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Oral dependent-dose toxoplasmic infection model induced by oocysts in rats: Myenteric plexus and jejunal wall changes

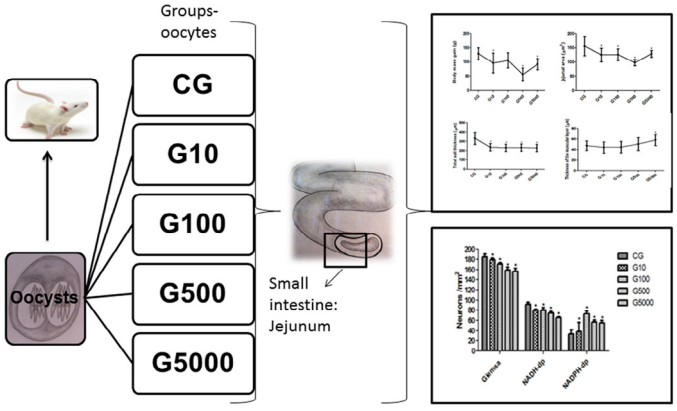
Suellen Laís Vicentino-Vieira [a], Gessilda de Alcantara Nogueira de Melo [a], Marcelo Biondaro Góis [a], Neide Martins Moreira [a], Luana Gabriela de Araujo Pereira [a], Eduardo José de Almeida Araújo [b], João Luiz Garcia [b], Débora de Mello Gonçales Sant'Ana [a,*]

[a] State University of Maringa, Av. Colombo, 5.790, 87020-900 Maringá, PR, Brazil
[b] State University of Londrina, Rodovia Celso Garcia Cid, PR 445, km 380, 86057-970 Londrina, PR, Brazil

HIGHLIGHTS

- We analyzed the jejunum of rats infected with different doses of *T. gondii*.
- The tested dose was from 10 to 5000 oocysts orally.
- NADHd-p and NADPHd-p neuronal atrophy was observed.
- Morphological and morphometric alterations were found in the infected groups.
- Hypertrophy of the muscle was observed in the group infected with 5000 oocysts.

GRAPHICAL ABSTRACT



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ABSTRACT

Toxoplasmosis is a widely distributed disease caused by the protozoan *Toxoplasma gondii* that is mainly transmitted orally. Once ingested, the parasite crosses the intestinal barrier to reach the blood and lymph systems to migrate to other regions of the host. The objective of this study was to evaluate the changes in the myenteric plexus and the jejunal wall of Wistar rats caused by oral infection with *T. gondii* oocysts (ME-49 strain). Inocula of 10, 100, 500 and 5000 oocysts were used. The total population of myenteric neurons and the most metabolically active subpopulation (NADH-diaphorase positive – NADH-dp) exhibited a decrease proportional to the dose of *T. gondii*. There was also a quantitative increase in the subpopulation of NADPH-diaphorase-positive (NADPH-dp) myenteric neurons, indicating greater expression of the NOS enzyme. Neuronal atrophy was observed, and morphological and morphometric alterations such as jejunal atrophy were found in the infected groups. Hypertrophy of the external muscle with the presence of inflammatory foci was observed in the group infected with 5000 oocysts. The changes observed in the infected groups were proportional to the number of oocysts inoculated.

* Corresponding author. Fax: +55 44 3011 4689.
E-mail address: dmgsantana@gmail.com.br (D. de Mello Gonçales Sant'Ana).

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1. Introduction

Toxoplasmosis is a widely distributed disease caused by *Toxoplasma gondii*, an obligate intracellular parasite in the phylum Apicomplexa, family Sarcocystidae (Grigg and Sundar, 2009; Kim and Weiss, 2004; Weiss and Kim, 2011). *T. gondii* has a heteroxenic cycle using felids as its definitive hosts and humans and other homeothermic animals as intermediate hosts (Dubey and Jones, 2008; Hill and Dubey, 2012). Intermediate hosts are mainly infected orally via ingestion of uncooked meat containing tissue cysts or oocysts present in contaminated water or food (Dubey and Beattie, 1988; Dubey and Jones, 2008; Weiss and Kim, 2011).

During chronic infection, *T. gondii* tissue cysts predominate in nervous and muscle tissues (Hill and Dubey, 2012). However, the parasite must cross the intestinal barrier to reach these tissues. During this invasion, the interaction between the host and the parasite can result in intestinal disorders that culminate in diarrhea and inflammation (Barragan and Hitziger, 2008).

Our research group was the first to show the effects of *T. gondii* infection on the enteric nervous system (ENS). Our previous studies have shown that the infection can cause changes in enteric neurons and in intestinal wall (da Silva Pde et al., 2010; Hermes-Uliana et al., 2011; Odorizzi et al., 2010; Sant'Ana et al., 2012; Shiraishi et al., 2009; Sugauara et al., 2008, 2009). These changes depend on the parasitic stage ingested, the duration of the infection, the host species and the part of the gastrointestinal tract evaluated. We hypothesize that the tissue response is dependent-dose to the parasite in chronic infections. Thus, this study is the first to show that there is a direct relationship between parasitic dose and alterations in the myenteric neurons and jejunal wall.

