, Sintec, Cotia, SP, Brasil) e sulfato de atropina 1% (0,22 mL/kg) (Atropion®, Ariston, São Paulo, SP, Brasil), por via intramuscular, segundo protocolo anestésico de Pachaly et al. (2003). Por meio de laparotomia, pela linha média, os segmentos intestinais foram retirados e o jejuno foi separado e suas dimensões foram mensuras da flexura duodeno-jejunal até a inserção da prega íleo-cecal.

2.4 Confirmação da infecção pelo *T. gondii*

A infecção pelo *T. gondii* foi confirmada pela detecção de anticorpos séricos anti-*T. gondii*. Colheu-se sangue dos animais anestesiados pela punção do plexo venoso retro-orbital, previamente a eutanásia, visando detecção de anticorpos séricos (IgG) anti-*T. gondii* pelo método de aglutinação direta (Desmonts and Remington, 1980), ensaio sensível para essa determinação (Ashbur et al., 1995; Cantos et al., 2000) .

2.5 Técnica de Giemsa

Realizou-se a coloração da população total de neurônios mientéricos com azul de metileno em conformidade com a técnica de Giemsa. Um segmento de cerca de 5 cm

do jejuno proximal de todos os animais foi lavado com solução salina a 0,9% (pH 7,2), preenchidos e imersos em solução fixadora de formol acético por 48h. Na sequência, foram dissecados e os preparados totais foram corados com solução corante de Giemsa (à base de azul de metileno) em tampão fosfato de Sorense (Barbosa, 1978).

2.6 Técnica de NADPH-diaforase

Outro segmento de cerca de 5 cm do jejuno proximal de todos os animais foram submetidos à técnica histoquímica da NADPH-diaforase, para a evidenciação dos neurônios mientéricos nitrérgicos (NADPH-d positivos). Para isso, uma parte do jejuno de todos os animais de cada grupo foi lavado e preenchido com tampão fosfato (pH 7,4), fixado com paraformaldeído (Merk, Darmstad, Germany) a 4% preparado em tampão fosfato 0,1 M (pH 7,4) por 30 minutos, imersos em Triton X-100 a 0,3% em PBS 0,01M pH 7,4, e depois lavado 10 vezes (10 min. cada) em PBS, e submerso durante 60 minutos, em meio de incubação contendo para cada 100 mL: 25 mg de NBT; 50 mg de β-NADPH (Sigma, Steinheim, Germany); 0,3 mL de Triton X-100 e tampão tris-HCl (GibcoBRIL, New York, USA) 0,1M (pH 6,0). Depois da incubação e da tríplice lavagem em PBS (5 min. cada), os segmentos foram abertos e imersos em solução de paraformaldeído a 4% (Scherer-Singler et al., 1983), em seguida dissecados para obtenção dos preparados totais.

2.7 Obtenção dos preparados totais

Os preparados totais foram confeccionados por dissecção sob estereomicroscópio com trans-iluminação (Motic SMZ-140), por meio da remoção da

túnica mucosa e da tela submucosa. A seguir, foram desidratados, em série crescente de solução aquosa de álcool etílico, diafanizados em xilol e montados entre lâmina e lamínula com resina sintética *Permount*® (Fisher Chemical, NewJersy, U.S.A).

Durante a dissecção foi avaliada a presença de focos de necrose ou hemorragia visíveis macroscópica e mesoscopicamente.

Para análise dos neurônios, os preparados totais foram divididos em três regiões: mesentérica (0º-60º e 300º-360º), intermediária (60º-120º e 240º-300º) e antimesentérica (120º-240º), considerando 0º como a inserção do mesentério.

2.8 Análise quantitativa

Com um microscópio óptico Motic BL, utilizando a objetiva de 40x, foram contados os neurônios presentes em 40 campos microscópicos de cada região, da circunferência intestinal de cada animal, totalizando uma área de 16,8 mm^2 para ambas as técnicas. Durante a contagem dos neurônios corados por Giemsa realizou-se a procura de cistos de *T. gondii*. O número estimado de neurônios colinérgicos (NADPH-d negativos) foi determinado reduzindo-se a média dos neurônios evidenciados pela marcação da NADPH-d da população total de neurônios (Giemsa) conforme proposto por Phillips et al., 2003.

2.9 Análise morfométrica

Na análise morfométrica, foram mensuradas as áreas do corpo celular e do núcleo de 100 neurônios, por região, de cada animal de todos os grupos em ambas as técnicas. A mensuração foi realizada com o auxílio de um microscópio fotônico, com objetiva de 40x, acoplado ao sistema de análise de imagem *Motic Images Plus,* versão 2.0. A área do citoplasma presente no corpo celular foi calculada a partir da diferença entre essas áreas. Além disso, determinou-se a razão entre a área do núcleo e a área do corpo celular, com o intuito de verificar a proporção ocupada por essa organela dentro do corpo celular.

2.10 Análise estatística

Os dados foram submetidos ao teste D´Agostino-Pearson ou de Shapiro-Wilk para verificar o tipo de distribuição. Dados com distribuição normal são apresentados como média ± desvio padrão. Neste caso, para comparar os grupos, utilizou-se análise de variância (ANOVA): seguida pelo teste de Tukey, para a comparação entre os três grupos. Dados com distribuição livre (não especificada) são apresentados como mediana (percentil 25; percentil 75), e a comparação entre os grupos foi realizada pelo teste de kruskal-Wallis, seguida do teste de Dunn. Em todos os testes estatísticos, valores de $p < 0,05$ foram considerados significantes. A análise da correlação foi verificada com o teste não paramétrico de Sperman r, valores positivos de r, demonstraram correlação positiva entre as variáveis e os valores de obedeceram a seguinte escala: 0,0 – 0,3 fraca; 0,3 – 0,6 regular; 0,6 – 0,9 forte; 0,9 – 1,0 muito forte.

3. RESULTADOS

Durante o experimento não foram observados sinais clínicos da toxoplasmose nos animais inoculados. Os animais do G30 e G90 apresentaram anticorpos anti-*T. gondii* (IgG), enquanto os animais do GC tiveram resultados negativos ao exame sorológico. Na análise macroscópica e mesoscópica não foram observadas lesões aparentes no jejuno.

Além disso, os animais dos grupos inoculados apresentaram maior ganho de peso corporal quando comparados ao GC, entretanto, não foram observadas alterações no comprimento, largura e área do jejuno (Tabela 1).

A análise quantitativa dos neurônios mientéricos evidenciados pela técnica de Giemsa e pela NADPH-d^+ (neurônios nitrérgicos), bem como, o número estimado de neurônios NADPH-d^- (neurônios colinérgicos) não apresentaram diferença significativa ($p>0,05$) (Tabela 2).

Os neurônios marcados com a técnica de Giemsa, dos animais do G30 apresentaram redução da área do núcleo, corpo celular, citoplasma e da proporção ocupada pelo núcleo dentro do corpo celular quando comparados ao GC. Já os animais do G90 apresentaram hipertrofia ($p<0,05$) (Tabela 3).

Com a técnica da NADPH-d os neurônios mientéricos apresentaram atrofia em todas as variáveis avaliadas aos 30 e 90 dias de inoculação comparados ao GC, no entanto, os do G90 demonstraram hipertrofia em relação aos do G30 ($p<0,05$).

