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ORIGINAL ARTICLE

EXPERIMENTAL STUDY OF NEURAL PLASTICITY IN CHAGASIC MEGACOLON IN RATS

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ABSTRACT

This is a cross-sectional and experimental study. The purpose of the study was to measure enteric neurons in rats with chagasic megacolon. Twenty-three male rats inoculated with 1,500,000 trypomastigotes of the Y strain of *Trypanosoma cruzi* were used. The animals were sedated intramuscularly at zero inoculation time (T_0) and at sixty days after inoculation (T_{60}), to perform a barium enema test to evaluate the dilatation of the different segments of the colon. Evidence of infection was performed with a blood smear collected from the animals' tails 18 days after inoculation with observation of blood forms. Membrane preparations underwent dual-label immunofluorescence of global and nitrergic neurons with HuC / HuD antibody and nNOS antibody, respectively. Subsequently, quantitative and morphometric analysis of cecal and proximal colon segments were performed. In the quantitative analysis of the proximal colon segment there was a significant decrease in the numbers of total neurons (Hucolo p=0.001), as well as in the number of nitrergic neurons (NOScolo p=0.032) in the population of rats with chagasic megacolon in relation to the control group. In the cecal segment, this difference was not observed in the result of the total neuron counts (Huceco p=0.289) and nitrergic neurons (NOSececo p=0.466). In the morphometric analysis, considering only the cell body area, a significant difference in the size of the neurons with p=0.000 was observed in the cecal segment. The extensive loss of total neurons that cause predominance of nitrergic neurons contributes to the development of megacolon and neuronal volume increase in the cholinergic neurons, this plasticity does not reestablish the lost equilibrium, causing megacolon.

KEY WORDS: Chagas disease; megacolon; enteric nervous system.

INTRODUCTION

Chagas disease (CD) is an infectious condition with an acute and chronic phase classified as a neglected illness by the World Health Organization (Dias et al., 2016). The World Health Organization estimates that approximately 8 million people are infected in 21 Latin American countries

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(WHO, 2017). According to recent estimates, in Brazil there are 1.9 million to 4.6 million people infected with *Trypanosoma cruzi* (Dias et al. 2016) and it is still considered a public health problem. The main transmission mechanisms, via vector and transfusion, were interrupted in Uruguay (1997), Chile (1999), Brazil (2006) and most of Central America (Guatemala, Honduras, El Salvador, Nicaragua) in 2009-2010, where chronic cases still prevail (WHO, 2012).

Chagas disease is characterized by two distinct phases: acute (short duration and, most of the time, asymptomatic) and chronic (prolonged duration with cardiac and digestive manifestations). Clinical manifestations of the chronic phase in CD exhibit regional variations that may be associated with differences in parasite and host factors. Most infected individuals (50-70%) are asymptomatic, which characterizes the latent or indeterminate form of the disease. Among those infected, 30-50% are affected by the cardiac form, of which main morbidity is chronic Chagas cardiomyopathy (Silva et al., 2015) which is more evident and severe in areas of Central Brazil as compared to the South of the country and / or Central America (Dias & Coura, 1997). The digestive form, characterized by megaesophagus and megacolon, is present in 10-12% of those infected. It is common in Central Brazil and Chile, although it is practically non-existent in the Amazon, Colombia, Venezuela and Central America (Macedo & Segatto, 2010; Andrade et al., 2015).

The chagasic megacolon (CM) is characterized by permanent dilation and increased muscle mass in the absence of any mechanical obstacle (Adad, 1997). Pathologically, both the esophagus and colon exhibit luminal enlargement and muscular hypertrophy. Microscopically, inflammatory infiltrates and fibrosis are found associated with lesions in muscle cells and in the intramural nervous system (Koberle, 1968).

