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Chronic infection with *Toxoplasma gondii* induces death of submucosal enteric neurons and damage in the colonic mucosa of rats

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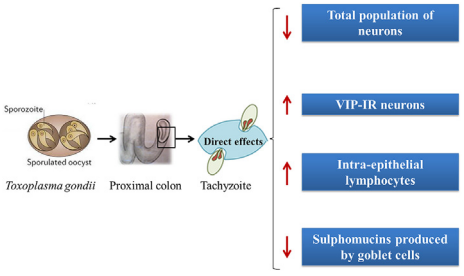
HIGHLIGHTS

- Effects of genotype II *Toxoplasma gondii* on neuronal populations of the submucosal plexus in the rat proximal colon are described.
- Findings contribute to the establishment of an experimental model of this infectious disease and inflammatory bowel disease.
- ME-49 *T. gondii* is very aggressive to submucosal neurons in rat proximal colon, causing uncommon death of enteric neurons.

GRAPHICAL ABSTRACT



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ABSTRACT

Intestinal epithelial secretion is coordinated by the submucosal plexus (SMP). Chemical mediators from SMP regulate the immunobiological response and direct actions against infectious agents. *Toxoplasma gondii* is a worldwide parasite that causes toxoplasmosis. This study aimed to determine the effects of chronic infection with *T. gondii* on the morphometry of the mucosa and the submucosal enteric neurons in the proximal colon of rats. Male adult rats were distributed into a control group (n = 10) and an infected group (n = 10). Infected rats received orally 500 oocysts of *T. gondii* (ME-49). After 36 days, the rats were euthanized and samples of the proximal colon were processed for histology to evaluate mucosal thickness in sections. Whole mounts were stained with methylene blue and subjected to immunohistochemistry to detect vasoactive intestinal polypeptide. The total number of submucosal neurons decreased by 16.20%. Vasoactive intestinal polypeptide-immunoreactive neurons increased by 26.95%. Intraepithelial lymphocytes increased by 62.86% and sulfomucin-producing goblet cells decreased by 22.87%. Crypt depth was greater by 43.02%. It was concluded that chronic infection with *T. gondii* induced death and hypertrophy in the remaining submucosal enteric neurons and damage to the colonic mucosa of rats.

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1. Introduction

Toxoplasmosis is one of the commonest zoonoses in humans as well as in animals and is broadly distributed geographically. It is estimated that one-third of the human population has been infected with *Toxoplasma gondii* (*T. gondii*), a protozoan from the *Apicomplexan phylum*, which causes this disease (Weiss and Kim, 2007; Dubey, 2010; Silva et al., 2010; Halliez and Buret, 2015). The intermediate hosts are infected mainly by the ingestion of oocysts present in the soil, water and foods (Speer and Dubey, 1998; Parlog et al., 2015). After ingestion, oocysts reach the gastrointestinal tract, where the epithelial cells of the intestinal mucosa have an important role in the mechanical barrier to aggressions from the external environment (Barragan and Sibley, 2003; Neunlist et al., 2013; Cohen and Denkers, 2014).

In the intestinal epithelium, intraepithelial lymphocytes (IELs) are in close contact with enterocytes and have an initial role in mucosal immunity against pathogens and other antigens because they are very close to the mucosal surface (Cohen and Denkers, 2014). Moreover, goblet cells are also found in this epithelium, and they are responsible for the production and release of mucins that form a film that lubricates and protects the intestinal epithelium (Deplancke and Gaskins, 2001; Gwynne et al., 2009). These cells and the composition of secreted mucins change in response to infection by microorganisms and parasites, including *T. gondii* (Moal and Servin, 2006; Shiraishi et al., 2009; Sant'Ana et al., 2012 Araújo et al., 2015; Halliez and Buret, 2015).

Our previous studies have shown that *T. gondii* infection induces several morphometric alterations in the intestinal wall and in myenteric neurons in different species such as cats (Da Silva et al., 2010), pigs (Silva et al., 2010; Odorizzi et al., 2010), chickens (Shiraishi et al., 2009; Bonapaz et al., 2010; Braga et al., 2011) and rats (Hermes-Uliana et al., 2011; Sant'Ana et al., 2012 Araújo et al., 2015; Vicentino-Vieira et al., 2015).