O grau de correlação entre a área do corpo celular, núcleo e citoplasma dos neurônios mientéricos está apresentado na Tabela 4. As Fig. 1 e 2 expressam,

respectivamente a distribuição de frequência dos neurônios mientéricos divididos por classes, de acordo com a área do corpo celular marcado com Giemsa ou NADPH-d^{+} .

4. DISCUSSÃO

Não foi observada diarreia nem outro sinal clínico de toxoplasmose nos animais inoculados, caracterizando uma infecção subclínica. A diarreia é um achado frequente em animais infectados pelo *T. gondii* (Dubey et al., 1979), porém, há poucos relatos de sinais clínicos entre ratos infectados (De Champs et al., 1998), os quais são considerados resistentes ao desenvolvimento da toxoplasmose (Dubey et al., 1999), e um bom modelo para o estudo do curso da infecção crônica desta doença. Outros animais são susceptíveis ao desenvolvimento de sinais clínicos da infecção toxoplásmica (Dubey et al., 1979). Por exemplo, suínos (Odorizzi et al., 2010) e frangos (Shiraishi et al., 2009), inoculados pela mesma cepa utilizada neste estudo apresentaram diarreia.

A sorologia positiva nos animais do G30 e G90 demonstra que foram infectados com o parasito, no entanto, o resultado negativo para o GC demonstra que esses animais permaneceram hígidos.

As cepas de genótipo III de *T. gondii* são consideradas de baixa virulência para camundongos e possuem alta capacidade cistogênica, viabilizando a cronificação da infecção (Howe and Sibley, 1995). Apesar de sua capacidade cistogênica, não foram encontrados cistos teciduais de *T. gondii* na amostra de neurônios mientéricos avaliada,

concordando com a observação de que cistos intestinais são achados raros (Dubey et al., 1998).

Os animais do G30 apresentaram ganho de peso corporal significativo (47,7%) em relação aos animais do GC. Já os do G90 foram semelhante ao do GC, e significativamente menor que o do G30 (22,9%). Esses resultados indicam que, durante o curso de infecção crônica, o parasito tenha induzido alterações metabólicas no rato, que levaram ao aumento de peso nos primeiros 30 dias após inoculação, seguido de redução nos meses seguintes. Sugere-se que alterações metabólicas ou comportamentais, tenham ocorrido nesses animais ao longo do experimento. Uma das hipóteses é que tenham ingerido mais ração durante os primeiros 30 dias, induzido por comprometimento do Sistema Nervoso Central (SNC). Vyas et al. (2007a) descrevem que cistos de *T. gondii* foram encontrados em áreas encefálicas relacionadas com a ansiedade em roedores. A presença de cistos e a alteração neuroquímica por eles induzida, pode resultar em mudanças comportamentais (Vyas et al., 2007 a, b). Desse modo, novos estudos são necessários, para que seja acompanhado o peso corporal ao longo do experimento assim como, o consumo de ração.

Igualmente, não foram observadas alterações na área do jejuno nos animais infectados, resultados semelhantes aos de estudos anteriores com infecção toxoplásmica em ratos (Soares et al., 2009; Sugauara et al., 2009; Pereira et al., 2010). Esses achados são indicativos que a infecção toxoplásmica em ratos provocada pela cepa utilizada neste estudo, não deve ter alterado a estrutura da parede intestinal, no entanto, sugere-se que estudos morfométricos avaliando os estratos da parede jejunal, sejam realizados para averiguar esta hipótese.

Neste estudo não houve alteração na densidade populacional total dos neurônios mientéricos, nem da projeção do número de neurônios para área total do jejuno, dados semelhantes a outros estudos no intestino delgado e grosso, de animais infectados por taquizoítos ou bradizoítos (Sugauara et al., 2008, 2009; Soares et al., 2009).

Além disso, os neurônios mientéricos da população total apresentaram atrofia nos animais do G30, e uma recuperação seguida de hipertrofia aos 90 dias (G90) independente da classe estudada. O alto grau de correlação observado entre a área do corpo celular e a área do citoplasma, indica que tanto a atrofia observada nos neurônios, 30 dias após a inoculação ($r=96$, $p<0{,}001$), quanto a hipertrofia dessas células, 90 dias após a inoculação ($r=97$, $p<0{,}001$), sejam decorrentes principalmente de alterações citoplasmáticas, embora nas duas situações, a área do núcleo também alterou de forma diretamente proporcional a esses eventos. Estudos preliminares realizados com ratos, também evidenciaram atrofia nos neurônios mientéricos do íleo, após 30 dias de inoculação com taquizoítos de outra cepa genótipo III (Sugauara et al., 2008). No intestino grosso de ratos, ao contrário, observou-se hipertrofia neuronal após 30 dias de inoculação por *T. gondii* causada por uma cepa genótipo III (Sugauara et al., 2008) e genótipo I (Soares et al., 2009). Este é o primeiro estudo que analisou os efeitos da inoculação toxoplásmica por 90 dias.

Na análise quantitativa dos neurônios nitrérgicos também não foram observadas alterações significativas. Não existem descrições de trabalhos que relacionem a infecção toxoplásmica e os neurônios mientéricos nitrérgicos em ratos, no entanto, em suínos inoculados durante 30 dias com a mesma cepa e forma infectante de *T. gondii* utilizada neste estudo foi observado aumento do número de neurônios NADPH-d^{+} por gânglio, o

que foi atribuído a um desvio fenotípico, com aumento do número de células nervosas que passaram a expressar esta enzima durante a infecção (Odorizzi et al., 2010). Os neurônios nitrérgicos normalmente são protegidos da morte (Cowen et al., 2000; Phillips et al., 2003). Possivelmente porque o óxido nítrico (NO) em cooperação com o Peptídeo Vasoativo Intestinal (VIP) sejam importantes para adaptação, manutenção e sobrevivência neuronal (Lin et al., 2004). Cowen et al. (2000) sugerem que, os neurônios que utilizam essencialmente o NO, podem ter desenvolvido mecanismos de defesa contra radicais livres, dentre estes podemos mencionar a neuroglobina, proteína expressa principalmente por neurônios centrais e periféricos (Burmester and Hankeln, 2009), que pode proteger células dos efeitos das espécies reativas do oxigênio (Milton et al., 2006).

O número estimado de neurônios colinérgicos (NADPH-d^{-}) não sofreu alteração numérica com a inoculação pelo parasito e permaneceu como subpopulação predominante em relação aos nitrérgicos, conforme descrita para roedores (Sang and Young, 1996). Neurônios colinérgicos são considerados vulneráveis a morte neuronal (Cowen et al., 2000), apesar disso os resultados deste experimento indicam que não sofreram alteração de densidade populacional com a inoculação pelo *T. gondii*.

A análise morfométrica dos neurônios NADPH-d^{+} demonstrou atrofia tanto no G30 como no G90 quando comparados ao GC, contudo a comparação dos neurônios do G30 aos do G90 demonstraram hipertrofia nos segundos, evidenciando que uma recuperação da área celular estava em curso. Semelhantemente ao que foi observado, para a população total, nesta subpopulação a área do corpo celular e do citoplasma apresentam alto grau de correlação que permite sugerir que as alterações morfométricas

constadas tenham sido decorrentes principalmente de modificações no citoplasma dessas células (r=95, $p<0,001$). Verificou-se que aos 30 dias de inoculação houve tendência de aumento da incidência de neurônios menores (de 101 a 150 μm^2) e redução de neurônios maiores (de 201 a >301 μm^2).