In the acute phase of the disease, when *T. cruzi* is present in a large number of tissues, the parasite may be responsible for neuronal lesions. The frequent occurrence of ganglionitis and periganglionitis in the chronic phase in chagasic patients with megacolon points to the participation of the immune system cells in these processes. In these patients there is an increase in the number of eosinophils and mast cells when compared to uninfected individuals and chagasic patients without megacolon. These cells can participate in tissue injury through the secretion of cytokines (IL-1, TNF-alpha and IL-6) that activate the cytotoxic process or cause damage by the secretion of enzymes, nitric oxide and free radicals (Dutra et al., 2009).

Chagasic megacolon is due to acquired hypoganglionosis (Jabari et al. 2011), a dramatic reduction in the number of enteric neurons in the affected segment itself (Koberle, 1968). Presenting topic dilatation as the pre-eminent macroscopic characteristic, it is to be expected that its main cause is the loss of excitatory neurons. Generally, these are cholinergic neurons while nitrergic neurons are an important part of the inhibitory innervation system (Jabari et al., 2011).

An experimental model in a laboratory animal was created in an attempt to develop therapeutic proposals. According to Fontes (2009) rats proved to be a good experimental model for the investigation of chagasic megacolon. The purpose of this study was to measure the enteric neurons in rats with chagasic megacolon.

MATERIAL AND METHODS

Ethical aspects

This research was approved by the Committee of Ethics in Animal Research of the State University of Maringá (UEM) (nº 021/2097) following the resolutions of the CNS - 196/96.

Parasite

Cryopreserved (liquid nitrogen) Y strain (TcII) (Silva & Nussenzweig, 1953; Zingales et al., 2009) was used in the experiments, provided by the Chagas disease trypanosomatid laboratory of UEM.

Animals and inoculum

Twenty-three Wistar rats (*Rattus novegicus albinus*, Wistar), all males, weighing between 180g and 200g, respectively, 20 for the experimental group (EG) and three for the control group (CG) were used. The animals were harvested from the central (UEM) room and kept in polypropylene cages with floors covered in dry sawdust under ideal temperature conditions (20 and 25°C), humidity (70%), light-dark cycle, with chlorinated water and rations (Nuvilab Cr-1® from Nuvital®) available at will. The inoculation was intraperitoneal with a concentration of 1,500,000 blood trypomastigotes / 0.1mL of blood (Brener, 1962).

Barium enema

The animals were sedated by intramuscular administration of ketamine hydrochloride and xylazine hydrochloride in a 1: 1 ratio and then placed on their own table. For the barium enema examination, a Nelaton n. 18 catheter was used rectally to administer 5.0 mL of barium sulfate with time of administration noted, and then radiographed. The evaluation of the dilatation of proximal, medial and distal colon segments was performed with a focus distance of 1.5 m and a 1s exposure time in a 50.000 Ma X-ray machine. A digital caliper was used to determine time zero (T_0). After the radiological examination the animals were inoculated as previously described. Eighteen days after inoculation 5 µL of blood were collected for the direct fresh smear (Brener, 1962). Sixty days after inoculation (T_{60}) the animals were re-sedated, and the barium enema was repeated to evaluate the dilatation of the different

segments of the colon; comparisons were made between the measurements obtained at T_0 and T_{60}.

Procedure

After finding megacolon by opaque enema 60 days after inoculation, the animals underwent surgery. The abdominal cavity was opened by ventral incision. The large intestine collected was washed in 0.1 M PBS pH 7.4, filled with Zamboni fixative and kept for 18 hours in the same solution under refrigeration. After this period, the segment was cut along the mesenteric border and washed successively in 80% alcohol until the excess fixative was removed. It was then dehydrated in alcohol (95% and 100%), bleached in xylol, hydrated in a decreasing series of alcohol (100%, 80%, 50%) and finally placed in 0.1M PBS. Subsequently, the segment was dissected to obtain the total preparation of the tunic. Immunofluorescence immunohistochemistry was performed with double labeling: HuC / HuD (neuronal human protein), pan-neuronal marker and nNOS (neuronal nitric oxide synthase) in the sub-population of nitrergic neurons.

