Characterization of enteropathy in mice infected with *Giardia duodenalis* and treated with differing anti-parasite drugs

**Resumo**

Nosso objetivo foi analisar a enteropatia do duodeno de camundongos infectados com *Giardia duodenalis* (assembleia A) e submetê-los a quatro tipos diferentes de tratamento parasitário. **Métodos:** Quarenta e oito camundongos Swiss machos, com 21 dias de idade, receberam 1000 cistos de parasitas por via oral. Os animais foram divididos em seis grupos: controle negativo (G1), controle positivo (G2), e grupos tratados com nitazoxanide (G3), albendazole (G4), metronidazole (G5), e secnidazole (G6). Após tratamento, os animais foram sacrificados e o duodeno foi examinado histopatologicamente. **Resultados:** Na entrada do grupo controle positivo (G2), observou-se um aumento de células goblet neutra e ácida, linfócitos intraepiteliais e células de Paneth, além de uma redução da altura dos vilos e aumento da largura; e criptas apresentaram aumentos de larguras e profundidades. Hiperтроfia em todas as camadas da parede intestinal e entérocytes foi observado. **Conclusões:** Metronidazole foi o mais eficaz em atenuar a enteropatia causada pela *G. duodenalis*, revertendo os aumentos de células linfócitas e células de Paneth, e atenuando os outros elementos investigados.

**Key words:** *Giardia* spp. *Giardiasis*. Parasitosis. Duodenum. Antiparasitic.
(G2) e tratamento com nitazoxanida (G3), albendazol (G4), metronidazol (G5) e secnidazol (G6). Eles foram então sacrificados e o duodeno foi examinado histopatologicamente. Resultados: No grupo G2, foram encontrados níveis aumentados de células caliciformes neutras e ácidas, linfócitos intra-epiteliais e células de Paneth. Houve redução da altura das vilosidades e aumento da largura das vilosidades; as criptas aumentaram em largura e profundidade. Hipertrofia em todas as camadas da parede intestinal e enterócitos também foi observada. Conclusões: O metronidazol foi mais efetivo na atenuação da enteropatia causada pela infecção por G. duodenalis, pois reverteu o aumento dos linfócitos intraepiteliais e das células de Paneth e atenuou os demais elementos investigados.


Introduction

According to the World Health Organization, *Giardia duodenalis* is one of the most common intestinal parasites in humans, with an estimated 400 million infected individuals and prevalence rates ranging from 0.9% to 42.9% in humans, and from 3.0% to 64.3% in animals (Feng & Xiao, 2011).

From a comprehensive description of giardiasis in Brazil in the last 20 years, one may observe that the disease remains neglected, with water sources being the main vehicles of contamination. Detection of new cases is restricted to the south and southeast regions of the country (Coelho et al., 2017).

In developed countries, Giardia causes often causes outbreaks of diarrhea associated with contaminated drinking water. In developing countries, it is a common cause of infectious diarrhea, especially in children, and it may impair development. Giardia is a neglected disease and is thus closely related to poverty, lack of basic sanitation, and poor water quality (Savioli, Smith, & Thompson, 2006; Karanis, Kourenti, & Smith, 2007).

Giardiasis is self-limiting and characterized symptomatically by diarrhea, abdominal cramps and bloating, weight loss, and mal-absorption. However, the asymptomatic form of the disease occurs frequently, especially in developing countries (Hellard, Sinclair, Hogg, & Fairley, 2000; Pupulin, Gomes, Dias, & Araujo, 2004; Eligio, Cortes, & Jimenes, 2008). Clinical changes, the result of enteropathy are characterized by reduced villi height and greater width (Muller & Allmen, 2005), increased crypt depth (Bartelt et al., 2013), increased mucin secretion by goblet cells (Shukla, Sidhu, & Verma, 2012; Ventura et al., 2013), greater numbers of Paneth cells (Eckmann, 2003; Tako, Hassimi, Li, & Singer, 2013), changes in intestinal epithelium renewal with changes in enterocyte morphometry (Linda et al., 2008), and an increased number of intraepithelial lymphocytes (IEL) (Arevalo et al., 2010) responding to the infection (Mohammad & Koshak, 2011).