Alterações nos neurônios mientéricos podem ser resultantes tanto de uma ação direta quanto indireta do parasito. Acredita-se que a atrofia dos neurônios nitrérgicos tenha sido causada pela ação do sistema imune em resposta à presença do parasito, provocando aumento do óxido nítrico produzido pela oxido nítrico sintase induzível (iNOS) (Brunet et al., 2001; Chandrasekharan and Shinivasan, 2007). O NO é uma molécula citotóxica que age inibindo enzimas essenciais mitocondriais e nucleares, é liberado juntamente com citocinas e quimiocinas durante a invasão do *T. gondii* (Kasper et al., 1994). Em quantidades excessivas o NO é citotóxico não somente aos parasitos, mas também às células do hospedeiro (Brunet et al., 2001). Neste estudo possivelmente tenha ocorrido o comprometimento de enzimas citoplasmáticas neuronais na fase inicial da inoculação, resultando em neurônios metabolicamente menos ativos e consequentemente com área nuclear reduzida.

Aos 30 dias de inoculação observou-se que, em ambas as técnicas ocorreram atrofia dos neurônios mientéricos, indicando que possivelmente o parasito tenha influenciado o seu metabolismo. No entanto, situação inversa sucedeu aos 90 dias após inoculação com uma tendência da recuperação da área desses neurônios. Sugere-se que essas alterações plásticas ocorreram em reposta ao processo inflamatório, que normalmente é provocado pela infecção causada pelo *T. gondii*, pois o parasito desencadeia uma cascata de reações imunológicas com grande liberação de mediadores

químicos e citocinas (Buzoni-Gatel and Werts, 2006), e, portanto, essa resposta imune possivelmente tenha causado as alterações morfométricas observadas neste estudo. Na medida em que ocorre a formação de cistos e, portanto a cronificação da infecção (Howe and Sibley, 1995) incide um restabelecimento do sistema imune, o que favorece a continuidade do parasitismo, e pode estar relacionado com as alterações ocorridas no decorrer da infecção, observadas neste estudo. Ou seja, sugere-se que aos 90 dias de infecção, tenha se estabelecido um equilíbrio na relação parasito-hospedeiro observada pela normalização do peso corporal e área neuronal. Isso pode ser corroborado pelo fato de que inicialmente o NO apresenta efeitos prejudiciais, no entanto à medida que se estabelece a cronificação da infecção, a produção do NO torna-se essencial para a sobrevivência do hospedeiro (Brunet et al., 2001).

Outra forma do parasito agir é por meio do parasitismo direto nos neurônios mientéricos, pois células infectadas pelo *T. gondii* sofrem mudanças fisiológicas e morfológicas, essas alterações podem ser em resposta a interação entre o parasito e a célula hospedeira. O *T. gondii* torna as células hospedeiras resistentes a indutores de apoptose por intermédio de sua capacidade de manipular respostas da célula hospedeira, possivelmente é uma estratégia do parasito para manter a viabilidade celular do hospedeiro (Weiss and Kim, 2007), o que pode ter contribuído para a manutenção na densidade neuronal observada neste estudo.

Conclui-se que a infecção por oocistos em ratos de uma cepa genótipo III de *Toxoplasma gondii* não provocou perda neuronal, bem como alterações na área do jejuno de ratos, contudo, causou atrofia em neurônios da população e da subpopulação

nitrérgica após 30 dias de inoculação, e que esses neurônios tenderam a sofrer hipertrofia após 90 dias.

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Tabela 1. Média ± desvio padrão do peso corporal, comprimento e largura e da área do jejuno de ratos do grupo controle (GC) e submetidos à inoculação por uma cepa genótipo III de *Toxoplasma gondii* durante 30 (G30) e 90 (G90) dias.

Grupo	Peso (g)	Comprimento (cm)	Largura (cm)	Área do jejuno (cm^2)
GC	$309{,}2^{a\,c}$ ± 24,1	102,0 ± 7,5	1,2 ± 0,2	122,2 ± 22.8
G30	$456{,}9^{b}$ ± 58,0	105,5 ± 9,1	1,0 ± 0,1	111,4 ± 20,5
G90	$352{,}4^{c}$ ± 35,4	106,6 ± 6,2	1,1 ± 0,1	117,1 ± 7,6

Médias seguidas de letras diferentes numa mesma coluna apresentam diferença significativa (p < 0,05).

Tabela 2. Média ± desvio-padrão da densidade populacional dos neurônios corados por Giemsa, NADPH-d positivos e do número estimado de neurônios NADPH-d negativos em $16{,}8mm^2$ e projeção do número de neurônios para área total do jejuno de ratos do grupo controle (GC) e submetidos à inoculação por uma cepa genótipo III de *Toxoplasma gondii* durante 30 (G30) e 90 (G90) dias.

		GC	G30	G90
GIEMSA	Em $16{,}8mm^2$	3.494,8 ± 308,3	3.858,2 ± 283,7	3.156,8 ± 905,1
	Projeção para área total	2.971.847,9 ± 929.781,0	2.541.749,9 ± 411.001,4	2.217.118,7 ± 659.885,4
NADPH-d^+	Em $16{,}8mm^2$	1.007,2 ± 262,6	1.146,0 ± 189,2	1.163,2 ± 193,2
	Projeção para área total	851.464,4 ± 309868,1	748.841,8 ± 122849,3	796.996,0 ± 133.375,1
NADPH-d^-	Em $16{,}8mm^2$	2.487,6 ± 248,6	2.712,2 ± 95,7	1.993,5 ± 1098,8
	Projeção para área total	2.120.385,3 ± 703632,9	1.792.908,1 ± 308.690,3	1.420.122,7 ± 769.753,7

Tabela 3. Mediana e percentis (P25; P75) das áreas do corpo celular, núcleo, citoplasma e da razão entre a área do núcleo e do corpo celular de neurônios corados por Giemsa e NADPH-d+ do jejuno de ratos do grupo controle (GC) e submetidos à inoculação por uma cepa genótipo III de *Toxoplasma gondii* durante 30 (G30) e 90 (G90) dias.

	GIEMSA			NADPH-d+		
	GC	G30	G90	GC	G30	G90
Área do corpo celular (μm^2)	144,0[a] (109,0; 187,0)	129,0[b] (102,0; 172,0)	154,0[c] (108,0; 207,0)	200,5[a] (164,6; 247,5)	162,4[b] (135,1; 202,0)	176,3[c] (143,8; 216,6)
Área do Núcleo (μm^2)	38,0[a] (27,0; 51,0)	34,0[b] (25,0; 47,0)	43,0[c] (30,0; 57,0)	58,5[a] (46,8; 71,6)	50,9[b] (39,8; 62,9)	53,4[c] (42,1; 66,0)
Área do Citoplasma (μm^2)	104,0[a] (77.7; 137,0)	97,0[b] (72,0; 128,0)	109,0[c] (74,0; 152,0)	142,1[a] (112.6; 179.1)	112,2[b] (89,3; 144,5)	121,8[c] (98,5; 154,8)
Razão (Núcleo/corpo celular)	0,27[a] (0,22; 0,33)	0,26[a] (0,1; 0,32)	0,28[b] (0,22; 0,34)	0,29[a] (0,24; 0,34)	0,30[b] (0,26; 0,36)	0,30[c] (0,25; 0,35)

Medianas seguidas de letras diferentes numa mesma linha para a mesma técnica apresentam diferença estatística significante ($p < 0,05$).