For the due labeling technique, the total preparations were washed twice (10 minutes each wash) in 0.1 M PBS with Triton X-100 (0.5%). They were then incubated in a blocking solution composed of 0.1 M PBS, 2% BSA, Triton X-100 (0.5%) and 10% goat serum for 1 hour at room temperature. After this procedure, the total preparations were incubated in the anti-HuC / HuD and nNOS primary antibodies in 0.1 M PBS solution containing 2% BSA, Triton X-100 (0.5%) and 2% goat serum for 48 hours at room temperature. They were then washed 3 times in PBS plus Triton X-100 (0.5%) and incubated with secondary antibody (Table 1) diluted in a solution composed of 0.1 M PBS, 2% BSA, Triton X-100 (0.5%) and 2% goat serum for 2 hours at room temperature. Finally, the preparations were washed again three times in PBS with Triton X-100 (0.5%) and slides mounted with glycerol.

Quantitative analysis

Images were made with an AxioCam high resolution camera (Zeiss, Jena, Germany), coupled to the Axioskop Plus (Zeiss) microscope, equipped with 40X objective filters for immunofluorescence (FITC). Subsequently, these were analyzed through the Image-Pro Plus program, version 4.5.029 (Media Cybernetics, Silver Spring, MD, USA) for neuronal quantification. For each membrane preparation, all the neurons present in 32 random fields were counted. The area of each image was approximately 0.093 mm^2 and the total area quantified per animal was 2.976 mm^2.

Table 1. Primary and secondary antibodies used in the immunoreaction for HuC / HuD and nNOS.

Primary	Host	Dose	Brand	Secondary	Dose	Brand
HuC/HuD	Rats	1:500	Invitrogen	Antimouse Alexa Fluor 488	1:500	Invitrogen
nNOS	Bunny	1:500	Santa Cruz	Antirabbit Alexa Fluor 546	1:500	Invitrogen

Morphometric Analysis

The morphometric analysis was performed using Image-Pro Plus software, version 4.5.029 (Media Cybernetics, Silver Spring, MD, USA) for the study of the neuronal area in different groups. The cell body profile (μm^2) of 100 random neurons from 6 chagasic animals and 200 random neurons from the 3 CG animals was measured, totaling 600 neurons in each group.

Statistical analysis

Cross-sectional and experimental study. The results obtained were analyzed using the Tukey test with the GraphPad Prism 5.04 program. The level of significance was 5% ($p < 0.05$).

RESULTS

Twenty Wistar rats that developed the intestinal form of Chagas disease after the inoculation of Y strain *T. cruzi* with radiological confirmation of megacolon were used in the EG, 10 of them (50%) did not survive the experiment.

In the quantitative analysis of the proximal colon segment there was a significant decrease in the numbers of total neurons (Hucolo p=0.001), as well as in the number of nitrergic neurons (NOScolo p=0.032) in the population of rats with chagasic megacolon in relation to the control group. In the cecal segment this difference was not observed both in the result of the total neuron count (Huceco p=0.289) and in nitrergic neurons (NOSececo p=0.466). Therefore, there was no significant decrease in the number of enteric plexus neurons of the EG rats in relation to the CG in the latter segment (Table 2).

Table 2. Neuron count between control (CG) and experimental (EG) groups.

	Hu Cecum	NOS Cecum	Hu Colo	NOS Colo
CG	4435 ± 365.9	2460 ± 222.9	3928 ± 954.8	3121 ± 1005
EG	3959 ± 696.5	2722 ± 573.9	1858 ± 544	1326 ± 1108
CG x EG	p = 0.289	p = 0.466	p = 0.001	p = 0.032

The results differed in the different markings. In the global population of Hu-neurons a decrease in the size of the cellular bodies in the group with megacolon was verified. On the other hand, in the superpopulation of NOS neurons there was an increase in the neuronal area of the experimental group in relation to the control (Figure 1 and 2). Figure 1 shows the labeling of total neurons (green) and Figure 2 shows the overpopulation of nitrergic neurons (red).