Treatment for giardiasis involves chemotherapy with either a single drug or a combination of drugs, being principally 5-nitroimidazole derivatives such as metronidazole (Rey, 2011; Neiva, et al., 2014). The drugs most widely used for treating giardiasis in Brazil whether by prescription or self-medication are metronidazole, tinidazole, and nitazoxanide (Venturini, Engrof, Ely, Tasca, & Cart, 2014). However, in the literature, the effectiveness of treatment is a source of controversy (Upcroft & Upcroft, 2001; Arguello, Cruz, Romero, & Ortega, 2004; Rossignol, 2010; Bailey et al., 2013; Gartner & Hiatt, 2007; Pérez et al., 2013; Shukla, Klar, & Sharma, 2013), and although analysis is important for understanding the remission of symptoms, no studies have investigated intestinal regeneration or restoration of intestine homeostasis after anti-Giardia drug therapy. This study uses induced Giardia enteropathy to analyze the activities of the principal antiparasitic drugs used in treating giardiasis.
Methods

The experimental protocol was approved by the Ethics Committee on Animal Experiments of the Centro Universitário Integrado, Paraná, under approval number 369.

Forty-eight male Swiss mice, 21 days of age, weighing about 20 g, from the Central Animal Laboratory of Maringá State University were used in this study. The animals were kept in the Animal Care Unit at the Centro Universitário Integrado, in polypropylene boxes (414 × 344 × 168 mm), capped with a galvanized grid, with a feed container, a bottle of mineral water, and a meshed cage floor to avoid contact between the animals and their feces (a possible source of reinfection during the experiment). The boxes were kept in an air-conditioned facility (temperature between 21°C and 23°C), with light/dark cycles of 12 h. After a week of adaptation at the animal facility, we began the experiment.

To confirm the absence of parasitic contamination before the experimental infection, all mice were submitted to fecal parasite examination in accordance with Faust et al. (1938).

Obtaining the inoculum

The Giardia duodenalis cysts used in this study were obtained from stool samples of patients in treatment at the Environmental and Food Parasitology Laboratory of the State University of Maringá (PLEF/UEM). The samples were processed using a spontaneous sedimentation method in water, together with a modified centrifugal sedimentation method in which formaldehyde was replaced by distilled water. A drop of the obtained sediment was analyzed under an optical microscope using 10× and 40× objective lenses. DNA was extracted from 100 μL of the sediment, and the remainder of the sediment was used for the inoculum to be administered to the animals.

DNA was extracted using a PureLink PCR Purification Kit (Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer’s instructions. A 432 bp fragment (approximately) of the GDH gene was amplified in a semi-nested PCR reaction, with modifications, using the primers GDHeF (TCA ACG TYA AYC GYG GYT TCC GT), GDHiF (CAG TAC AAC TCY GCT CTC GG), and GDHiR (GTT RTC CTT GCA CAT CTC C) (Read, Monis, & Thompson, 2004). Each amplification reaction was performed in a final volume of 11 μL, containing 10× buffer (200 mM Tris-HCl [pH 8.4] - 500 mM KCl), 1.5 mM MgCl₂, 0.5 U of Taq DNA polymerase platinum (Invitrogen, USA), 200 μM deoxyribonucleotide triphosphates (dNTPs), 2 pmol of each primer (GDHeF and GDHiR in the first reaction, with GDHiF and GDHiR in the second reaction), sterile H₂O (Milli-Q), and 2 μL of total DNA. The following reaction conditions were used to amplify the GDH gene: initial denaturation at 94°C for 2 min; 35 cycles at 94°C for 45 s, 55°C for 30 s, 72°C for 45 s, and 72°C for 5 min, for both reactions.

The products amplified from the GDH gene were digested using the enzymes Nla IV and Rsa I. The Nla IV enzyme differentiates G. duodenalis assemblages A1, A2, B, C, D, and E while the RSA I enzyme differentiate assemblages B3 and B4. Parasites from assemblage A2 were used in this study.