The enteric nervous system (ENS) is formed by two ganglionated plexuses: the myenteric (MP) and submucosal (SMP) plexi (Hoyle and Burnstock, 1989; Furness, 2006), which are made up of neurons and glial cells (Furness, 2006; Neunlist et al., 2013). MP controls intestinal motility, and SMP regulates blood flow and mucosal secretion (Hoyle and Burnstock, 1989; Sant'Ana et al., 2012; Vicentino-Vieira et al., 2015). The functions of SMP depend on some neuromediators, including vasoactive intestinal polypeptide (VIP), which induces chloride secretion (Cooke, 1998; Furness, 2006) and vasodilatation, modulates the release of mucins (Toumi et al., 2004), and controls the proliferation of goblet cells, enterocytes and lymphocytes in the lamina propria and submucosa (Balemba et al., 1998; Weber et al., 2001; Sandgren et al., 2003; Kim and Ho, 2010; Halliez and Buret, 2015).

VIPergic neurons represent 45% of the submucosal neuronal population in the small intestine of guinea pigs (Furness, 2006). However, there are few studies that evaluate the alterations of the mucosa and submucosal neurons considering quantitative parameters related to goblet cells, enterocytes and IELs in chronic infections caused by *T. gondii* oocysts. In addition, the existing studies are restricted to the small intestine (Sant'Ana et al., 2012).

In view of the interest in understanding the damage caused by this parasite and the local response to *T. gondii* infection, our study aimed to evaluate the damage occurring in the mucosa and submucosal neurons of the colon during the chronic phase of infection. We also tried to determine the adaptive and morphological defensive responses as well as the quantitative ones found in the total population of neurons and the VIPergic subpopulation in the integration with IELs, goblet cells and enterocytes.

2. Material and methods

The experimental protocol was approved by the Ethics Committee in Research Involving Animal Experimentation (CEPEEA) of the Universidade Paranaense (Protocol No. 12361/08).

2.1. Experimental design

Twenty 60-day-old male Wistar rats (*Rattus norvegicus*), weighing 258.58 ± 13.64 g, were used. The animals were randomly distributed into a control group (CG, n = 10) and infected group (IG), (n = 10), kept for 36 days and provided with commercial chow for rodents and water *ad libitum*.

Cats (*Felis catus*) were inoculated orally with *T. gondii* tissue cysts (strain ME-49 genotype II) isolated from infected mice (*Mus musculus*) to obtain oocysts (Dubey, 1995). The oocysts were concentrated using Sheather's method and allowed to sporulate in 2% sulfuric acid (Sloss et al., 1999). Each IG rat received orally 500 sporulated *T. gondii* oocysts resuspended in 1 mL of sterile saline, whereas CG animals received only sterile saline.

2.2. Sample collection

At 36 days post-infection, the rats were euthanized in a chamber saturated with halothane (Vivas et al., 2007). Necropsy was immediately performed, and the proximal colon was removed and subjected to various procedures.

2.3. Confirmation of infection with T. gondii

Serological testing - Blood from all animals was collected at the beginning and end of the experiment by retro–orbital puncture. The serum was subjected to the modified agglutination test (Desmonts and Remington, 1980) to verify the presence of serum anti-*T. gondii* antibodies. Sera were considered positive when titers were greater than or equal to 25.

2.4. Analysis of IELs and goblet cells

Histological processing - A 1-cm ring from the proximal colon of each rat from each group was collected and subjected to routine histological processing (paraffin embedding). Semi-serial transverse sections stained with hematoxylin and eosin (H&E) were used for morphometric analysis of the mucosa and quantification of IELs. Periodic acid shiff (PAS) plus diastasis solution was used to detect neutral mucins and labile sialomucins; alcian-blue (AB) at pH 2.5 was utilized to detect sialomucins and sulfomucins; and AB at pH 1.0 was for detecting sulfomucins (Myers et al., 2008). Images were obtained and analyzed using a digital camera (Moticam 2000, 2.0 Megapixel) coupled to a trinocular light microscope, equipped with an image analysis system (Motic Image Plus, version 2.0).

Counting id IELs and goblet cells - In each specimen, 2500 consecutive IELs and enterocytes were counted aiming to calculate the proportion of IELs/100 epithelial cell. This procedure allowed determining the number of goblet cells/100 epithelial cells. For this, we used sections stained by PAS, AB pH 2.5 and AB pH 1.0 (Sant'Ana et al., 2012).

2.5. Histochemical Giemsa technique

A 2-cm proximal colon segment was first washed in saline. The segment was then filled and immersed in acetic formalin fixative solution for 48 h. The proximal colon was cut into samples of one cm length to make whole mounts consisting of the submucosa layer. For that, the mucosa was removed by gentle scraping, and the