Tabela 4 – Grau de correlação entre a área do corpo celular, do citoplasma e do núcleo de neurônios mientéricos corados por Giemsa e NADPH-d+ do jejuno de ratos do grupo controle (GC) e submetidos à inoculação por uma cepa genótipo III de *Toxoplasma gondii* durante 30 (G30) e 90 (G90) dias.

	GIEMSA			NADPH-d+		
Área	GC	G30	G90	GC	G30	G90
Corpo Celular x Núcleo	0,75	0,69	0,79	0,69	0,72	0,68
Corpo Celular x Citoplasma	0,96	0,96	0,97	0,96	0,95	0,95
Núcleo x Citoplasma	0,54	0,48	0,63	0,48	0,48	0,43

Todos os valores são significativos com $p< 0, 0001$.

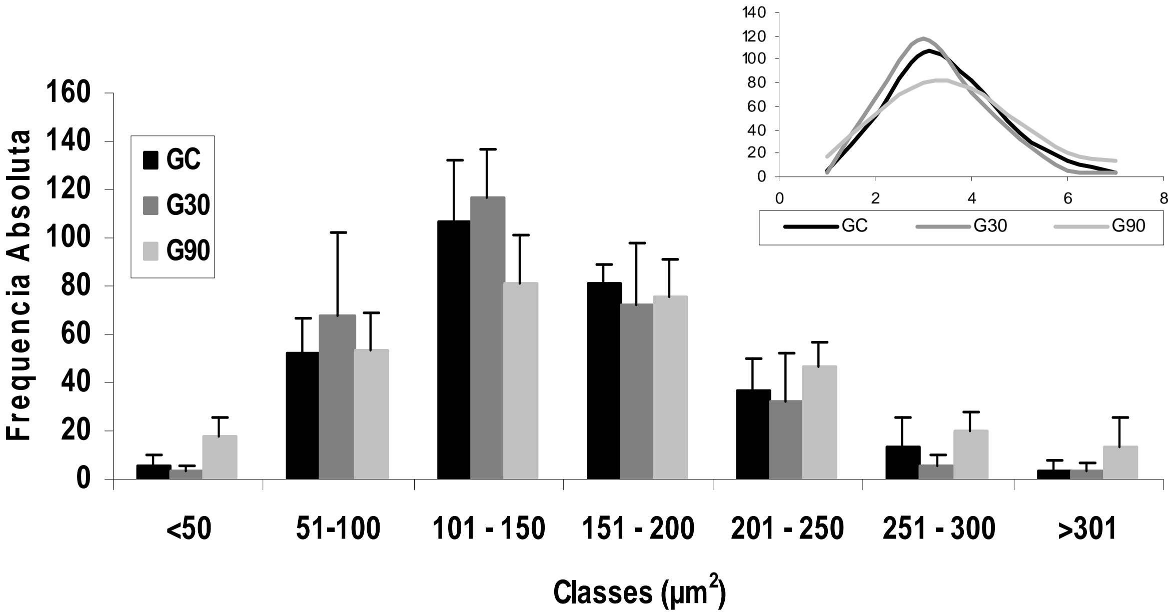

Fig. 1 – Distribuição dos neurônios mientéricos corados por Giemsa do jejuno de ratos do grupo controle (GC) e submetidos à inoculação por uma cepa genótipo III de *Toxoplasma gondii* durante 30 (G30) e 90 (G90) dias, distribuídos segundo classes da área do corpo celular.

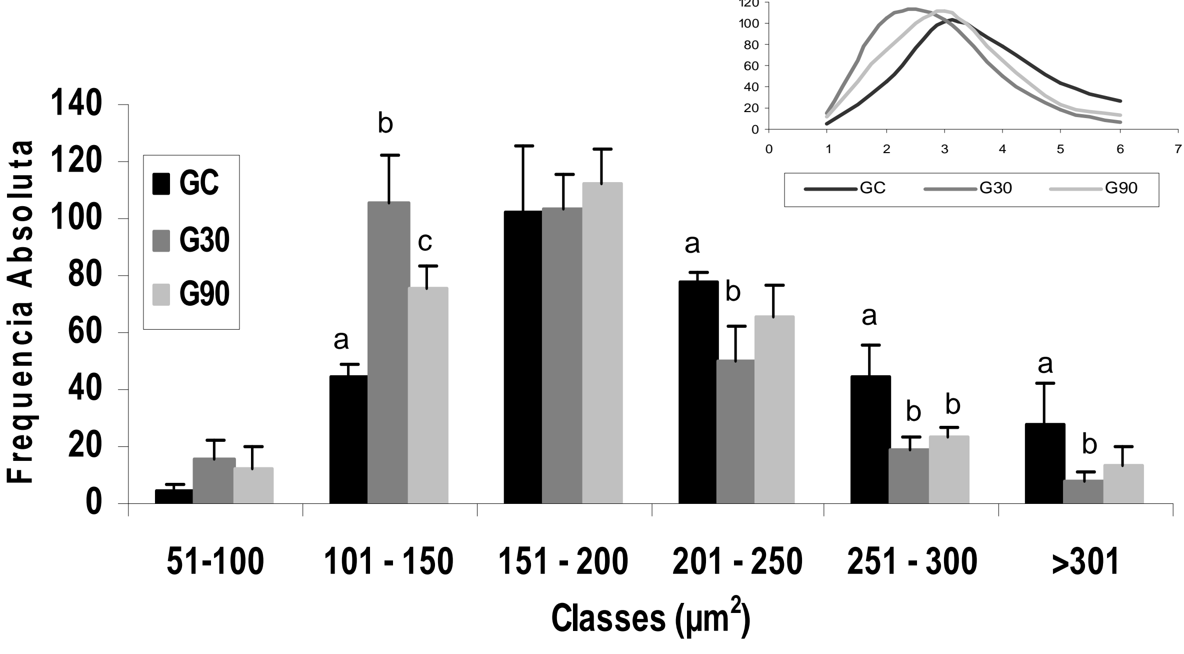

Fig. 2 - Distribuição dos neurônios mientéricos corados por NADPH-d do jejuno de ratos do grupo controle (GC) e submetidos à inoculação por uma cepa genótipo III de

Toxoplasma gondii durante 30 (G30) e 90 (G90) dias, distribuídos segundo classes da área do corpo celular. Colunas marcadas com letras diferentes para uma mesma classe diferem significativamente ($p < 0,05$).

Chronic infection with *Toxoplasma gondii* causes myenteric neuroplasticity of the jejunum of rats

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Abstract: The *Toxoplasma gondii* is an etiological agent of toxoplasmosis which commonly causes diarrhoea in a number of species. This finding and the parasite's affinity for the nervous tissue are evidences that the *T. gondii* infection may affect the myenteric neurons. The aim of this study was to evaluate the changes caused by *Toxoplasma gondii* (Genotype III) in the myenteric neurons of the jejunum of rats. Fifteen rats were distributed into 3 groups: control (CG), inoculated for 30 days (G30) and inoculated for 90 days (G90). Rats from the G30 and G90 received an oral inoculum with 500 oocysts from a genotype III (M7741) *T. gondii* strain. At 180 days of age, all animals were anesthetised and killed. Whole mounts were stained by using Giemsa and NADPH-diaphorase histochemistry. Maintenance of the width, length, area and neuronal density were observed, whereas there was neuronal atrophy for the G30 and a tendency to hypertrophy for the G90.