The restriction fragments were viewed on 5% polyacrylamide gel stained with silver. The isolated G. duodenalis cysts were quantified in a Neubauer chamber. We counted cysts present in the four side quadrants of the chamber and multiplied the number of cysts by the corresponding sample dilution correction factor.

The experimental and treatment groups

The mice were randomly divided into six groups of eight animals each. In a single dose by gavage, the negative control animals in group G1
were fed 0.5 mL of 0.85% NaCl. The animals in the other groups were infected orally with 1000 G. duodenalis assemblage A2 cysts. The infected G3–G6 groups were treated with commercially available antiparasitic drugs at the dosages established in the literature: nitazoxanide (G3), 10 mg/kg twice a day for 3 days (Pavanelli, Garcia, & Colli, 2016); albendazole (G4), 4 mg/day for 5 days (Reynoldson, Thompson, & Meloni, 1991); metronidazole (G5), 500 mg/kg, three times a day for 7 days (Reynoldson et al., 1991); secnidazole (G6), 200 mg/kg, in a single dose (Cruz, Ferrari, & Sogayar, 1997). The animals in group G2 (positive control) also received 0.5 mL of 0.85% NaCl by gavage.

Confirmation and monitoring of infection

One week after infection, the animals were placed in individual cages for 2 h for feces collection; parasitological examination was done using the Faust et al. (1938) technique. To confirm the infection, the procedure was performed on all animals on alternating days. To confirm the assemblage used, samples of the animals’ fecal material were analyzed using the molecular biology techniques described above.

Specimen collection

At 45 days post infection (dpi), the animals were sacrificed in deep anesthesia using halothane vapor (Vivas, Jamel, & Refinetti, 2007). After vertical laparotomy, the duodena were removed and processed for histological analysis. The distal anatomic limit for the specimens was the duodenojejunal flexure.

Histologic processing

Sections of proximal duodena from all animals, measuring 2 cm, were collected and fixed in Bouin solution for 6 h. They were embedded in paraffin to prepare semi-serial 4-μm sections, which were cut using a microtome, and then mounted on glass slides. The sections underwent de-paraffinization and serial hydration, and were then stained with hematoxylin and eosin to quantify IELs, and Paneth cells, and to investigate the morphometry of the villi, crypts, enterocytes, submucosa, muscle layer, and intestinal wall. We also prepared slides with periodic acid-Schiff (PAS) staining to detect neutral mucins and labile sialomucins (PAS+). Alcian blue (pH 2.5; AB 2.5+) was used to detect sialomucins and sulfomucins. And Alcian blue (pH 1.0, AB 1.0+) was used to detect sulfomucins. All analysis was performed with the examiner blind to the type of treatment.

Duodenal epithelium analysis

Morphometric analysis of enterocytes

The width and height of 50 enterocytes randomly present in the villi and crypts of each animal were measured together with their smallest and largest nuclei diameters. To perform this, we captured four images from each section using a 40× digital camera (Pro series 3CCD camera) coupled to an optical microscope (Olympus BX50). The widths and heights of the enterocytes and their nuclei were measured using image analysis software (Image Pro Plus, Media Cybernetics).

Counting of goblet cells and intraepithelial lymphocytes

Four sections were stained and 2500 cells from the mucosa of each animal were counted for each staining technique (PAS, AB 1.0, and AB 2.5). The left half of the villus was chosen for analysis using an optical microscope (Olympus BX50) with a 40× objective lens. For each technique, we quantified the number of goblet cells present and calculated the goblet to epithelial cell (100) ratio. The procedure was repeated for IELs.
Paneth cell count

Each section was divided into four quadrants. We counted Paneth cells present at the base of four crypts of the four quadrants, totaling 64 crypts per animal.