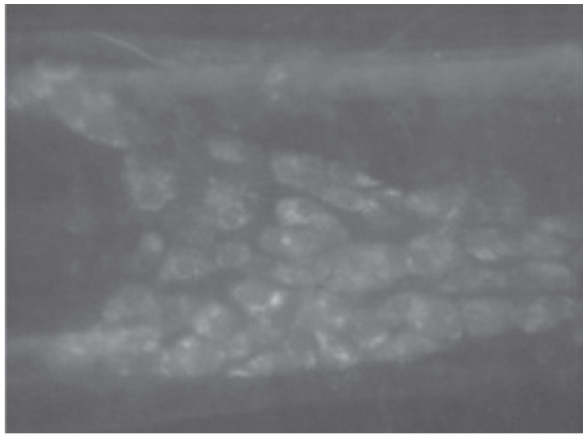

Figure 1. Photomicrography of proximal colon segment presenting supernumerary population of nitrergic neurons by immunofluorescence polarized light and increase of 40x.

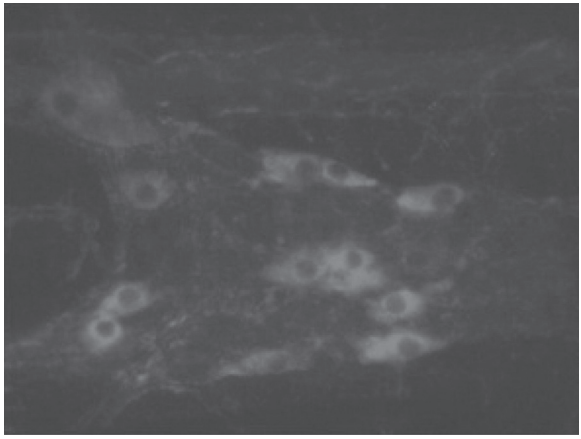

Figure 2. Photomicrography of chagasic proximal colon segment by immunofluorescence polarized light and increase of 40x showing increase of cholinergic neuronal cell body.

In the segment of the proximal colon, no significant difference was observed when compared to the cell body area of the Hu-neuron population between the studied groups p=0.001. However, in the comparison of this measurement in the population of NOS neurons, a significant increase in size was noted in the group with megacolon in relation to the control group (Table 3). In Table 3 the mean ± standard deviation of the cell body areas of neurons (in μm) of the control group (CG) and experimental group (EG) was used. Six hundred neurons from each group were evaluated.

Table 3. Morphometry of the cecal and proximal segment of the control group and the experimental group.

	Hu Cecum	NOS Cecum	Hu Colo	NOS Colo
CG	349.3 ± 156.5	326.9 ± 156.7	268.1 ± 127.2	326.4 ± 144.1
EG	311.8 ± 137.5	354 ± 160	280.9 ± 205.2	361.1 ± 161.8
CG x EG	p = 0.000	p = 0.003	p = 0.195	p = 0.000

DISCUSSION

American trypanosomiasis still affects millions of people in Latin America (WHO, 2017). Megacolon is one of the clinical manifestations of this chronic disease. The rectum-sigmoid transition of the colon dilates more than any part of the large bowel and is usually affected by complications such as fecal impaction and sigmoid volvulus (Koberle, 1968). Neuronal lesions may impair motility involved with colonic transit and colonic outlet mechanisms (Adad et al., 2001).

Chagasic megacolon has been traced back to extensive neuron loss in the affected gut segment (Koberle, 1968; Fernandez et al., 1992). CM is the result of the destruction of the enteric neurons (Da Silveira et al., 2008). These neurons are located in the wall of the colon, organized into ganglia and interconnected by bundles of nerve fibers that constitute the myenteric and submucosal plexus (Furnes, 2006).