Keywords: enteric nervous system, morphometry, neural plasticity, small intestine, toxoplasmosis.

1. INTRODUCTION

Toxoplasma gondii (*T. gondii*) is an obligate intracellular parasite, etiological agent of toxoplasmosis, a zoonosis of worldwide distribution (Tenter et al., 2000). In animals infected with this parasite, diarrhoea is one of the clinical signs (Dubey et al., 1979). Another common finding is the presence of brain-tissue cysts, which may cause functional and behavioral changes in the immunodepressed (Ascenzi et al., 2005). Prevalence of diarrhoea among infected animals andthe affinity of the parasite for the nervous tissue suggest that the enteric nervous system (ENS) may be a target for *T. gondii*.

Cell bodies of ENS neurons are organized in the submucosal and myenteric plexus (Furness, 2006). Recent studies showed that the infection with *T. gondii* causes morphometric changes in the myenteric neurons of rats (Sugauara, et al., 2008, 2009; Soares et al., 2009; Pereira et al., 2010) and pigs (Odorizzi et al., 2010). However, the total population and the NADH-diaphorase positive myenteric neurons were evaluated and there are no studies involving the two most prevalent subpopulations of myenteric neurons: nitrergic and cholinergic (Phillps et al., 2003).

T. gondii has three forms of infective life: tachyzoite, bradizoite and sporozoite. The sporozoites are located within the oocysts which remain free in the environment, therefore representing the most common infectious form (Dubey et al., 1998; Tenter, 2009). The only study evaluating the infection caused with the oral inoculation of oocysts in rats analyzed the subpopulation of NADH-dp myenteric neurons (Pereira et al., 2010). In other preliminary studies on rodents, the effects of the infection caused by

tachyzoites (Sugauara et al., 2008; Soares, et al., 2009) and bradyzoites (Sugauara et al., 2009) was evaluated without analyzing the infection with oocysts from this parasite.

Because of the existing gap regarding the studies on the other subpopulations of myenteric neurons in infected rats, this study aimed to evaluate the possible changes caused during a chronic infection with oocysts from a genotype III (M7741) *T. gondii* on the myenteric neurons of the total neuronal population, NADPH-d positive and NADPH-d negative neurons in the jejunum of rats.

2. MATERIAIS AND METHODS

All experimental procedures were previously approved by the Ethics Committee Involving Experimental Animals of the *Universidade Paranaense* (UNIPAR, Umuarama, Paraná, Brazil; Protocol: 12361/2008), according to the *Sociedade Brasileira de Ciência em Animais de Laboratório* (SBCAL). Orientations from the NRC *Guide for the Care and Use of Laboratory Animals* were also followed.

2.1 Experimental outline and groups

Fifteen male, adult, 90-days-old, 286.0±39.2g Wistar rats (*Rattus novergicus*) from the *Universidade Paranaense* – UNIPAR vivarium were used. They were randomly distributed into 3 experimental groups: control (CG, n=5), inoculated for 30 days (G30, n=5) and inoculated for 90 days (G90, n=5). They were allocated in a temperature-controlled room (22-24°C) with a 12h dark/bright cycle. They received

water (chlorinated) and a commercial rodent diet (Nuvilab®, Nuvital, Colombo, Paraná, Brazil) *ad libitum* and were housed in polypropylene rodent cages. Animals from the CG received an oral saline solution, whereas those from the G30, at 150 days of age, and G90, at 90 days of age, were orally inoculated with 500 sporulated oocysts from a genotype III (M7741) *T. gondii* strain.

2.2 Inoculum

We used oocysts from the genotype III (M7741) strain of *T. gondii*, from the Laboratório de Medicina Veterinária Preventiva e Saúde Pública of Universidade Paranaense, originally isolated by Jacobs et al. (1960) from the ovine diaphragm.Theywere obtained through the oral inoculation of cats (*Felis cattus*) with tissue cysts of mice (*Mus musculus*) previously infected with the parasite. From the fifth day, cat fecal samples were collected and examined using Sheather's flotation method (Sloss et al., 1999) for the concentration of oocysts, which were counted in a Newbauer chamber and sporulated in 2% sulfuric acid solution. Five hundred sporulated oocysts were re-suspended in 1 mL of phosphate buffered saline solution (pH 7.4) for the inoculation of the rats.

2.3 Sample collection

After 90-day-old all rats were intramuscularly anesthesized with acepromazine maleate 2% (1,26 mL/kg) (Acepram®, Univet, São Paulo, SP, Brazil) + ketamine chloridate 10% (1,26 mL/kg) (Ketalar®, Pfizer, Santo Amaro, SP, Brazil) xylazine

chloridate 2,5% (0,42 mL/kg) (Xilasin®, Sintec, Cotia, SP, Brazil) e atropine sulphate 1% (0,22 mL/kg) (Atropion®, Ariston, São Paulo, SP, Brazil) (Pachaly et al., 2003). Laparotomy was conducted and the jejunum removed by having the pylorus and the duodenal-jejunal flexure as anatomical limits.

2.4 Confirmation of infection with *T. gondii*

Infectionwith *T. gondii* was confirmed through the detection of anti-*T. gondii* seric immunoglobulins. Blood sampleswere collected fromall animals and submitted to direct agglutination for the detection of IgG against *T. gondii* (Desmonts and Remington, 1980), sensitive test for this determination (Ashbur et al., 1995; Cantos et al., 2000).

2.5 Giemsa technique

Methylene blue solution according to Giemsa was used to stain the total population of myenteric neurons. A 5cm segment of the proximal jejunum of all animals was washed in a 0.9% saline solution (pH 7.2), filled and fixed in formalin-acetic acid solution for 48h. These segments were then dissected and the whole mounts were stained with Giemsa (methylene blue) in a Sorensen's phosphate buffer (Barbosa, 1978).

2.6 NADPH-diaphorase technique

Another segment, with around 5cm, from the proximal jejunum of all animals was submitted to NADPH-diaphorase histochemistry to evidence the nitrergic myenteric neurons (NADPH-d positive). Segments washed in PBS were washed and filled with phosphate buffer (pH 7.4), fixed with 4% paraformaldehyde (Sigma, St. Louis, MO, EUA) in phosphate buffer 0.1 M pH 7.4 for 30min, immersed in 0.3% Triton X-100 (Sigma, St. Louis, MO, EUA) in PBS and submersed for 60 min in an incubation medium containing in each 200 mL: 200 mL tris–HCl (GibcoBRL®, Grand Island, NY, EUA); 0.05 g NBT (Sigma, St. Louis,MO, EUA), 0.1 g β-NADPH (Sigma, St. Louis,MO, EUA) and 0.6 mL Triton X-100) (Scherer-Singler et al., 1983).

2.7 Whole mounts

Whole mounts were dissected under a stereomicroscope with transillumination (Motic SMZ-140) by removing the tunica mucosa and the tela submucosa. They were dehydrated through an ascending series of aqueous ethanol solution, cleared in xylene and mounted between the cover slip and the slide with *Permount*® (FisherChemical, NewJersy, U.S.A).