Morphometric analysis of the intestinal wall

Images taken using a 4× objective were used to measure the villi width and height, and crypt depth and breadth at their midpoints. Images taken with a 10× objective were used to measure the submucosa and muscle layer. Images taken with a 4× objective were also used to measure the total intestinal thickness, using the distance from the tunica serosa to the base of the villi. Four images of each of the four sections were obtained. Sixteen measurements were obtained per animal for each of the intestinal wall parameters. These were evaluated using Image Pro Plus software.

Statistical analysis

Statistical analysis was performed using Bioestat 5.3 software. To verify the distribution of the data, the results were tested for D’Agostino-Pearson normality. All of the data were found to be normally distributed and are presented as the mean ± standard deviation. Analysis of variance (ANOVA) testing was also used, followed by the Student’s t test. The significance level was set at 5%.

Results

As compared with the control animals in G1, the size of the enterocytes and of their nuclei increased in the infected and treated animals (p<0.05) (Table 1).

Significantly increased numbers of goblet cells (using the PAS, AB 1.0, and AB 2.5 stain techniques) were observed in all groups, as compared to G1, with the smallest increase observed for G5. In quantification of IELs (except for G5) and Paneth cells, (p<0.05) (Table 1, Figures 1 and 2), the results were equivalent.

Table 1

Goblet cells and intraepithelial lymphocytes/100 epithelial cells, Paneth cells and morphometry of the duodena of Swiss mice infected by G. duodenalis and treated with antiparasitic drugs

<table>
<thead>
<tr>
<th>GROUPS</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
<th>G5</th>
<th>G6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goblet Cells/100 Epithelial cells</td>
<td>Negative Control</td>
<td>Positive Control</td>
<td>Nitazoxanide</td>
<td>Albendazole</td>
<td>Metronidazole</td>
<td>Secnidazole</td>
</tr>
<tr>
<td>PAS+</td>
<td>7.04 ± 0.36</td>
<td>9.05 ± 0.79</td>
<td>8.54 ± 1.08</td>
<td>8.42 ± 1.07</td>
<td>8.36 ± 0.71</td>
<td>8.39 ± 0.79</td>
</tr>
<tr>
<td>AB 1.0+</td>
<td>0.80 ± 0.38</td>
<td>3.41 ± 0.38</td>
<td>0.80 ± 0.38</td>
<td>2.70 ± 0.39</td>
<td>1.22 ± 0.19</td>
<td>2.38 ± 0.33</td>
</tr>
<tr>
<td>AB 2.5+</td>
<td>1.16 ± 0.42</td>
<td>3.08 ± 0.46</td>
<td>2.85 ± 0.56</td>
<td>2.49 ± 0.45</td>
<td>1.89 ± 0.27</td>
<td>2.34 ± 0.42</td>
</tr>
<tr>
<td>IEL</td>
<td>2.14 ± 0.47</td>
<td>5.56 ± 0.53</td>
<td>3.21 ± 0.53</td>
<td>4.27 ± 0.61</td>
<td>2.73 ± 0.76</td>
<td>3.19 ± 0.77</td>
</tr>
<tr>
<td>PANETH</td>
<td>48 ± 6.43</td>
<td>82.14 ± 10.14</td>
<td>71.71 ± 5.15</td>
<td>81.25 ± 15.45</td>
<td>66 ± 10.14</td>
<td>76.75 ± 15.35</td>
</tr>
</tbody>
</table>

Mean ± standard deviation followed by equal letters in the same column present significant difference (p <0.05). Analysis of Variance ANOVA, t test. PAS + (Periodic Acid Shiff) AB 1.0+ and AB 2.5+ (Alcian-Blue).
Figure 1. Micrograph of duodenal sections from mice in groups G1, G2, G3, G4, G5, and G6, stained using HE. The arrows show Paneth cells. Increased numbers of these cells can be seen in the infected groups, by different treatment. 400x magnification; bar: 20 µm.
Figure 2. Micrograph of duodenal sections from mice in groups G1, G2, G3, G4, G5, and G6, stained using HE. The arrows show intraepithelial lymphocytes. Increased numbers of these cells can be seen in the infected group, by different treatment. 400x magnification; bar: 20 µm.