In the quantitative analysis of the proximal colon segment there was a significant decrease in the numbers of total neurons, as well as in the number of nitrergic neurons in the population of rats with chagasic megacolon in relation to the control group. Data are confirmed by the literature (Da Silveira et al. 2007), which showed a decrease in the number of NOS neurons in patients with CM when compared to uninfected individuals. Da Silveira et al. (2007) reports that inhibitory motor neurons (VIP and immunoreactive NOS) are preferentially destroyed by *Trypanosoma cruzi* and / or by the inflammatory process. These results suggest a selective destruction of enteric neurons in the colon of chagasic patients with megacolon.

In the cecal segment there was no significant decrease in the number of enteric plexus neurons of the EG rats in relation to the CG. In patients with a disorder of the digestive system, the destruction of ganglion cells leads to more evident intestinal motility disorders in the distal, sigmoid and rectal segments (Guillén-Pernía et al., 2001).

A manometric study in chagasic patients demonstrated that the basal motility index and the sigmoid and rectum colon wave frequency was lower than in normal subjects. Moreover, it demonstrated the lack of relaxation of the internal sphincter of the anus in chagasic patients, absence of the rectal inhibitory reflex in CM similar to that of Hirschsprung's disease (Matsuda et al., 2009). The same study reported that the incidence of diverticula in the sigmoid colon of the non-chagasic group was higher than in the chagasic group with and without megacolon. Among the patients with megacolon and diverticular disease, the diverticula was always located in the non-dilated portions of the large intestine, suggesting that there are unfavorable conditions for the genesis or maintenance of diverticula in the dilated colon of the chagasic patients.

Evidence pointed out by Jabari et al. (2011) demonstrates an apparent resistance of nitrergic neurons against the pathological factors causing neuronal

loss in relation to the cholinergic neurons. Thus, there is a relative increase of nitrergic neurons as a consequence of the selective loss of cholinergic neurons. Nitrergic neurons exert an inhibitory function on the intestinal musculature, so the development of chagasic megacolon could be explained, since the preponderance of NOS neurons would lead to greater muscle relaxation.

Different authors reported that the sigmoid colon of humans with Chagas disease presents a neuronal decrease from 66.0% to 55.6%. The differences in the percentages obtained by different researchers are probably due to the different neuronal classes investigated, the deformation of the parasite, the inoculum and the time of infection (Da Silveira et al. 2007 and 2008).

In the morphometric analysis, considering only the cell body area, a significant difference in the size of the neurons was observed in the cecal segment. However, results were not similar in the different markings. In the global population of Hu-neurons a decrease in the size of the cellular bodies in the group with megacolon was verified. On the other hand, in the sub-population of NOS neurons there was an increase in the neuronal area of the experimental group in relation to the control. In the segment of the proximal colon, no significant difference was observed when comparing the cell body area of the Hu-neuron population between the studied groups. However, in the comparison of this measurement in the population of NOS neurons, a significant increase in size was observed in the group with megacolon in relation to the control group.

Neuronal plasticity can cause intense neuronal loss in the organ dilatation region. Once the neurons lack replicative capacity, they probably increase the regeneration of their fibers and extend their reach to the affected areas in an attempt to maintain the organ's physiological functions. Plasticity increase in neurons of the enteric nervous system is presumably responsible for the late development of the chagasic megacolon (Da Silveira et al., 2008).

The inflammatory process is followed by cytokine secretion that acts directly on neurons and, together with neuronal destruction, may contribute to more intense neuronal regeneration (Da Silveira et al., 2008). However, the results showed that even with neuronal plasticity there is no morphological compensation and the colon remains relaxed causing megacolon.

We conclude that the extensive loss of total neurons causing predominance of nitrergic neurons contributes to the development of megacolon and neuronal volume increases in the cholinergic neurons, this plasticity does not reestablish the lost balance therefore provoking megacolon.

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