The presence of foci of necrosis and hemorrhage visible macroscopically and mesoscopically was evaluated during dissection.

2.8 Quantitative analysis

The whole-mounts were assigned into three regions: mesenteric (0º-60º and 300º-360º), intermediary (60º-120º and 240º-300º) e antimesenteric (120º-240º),

considering 0º as the insertion of the mesentery. The neurons within 40 microscopic fields from each region, in the intestinal segment of each animal, totalizing an area of 16.8 mm^2, were counted by using a 40X optical microscope MOTIC BL, totalizing 16,8 mm^2. The search for *T. gondii* cysts was conducted while counting the Giemsa-stained neurons. The estimated amount of cholinergic neurons was determined by reducing the mean of neurons evidenced by the NADPH-d of the total population stained with Giemsa, according to Phillips et al. (2003).

2.9 Morfphometric analysis

The area of the body cell and the nucleus of 100 neurons, per region, per animal, in each group were measured in the morphometric analysis. The measurement was carried out with the aid of a 40X optical microscope and *Motic Images Plus* 2.0. The cytoplasm area was calculated from the difference among these areas. Moreover, the ratio between the area of the nucleus and the body cell was determined in order to verify the proportion occupied by that organelle within the body cell.

2.10 Statistic analysis

Student's t-Test was used to compare the groups. Data presenting free distribution are presented as median (percentile 25, percentile 75). The comparison between the groups was performed by the Mann-Whitney test here. For all statistic tests, P values less than 0.05 were considered significant.

Numeric data collected initially were submitted to D´Agostino-Pearson or Shapiro test in order to verify the type of distribution. Data presenting normal

distribution was presented as mean±standard deviation. Analysis of variance (ANOVA), followed by Tukey's test, was used to compare the groups. Data presenting free distribution are presented as median (percentile 25, percentile 75). The comparison between the groups was performed by the kruskal-Wallis test here. For all statistic tests, P values less than 0.05 were considered significant. Spearman's rank correlation was used for the correlation analysis as *r* positive values showed positive correlation between the variables and $r > 0.90$ was considered very strong.

3. RESULTS

No clinical sign of toxoplasmosis in the inoculated animals was observed throughout the experiment. The G30 and G90 animals presented anti-*T. gondii* antibodies (IgG) whereas the CG animals had negative results for the serology. No apparent lesions in the jejunum were observed in the macroscopic and mesoscopic analyses.

The animals from the inoculated group had higher body weight gain when compared to the CG. No changes in length, width and area of the jejunum were observed (Table 1).

The quantitative analysis of the myenteric neurons evidenced with Giemsa and NADPH-d^+ as well as the number of the estimated NADPH-d^- (cholinergic neurons) showed no significant difference (Table 2).

The morphometric analysis of the neurons stained with Giemsa of the G30 animals showed a decrease of the area of the nucleus, body cell, cytoplasm and the

nucleus/body cell proportion when compared to the CG (Table 3). The G90 animals had hypertrophy.

NADPH-d in the myenteric neurons showed atrophy of the variables evaluated at 30 and 90 days of inoculation in relation to the CG, whereas the G90 showed hypertrophy in relation to the G30.

The degree of correlation among the area of the body cell, nucleus and cytoplasm of the myenteric neurons is showed in Table 4. Figures 1 and 2 show the frequency distribution of the myenteric neurons divided into classes according to the body cell area stained with Giemsa (Fig. 1) or NADPH-d$^+$ (Fig. 2).

4. DISCUSSION

Neither diarrhoea nor any other clinical sign of toxoplasmosis characterizing a subclinical infection was observed in the inoculated animals. Diarrhoea is often observed in animals infected with *T. gondii* (Dubey et al., 1979), however, there are a few reports of clinical signs among infected rats (De Champs et al., 1998), thus, they are considered resistant to the development of toxoplasmosis (Dubey et al., 1999) and a good model for the study of the course of the chronic infection. Other animals are susceptible to the development of clinical signs of the toxoplasmic infection (Dubey et al., 1979), such as pigs (Odorizzi et al., 2010) and chickens (Shiraishi et al., 2009), which presented diarrhoea when inoculated with the same strain used in this study.

The positive serology ensures that these animals are infected with the parasite, however, negative results for the CG ensure that the animals remain healthy.

The genotype III strains of *T. gondii* are considered low virulent for mice and have high cystogenic capacity enabling the chronification of the infection (Howe and Sibley, 1995). Despite its cystogenic capacity, no tissue cysts of *T. gondii* were found in the myenteric neuron samples evaluated, what matches the observation that intestinal cysts are rarely found (Dubey et al., 1998).

The animals from the G30 had significant body weight gain (47.7%) in relation to the CG. The G90 was similar to the CG and significantly lower than the G30 (22.9%). These results show that during the chronic infection, the parasite induced metabolic changes in the rat, resulting in the increase of the weight at the first 30 days after inoculation, followed by loss of weight in the following months. Metabolic and behavioral changes may have occurred in these animals throughout the experiment. One of the hypotheses is that they had ingested more rodent diet during the first 30 days induced by the compromising of the Central Nervous System (CNS). Vyas et al. (2007a) describe that cysts of *T. gondii* are found in the brain areas related to the anxiety of the rodents. The presence of the cysts and the neurochemical changes induced by them may result in behavioral changes (Vyas et al., 2007 a, b). New studies are needed so that body weight and diet ingestion can be followed throughout the experiment.

No changes in the jejunum area in the animals infected were observed. The results are similar to those of former studies involving the toxoplasmic infection in rats (Soares et al., 2009; Sugauara et al., 2009; Pereira et al., 2010). These findings indicate that the toxoplasmic infection caused by the strain used in this study may not have changed the structure of the intestinal wall; however, we suggest that morphometric

studies evaluating the strata of the jejunal wall are investigated in order to verify such hypothesis.

The decrease of neither the total population density of the myenteric neurons nor the projection of the number of neurons in the total area of the jejunum was observed. The data were similar to those found in other studies on the small and large intestine of animals infected by tachyzoites and bradyzoites (Sugauara et al., 2008, 2009; Soares et al., 2009).

The myenteric neurons of the total population atrophied in the G30 animals and had a recovery followed by hypertrophy at 90 days (G90) – in spite of the class studied. The high correlation degree observed between the area of the body cell and the area of the cytoplasm indicate that both the atrophy observed in the neurons, 30 days after inoculation ($r=96$, $p<0.001$) and the hypertrophy of those cells, 90 days after inoculation ($r=97$, $p<0.001$), resulted mainly from the cytoplasmic changes although, in both situations, the area of the nucleus also changed directly proportionally to those events. Preliminary studies conducted in rats also evidenced atrophy in the ileum myenteric neurons 30 days after inoculation with tachyzoites from another genotype III strain (Sugauara et al., 2008). Within the large intestine of rats, on the other hand, neuronal hypertrophy 30 days after inoculation with *T. gondii* caused by a strain genotype III (Sugauara et al., 2008) and genotype I (Soares et al., 2009) was observed. This is the first study to analyze the effects of toxoplasmic inoculation for 90 days.