The villi of all groups presented reduced heights and increased widths as compared to G1 (p<0.05). The crypts presented increased depths and widths in all groups as compared with G1 (p<0.05). And G5 presented the smallest changes in both villi and crypts (p<0.05) (Figure 3).
Figure 3. (A) Villus height and crypt depth, and (B) villus width and crypt width, in the duodena of mice in groups G1, G2, G3, G4, G5, and G6, stained using HE. The statistical difference from G1 is represented by * (p<0.05).

The intestinal wall morphometry revealed hypertrophy in all groups as compared to G1 (p<0.05) (Table 1, Figure 4).
Discussion

This study demonstrates that in infections caused by *G. duodenalis*, morphologic changes to the intestinal and epithelial cell lining of the duodena of Swiss mice may well occur. Various changes were observed in the epithelium such as hypertrophy of enterocytes and of their nuclei. This may be indicative of apoptosis, in which the cell and its respective nuclei increase in size, as has already been described (Linda et al., 2008; Alberts et al., 2008). Similar hypertrophy has been related in the duodena of mice and their offspring infected with *G. duodenalis* in previous studies (Linda et al., 2008; Troeger et al., 2013). In the present study, we demonstrate that the principal antiparasitic drugs do not prevent morphometric changes, and thus,
alterations in function may affect the entire duodenal epithelium. Enterocytes, the most numerous cells in this epithelium, present both absorptive and digestive functions, and any changes found in them may indicate impaired organ function as a whole (Gartner & Hiatt, 2007).

The greater numbers of goblet cells found in all of the infected groups, using three distinct techniques, reflect a demand for more mucin secretion, possibly an attempt to form a thicker mucus layer to protect the epithelium against the infection (whether parasitic or secondary) (Myers, 2008). Goblet cells are responsible for producing the mucin precursor of intestinal mucus which has the function of protecting the epithelium against chemical, physical, and biological attacks. Of the mucins secreted by goblet cells, neutral mucin (PAS+) presents higher density, whereas acidic mucins (AB+) confer fluidity to the mucus (Beamish, 1972; Zieske & Bernstein, 1982). This increased number of goblet cells has been found in dogs, rats, mice, and gerbils, and also in children infected with *G. duodenalis*; protects the intestinal epithelium against invasion by preventing parasitic attachment and/or expelling the parasite (Shukla et al., 2012; Ponce-Macotela, Gonzalez, Reunoso, & Martinez, 2008; Mokrzycka, Kolasa, Kosierkiewicz, & Wiszniewska, 2010; Hensel et al., 2014). In the infected animals there was a 28% increase in PAS+ goblet cells. The antiparasitic treatments did not correct this epithelial modification, although metronidazole induced a smaller increase of 18% (p>0.05) as compared to the other drugs. The greater numbers of cells producing acid mucins (i.e. by 326% according to AB 1.0, and 165% according to AB 2.5) in the untreated infected animals is noteworthy. Metronidazole did not correct the increase in the number of cells, but promoted smaller increases of 52% (AB 1.0) and 62% (AB 2.5), being the most efficient agent for evolution toward normality. However, the chemical composition of the mucus remained altered, thus potentially compromising its function. Goblet cell hyperplasia is detrimental to the epithelial barrier, giving rise to passages that allow tissue invasion by the parasite (Ponce-Macotela et al., 2008).

Infection also caused a significant increase in the number of IELs (159%) in the untreated animals. Only metronidazole (G5) reestablished the IEL/epithelial cell index as compared to the negative control (G1). Accumulation of leukocytes in the intestinal mucosa is a pathologic feature of inflammatory digestive system processes. For their proximity to the mucosal surface, IELs seem to play an initial role in the immune response. The cells present facilitating functional characteristics, such as selective survival, local proliferation, and retention of subpopulations in the epithelium. In the presence of mucosal inflammation, the IEL ratio is altered (Elia & Souza, 2001). Increased IEL levels have been observed in experimental infections in rats, mice, and calves, and in human biopsy specimens, and reveal active local immunologic processes aimed at increasing the animals' protection against infection (Arevalo et al., 2010; Mohammad & Koshak, 2011).