2. Materials and methods

2.1. Experimental design

The experimental protocol was conducted in accordance with international standards of ethical conduct in experiments and was previously approved by the Committee on Ethical Conduct in the Use of Experimental Animals (Comitê de Conduta Ética no Uso de Animais em Experimentação – CEAE) of the State University of Maringá (Universidade Estadual de Maringá – UEM) (document number 081/2012).

Male Wistar rats (*Rattus norvegicus*) (233.61 ± 5.26 g) were randomly distributed into five groups of seven animals each ($p > 0.05$). The work follows a fully randomized experiment design.

Blood samples were collected by puncture of the retro-orbital plexus before and after inoculation of *T. gondii* oocysts to check for the presence of serum anti-*T. gondii* IgG antibodies using the direct agglutination test, with samples with titers above 25 considered positive (Desmonts and Remington, 1980).

2.2. Experimental infection

The control group (CG) received sterile 0.9% NaCl aqueous solution, and the infected groups received a solution containing 10 (G10), 100 (G100), 500 (G500) or 5000 (G5000) sporulated oocysts via gavage. The animals were maintained in individual cages in a maintenance room with controlled temperature and humidity, a light–dark cycle of 12 h and food and water *ad libitum*. The ME-49 strain oocysts (genotype II) used in this study was obtained from the Laboratory of Veterinary Parasitology of the Londrina State University (Universidade Estadual de Londrina – UEL). These oocysts were originally isolated from sheep muscle in the USA (Lunde and Jacobs, 1983).

2.3. Euthanasia and collection of biological material

After 30 days of infection, the animals were euthanized under deep anesthesia with halothane vapor (Tanohalo®, Cristália, Itapira, São Paulo, Brazil) (Vivas et al., 2007). Vertical laparotomy was performed, and the jejunum was removed and measured. The duodenojejunal flexure (proximal) and the ileocecal fold (distal) were used as anatomical references for measurement of the jejunum. The proximal jejunum was used for the analysis.

2.4. Analysis of the intestinal wall

A 2-cm ring of the jejunum was collected and subjected to routine histological examination. Semi-serial 4 μm transverse sections were cut and stained with hematoxylin and eosin (HE).

Morphometric analysis of the intestinal wall was performed from images captured by a digital camera (Pro series 3CCD camera) coupled to an optical microscope (Olympus BX50). Images captured with the 4× objective lens were used to measure the total thickness of the intestinal wall, and images captured with the 20× objective lens were used to measure the muscular tunic. Each histological section was divided into four quadrants, and each quadrant was measured along the circumference. Four sections from each animal in each group were analyzed, totaling 16 measurements per animal. The analyses were performed with the aid of the Image Pro Plus software (Media Cybernetics).

2.5. Analysis of the myenteric plexus

With the aid of a stereomicroscope with transillumination (Olympus Micronal SZ40) the jejunum was microdissected with the removal of the tunica mucosa and the tela submucosa. The whole mounts formed by the muscular and serous tunics were subjected to the following techniques: Giemsa stain (Barbosa, 1978), to show the total neuronal population; NADH-diaphorase (NADH-d) (Gabella, 1969), to mark the metabolically more active subpopulation; and NAPDH-diaphorase (NADPH-d) (Scherer-Singler et al., 1983) to show the subpopulation that produces nitric oxide.

2.5.1. Giemsa staining

A 5-cm jejunum segment was washed in NaCl solution (0.9%), filled and immersed in acetic formalin fixative solution for 48 h. The jejunum was cut into samples of roughly 1 cm length to prepare whole mounts of the myenteric plexus. The whole mounts were Giemsa stained using a protocol that is based on the visualization of neurons by methylene blue (Barbosa, 1978).

2.5.2. NADH-d histochemistry

Jejunum segments (5 cm) was washed with Krebs buffer (pH 7.3) and immersed for 5 minutes in 0.3% Triton X-100 (Sigma, St. Louis, MO, USA) in Krebs buffer. Afterwards, the jejunum segments were washed twice (10 minutes each wash) with Krebs solution and immersed for 45 min in a solution containing the following per 100 mL: 0.05 g of β-NADH (Sigma, St. Louis, MO, USA), 25 mL of nitroblue tetrazolium (NBT) stock solution (Sigma, St. Louis, MO, USA), 25 mL of 0.1 M phosphate buffer (pH 7.3) and 50 mL of distilled water (Gabella, 1969). The reaction was stopped by the addition of 10% buffered formalin.

2.5.3. NADPH-d histochemistry

Approximately 5 cm of the jejunum was washed with 0.1 M PBS (pH 7.3), immersed for 30 min in 4% buffered paraformaldehyde (Sigma, St. Louis, MO, USA), immersed for 10 min in 0.3% Triton X-100 (Sigma, St. Louis, MO, USA) in 0.1 M PBS and washed 10 times (10 min each wash) with 0.01 M PBS solution. Subsequently, the sections were immersed for 2 h in a solution containing the following