No significant changes were observed in the quantitative analysis of the nitrergic neurons. There are no descriptions of studies relating the toxoplasmic infection and the nitrergic myenteric neurons in rats, however, the increase of the number of NADPH-d^{+}

neurons was observed in pigs inoculated for 30 days with the same strain of *T. gondii* used in this study, what was assigned to a phenotypic deviation with increase of the number of nervous cells which began to express that enzyme during the infection (Odorizzi et al., 2010). The nitrergic neurons are usually protected from death (Cowen et al., 2000; Phillips et al., 2003) possibly because the Nitric Oxide (NO) in cooperation with the vasoactive intestinal peptide (VIP) are important for neuronal adaptation, maintenance and survival (Lin et al., 2004). Cowen et al. (2000) suggest that the neurons essentially using NO may have developed a defense mechanism against the free radicals, including the neuroglobin – a protein expressed mainly by the central and peripheral neurons (Burmester and Hankeln, 2009) – which can protect the cells from the reactive effects of the oxygen (Milton et al., 2006).

The estimated number of cholinergic neurons (NADPH-d$^-$) had no numeric change with the inoculation of the parasite and remained as the predominant subpopulation in relation to the nitrergic neurons, as described for rodents (Sang and Young, 1996). Cholinergic neurons are considered vulnerable to neuronal death (Cowen et al., 2000), nevertheless, the results of this experiment showed no changes in the population density with the inoculation with *T. gondii*.

The morphometric analysis of the NADPH-d$^+$ neurons showed atrophy for both the G30 and G90 when compared to the GC, however, the comparison of the neurons in the G30 with those in the G90 showed hypertrophy in the latter, evidencing that a recovery of the cellular area was happening. Similarly to what was observed for the total population, in this subpopulation the area of the body cell and cytoplasm showed high degree of correlation, suggesting that the morphometric changes found resulted

mainly from the changes in the cytoplasm of those cells (r=95, p<0.001). At 30 days of inoculation, a tendency to increase the incidence of smaller neurons and the decrease of the larger neurons, what was not inverted at 90 days of infection, was observed.

Changes in the myenteric neurons may result from either the direct or indirect action of the parasite. The atrophy of the nitrergic neurons is believed to have been caused by the action of the immune system in response to the presence of the parasite, causing the increase of NO produced by the iNOS (Brunet et al., 2001; Chandrasekharan and Shinivasan, 2007). NO is a cytotoxic molecule which inhibits the mitochondrial and nuclear essential enzymes. It is triggered together with cytokines and chemokines during the invasion by *T. gondii* (Kasper et al., 1994). Excessive amounts of NO are cytotoxic not only for the parasite but also to the host cells (Brunet et al., 2001). Compromising of the neuronal cytoplasmic enzymes may have occurred in the initial stage of inoculation in this study, resulting in neurons metabolically less active and consequently with a decreased cellular area.

At 30 days of inoculation, we observed that atrophy of the myenteric neurons had occurred with both techniques, indicating that the parasite may have influenced its metabolism. Nonetheless, an inverse condition occurred at 90 days of inoculation with a tendency to recover the area of those neurons. Such alterations might have occurred in response to the inflammatory process usually caused by the infection with *T. gondii*, as the parasite triggers a cascade of immunological reactions with great release of chemical mediators and cytokines (Buzoni-Gatel and Werts, 2006), and, therefore, that immune response may have caused the morphometric changes observed in this study. As the formation of cysts occurs, and therefore the chronification of the infection (Howe and

Sibley, 1995), there is the reestablishment of the immune system, what favors the continuity of the parasitism and may be related to the changes throughout the infection in this study. That is, at 90 days of infection, there should have been an unbalance in the parasite-host relation which had been showed by the normalization of the body weight and the neuronal area. This may have corroborated by the fact that, initially, the NO presents harmful effects, on the other hand, as it established the chronification of the infection, the production of NO becomes essential for the survival of the host (Brunet et al., 2001).

Another way of the parasite to act is through direct parasitism of the myenteric neurons as the cells infected with *T. gondii* suffer physiological and morphological changes. Such changes occur as a response to the interaction between the parasite and the host cell. *T. gondii* makes the host cells resistant to apoptosis inducers through its capacity to manipulate the responses of the host cells. It is possibly a strategy of the parasite to keep the cellular viability of the host (Weiss and Kim, 2007), what may have contributed to the maintenance of the neuronal density observed in this study.

We concluded that the infection with oocysts from a genotype III strain of *Toxoplasma gondii* in rats neither did cause neuronal loss nor changed the area of the jejunum of rats; however, it caused atrophy in neurons from the nitrergic population and subpopulation after 30 days of inoculation and that these neurons tend to hypertrophy after 90 days.

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Table 1. Mean ± Standard deviation of body weight, length and width, and the area of the jejunum of rats from the CG and the groups inoculated with a genotype III strain of *Toxoplasma gondii* for 30 (G30) and 90 (G90) days.

Group	Body weight (g)	Length (cm)	Width (cm)	Jejunum area (cm^2)
CG	$309.2^{a\,c}$ ± 24.1	102.0 ± 7.5	1.2 ± 0.2	122.2 ± 22.8
G30	456.9^{b} ± 58.0	105.5 ± 9.1	1.0 ± 0.1	111.4 ± 20.5
G90	352.4^{c} ± 35.4	106.6 ± 6.2	1.1 ± 0.1	117.1 ± 7.6

Values denoted by different letters in the same column are significantly different ($p< 0.05$).

Table 2. Mean ± Standard deviation of the population density of neurons stained with Giemsa, positive NADPH-d neurons and the estimated number of negative NADPH-d neurons in 16.8mm^2, and projection of the number of neurons in the total area of the jejunum of rats from the CG and the groups inoculated with a genotype III strain of *Toxoplasma gondii* for 30 (G30) and 90 (G90) days.

		CG	G30	G90
GIEMSA	In 16.8mm^2	3,494.8 ± 308.3	3,858.2 ± 283.7	3,156.8 ± 905.1
	Projection in the total area	2,971.847.9 ± 929,781.0	2,541.749.9 ± 411,001.4	2,217,118.7 ± 659,885.4
NADPH-d$^+$	In 16.8mm^2	1,007.2 ± 262.6	1,146.0 ± 189.2	1,163.2 ± 193.2
	Projection in the total area	851,464.4 ± 309,868.1	748,841.8 ± 122,849.3	796,996.0 ± 133,375.1
NADPH-d$^-$	In 16.8mm^2	2,487.6 ± 248.6	2,712.2 ± 95.7	1,993.5 ± 1098.8
	Projection in the total area	2,120,385.3 ± 703,632.9	1,792,908.1 ± 308,690.3	1,420,122.7 ± 769,753.7

Table 3. Median and percentiles (P25; P75) of the areas of the body cell, nucleus, cytoplasm and the ratio between the area of the nucleus and the body cell of neurons stained with Giemsa and NADPH-d^{+} in the jejunum of rats from the CG and the groups inoculated with a genotype III strain of *Toxoplasma gondii* for 30 (G30) and 90 (G90) days.