In this study, the animals treated with metronidazole (G5) presented normal IEL counts as compared with the negative controls. The changes seen in the animals treated with the other antiparasitic drugs may be a response to persistence of the parasite in places without complete elimination, making its spread therefore possible. Metronidazole drug treatment also plays an immunomodulatory role that might have expanded its specific action in relation to IELs (Venturini et al., 2014).

By producing anti-Giardia defensins, Paneth cells play a critical role in local defense (Gartner & Hiatt, 2007). The animals infected with *G. duodenalis* presented 71% more Paneth cells than the negative controls. The protozoan provokes increases in the quantity and activation of these cells, which results in parasite lysis and infection control. Previous studies both in vitro and in vivo
have revealed an increase in both the activity and/or number of Paneth cells, emphasizing the importance of defensins, and their potential for future use as an anti-Giardia medicine (Eckmann, 2003; Tako et al., 2013).

In this study, at 30 days after the final treatment with the antiparasitic drugs, Paneth cell numbers had not been reestablished in any group. Comparing the treated groups, the lowest increase in the numbers of these cells (p<0.05) was observed in animals treated with metronidazole (a 37.5% increase).

The classic histopathological description of giardiasis includes villi atrophy, chronic inflammation of the mucosa, and crypt hyperplasia. These together account for the symptomatic malabsorption and diarrhea (Mokrzycka et al., 2010; Elia & Souza, 2001). We also observed a decrease in the height of the villi, which may indicate (as a protection mechanism) reduced contact between the epithelial surface and the intestinal lumen. The increase in villi width in the infected animals may also be explained by the increased immune response against the parasite and the presence of an inflammatory process mediated primarily by eosinophils and other leukocytes (Zieske & Bernstein, 1982). The antiparasitic drugs used in the present study did not reestablish the villi to their normal height. In comparison with the other drugs, metronidazole-(G5) caused less harm to the animals (p<0.05). The villi width remained augmented in animals treated with nitazoxanide, albendazole, and secnidazole, but was restored with metronidazole treatment (p<0.05).

We believe that this normalization of the villus width reflects control over the local inflammatory process, and of a reduction in leukocyte migration, which is corroborated by the standardization of the IEL ratio presented above.

The increased width of the crypts, and alterations in their depth may be explained by a possible need to rebalance the intestinal epithelium in response to the presence of parasites and parasitic attacks on the mucosa. Crypts are a site of continual renewal and cell proliferation (Elia & Souza, 2001). The changes were present in all of the treated groups in our study (p<0.05). The depth of the crypts in the animals treated with metronidazole and albendazole presented smaller changes than in the other animals (p<0.05), thus revealing a trend toward normalization.

In all groups, the intestinal wall layers of the duodena presented hypertrophy (p<0.05), this is likely because during inflammation, the cells of the intestinal wall increase their interactions with those of the immune system. Their exposure to cytokines may alter their metabolisms, and leave them hyper- or hypo-trophic (Ponce-Macotela et al., 2008). This is the first study to separately analyze the intestinal layers of animals infected by G. duodenalis.

Our results suggest that G. duodenalis infection causes enteropathy, characterized by reduced height and increased width of the villi, increased height and width of the crypts, increased size of enterocytes and their nuclei, increased numbers of goblet cells secreting neutral and basic mucins, increased numbers of IELs and Paneth cells, and hypertrophy of intestinal wall layers. Of the tested antiparasitic drugs, all minimized the enteropathy caused by the infection, yet metronidazole was the most efficient treatment.

Effective treatment against giardiasis should involve monitoring not only elimination of parasites in stools, but also regulation of intestinal functions. In immunocompromised individuals or those with recurrent infections, intestinal histological analysis and treatment to control inflammation may be required.

**Conflict of Interest**

The authors declare no conflicts of interest.
References


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