	GIEMSA			NADPH-d^{+}		
	CG	G30	G90	CG	G30	G90
Body cell area (μm^2)	144.0^{a} (109.0; 187.0)	129.0^{b} (102.0; 172.0)	154.0^{c} (108.0; 207.0)	200.5^{a} (164.6; 247.5)	162.4^{b} (135.1; 202.0)	176.3^{c} (143.8; 216.6)
Nucleus area (μm^2)	38.0^{a} (27.0; 51.0)	34.0^{b} (25.0; 47.0)	43.0^{c} (30.0; 57.0)	58.5^{a} (46.8; 71.6)	50.9^{b} (39.8; 62.9)	53.4^{c} (42.1; 66.0)
Cytoplasm area (μm^2)	104.0^{a} (77.7; 137.0)	97.0^{b} (72.0; 128.0)	109.0^{c} (74.0; 152.0)	142.1^{a} (112.6; 179.1)	112.2^{b} (89.3; 144.5)	121.8^{c} (98.5; 154.8)
Nucleus/Body Cell ratio	0.27^{a} (0.22; 0.33)	0.26^{a} (0.1; 0.32)	0.28^{b} (0.22; 0.34)	0.29^{a} (0.24; 0.34)	0.30^{b} (0.26; 0.36)	0.30^{c} (0.25; 0.35)

Values denoted by different letters in the same column are significantly different ($p < 0.05$).

Table 4 –Degree of correlation among the area of the body cell, cytoplasm and nucleus of the myenteric neurons stained with Giemsa and NADPH-d^{+} in the jejunum of rats from the CG and the groups inoculated with a genotype III strain of *Toxoplasma gondii* for 30 (G30) and 90 (G90) days.

	GIEMSA			NADPH-d^{+}		
Area	CG	G30	G90	CG	G30	G90
Body Cell x Nucleus	0.75	0.69	0.79	0.69	0.72	0.68
Body Cell x Cytoplasm	0.96	0.96	0.97	0.96	0.95	0.95
Nucleus x Cytoplasm	0.54	0.48	0.63	0.48	0.48	0.43

All the values are significant, with $p < 0.0001$.

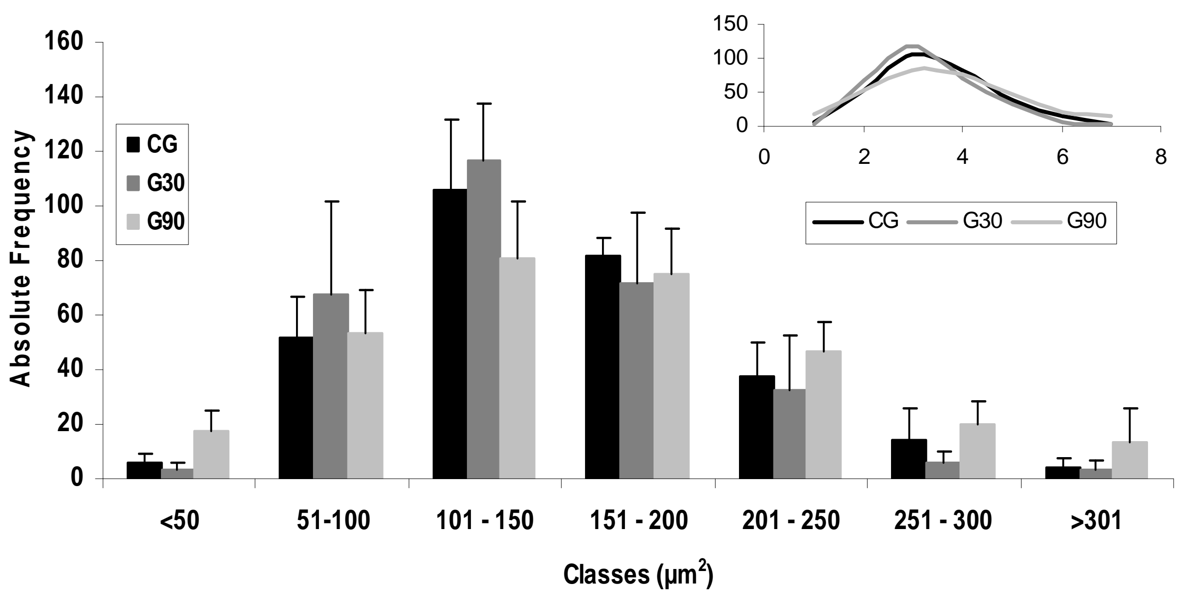

Fig. 1 – Distribution of the Giemsa-stained myenteric neurons in the jejunum of rats from the CG and the groups inoculated with a genotype III strain of *Toxoplasma gondii* for 30 (G30) and 90 (G90) days. There is no significant difference between the groups for any classes.

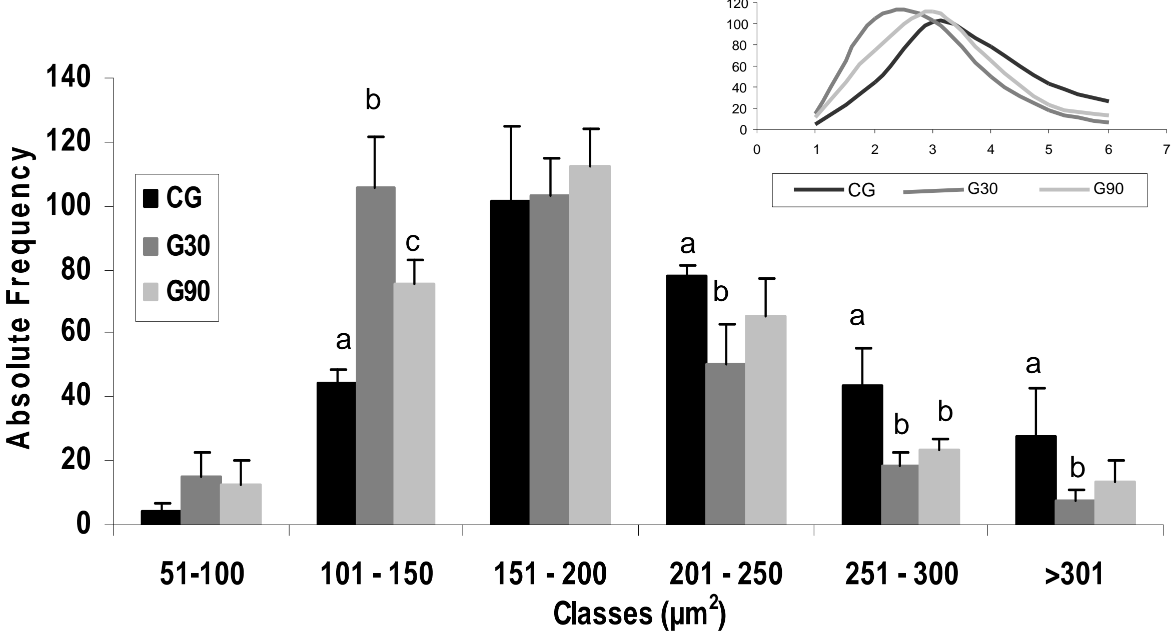

Fig. 2 – Distribution of the NADPH-d-stained myenteric neurons in the jejunum of rats from the CG and the groups inoculated with a genotype III strain of *Toxoplasma gondii*

for 30 (G30) and 90 (G90) days. Columns denoted with different letters differ significantly ($p < 0.05$).

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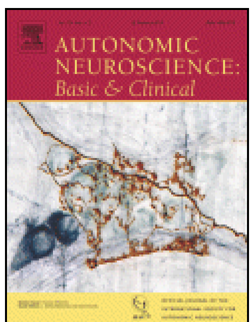

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