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REGIONAL DIFFERENCES IN THE NUMBER AND TYPE OF MYENTERIC NEURONS IN THE DESCENDING COLON OF RATS

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ABSTRACT - The purpose of this study was to analyze the neuronal density of the myenteric plexus of the intermediate and antimesocolic regions of the descending colon of rats. Whole-mounts were stained with three different techniques of neuronal evidenciation. Through counts of the number of neurons in an area of 6.64 mm^2 under light microscopy, we found 1,271 ± 227.54 neurons with Giemsa in the intermediate region and 1,234 ± 225.92 neurons in the antimesocolic region; with the NADH-diaphorase technique we found 530 ± 92.97 neurons in the intermediate region and 539 ± 146.72 neurons in the antimesocolic region; and through the NADPH-diaphorase histochemistry, we found 417 ± 34.42 neurons in the intermediate region and 547 ± 84.01 neurons in the antimesocolic region. We conclude that there is a variation in the density of NADPH-diaphorase positive neurons in the intestinal circumference; that the NADH-diaphorase positive neuronal subpopulation represented 42.7% of that stained with Giemsa; and that the NADPH-diaphorase positive neurons represented 37.8% of the whole myenteric population.

KEY WORDS: myenteric plexus, Giemsa, NADH-diaphorase, NADPH-diaphorase, rat, descending colon.

Diferenças regionais no número e tipo de neurônios mioentéricos do colo descendente de ratos

RESUMO - O objetivo deste estudo foi analisar a densidade neuronal do plexo mioentérico das regiões intermediária e antimesocólica do colo descendente de ratos. Preparados de membrana foram corados com três técnicas diferentes de evidenciação neuronal. Através da contagem do número de neurônios, em uma área de 6,64 mm^2, sob microscopia de luz, encontramos com a coloração de Giemsa: 1271 ± 227,54 neurônios na região intermediária e 1234 ± 225,92 na região antimesocólica. Utilizando a técnica de marcação da NADH-diaforase, encontramos 530 ± 92,97 neurônios na região intermediária e 539 ± 146,72 na região antimesocólica. Através da histoquímica da NADPH-diaforase, encontramos 417 ± 34,42 neurônios na região intermediária e 547 ± 84,01 na região antimesocólica. Concluímos que há uma variação da densidade de neurônios NADPH-diaforase positivos ao redor da circunferência intestinal; que a subpopulação neuronal NADH-diaforase positivos representou 42,7% da população evidenciada no Giemsa e que os neurônios NADPH-diaforase positivos representam 37,8% do total.

PALAVRAS-CHAVE: plexo mioentérico, Giemsa, NADH-diaforase, NADPH-diaforase, rato, colo descendente.

For most mammals, the large intestine is very versatile, once it contributes to the hydroelectrolytic balance, is a potential site of nutrient absorption, controls the velocity of formation and elimination of feces, and is the habitat of billions of microorganisms[1]. The large intestine of rats has the capacity to mix, store and propel feces, as well as absorb fluids[2]. It is composed of cecum, ascending colon, descending colon and rectum. In the ascending colon the feces are temporarily stored, favoring the absorption of the excess fluid[1-3]. The descending colon, on the other hand, expels the fecal matter and exhibits a pattern of intense peristaltic movements, controlled trough reflexes triggered by the action of the dehydrated faces[2-5], so that it stores them for a short period[1,2]. The colonic activities are coordinated

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mainly by the nervous system and modulated by secretions such as hormones, bacterial enterotoxins, long-chain fatty acids, and endogenous stimulants[1].

The intrinsic nervous control of the colon is made by the Enteric Nervous System, the control of motility being carried out by the myenteric plexus. When we analyze the literature on the myenteric plexus, we verify that many studies approach only the descriptive aspects[6-9], and a smaller number of authors were concerned with the quantitative features[10-12]. It is scarce the number of authors who observed the neuronal density considering the regional differences in the circumference of an organ when carrying out their quantitative analyses[13-18]. Still smaller is the number of studies directed to the quantification in the descending colon. In these studies, 359 neurons/cm^2 were found in the descending colon of cats[19], and in the descending colon of guinea pigs it was found that 25% of the enteric neurons are positive to the technique of the NADPH-diaphorase[11]. Studies carried out in rats evidenced 2,227 neurons in an unreported area[10].

Considering the importance of knowing the enteric neurons of the descending colon and the paucity of studies on this organ, as well as aiming at providing support for physiological and pharmacological investigations, we propose to analyze, in both qualitative and quantitative terms, the distribution of the population of enteric neurons, in addition to subpopulations stained with NADH-diaphorase and NADPH-diaphorase, in the intermediate and antimesocolic regions of the descending colon.

METHOD

We used 14 male Wistar rats (*Rattus norvegicus*) in age of seven months (body weight 456.03 ± 33.48 g). The animals were handled according to the rules of ethic conduct in animal experimentation[20].

The rats were killed through inhalation of ethylic ether. All the experiments were made at the same daily hour and season of the year. The large intestine was removed, measured with millimeter ruler and the descending colon was collected.

The descending colon of five animals was washed in 0.9% saline solution, filled and immersed in fixative solution of acetic formol for 48 hr. After that the segments were dissected and stained with Giemsa (methylene blue) staining solution in Sorensen phosphate buffer (pH 7.0)[21].

The descending colon of other five animals was filled with Krebs solution (pH 7.3), washed twice (10 min. each) in the same solution and immersed for 5 min. in 0.3% Triton X-100 in Krebs solution. They were again washed twice (10 min. each) in Krebs solution and immersed for 45 min. for evidenciation of the NADH-diaphorase enzyme. This incubation medium contained in each 100 ml: 25 ml of a 0.5% Nitro Blue Tetrazolium stock solution (NBT: Sigma, St. Louis, USA); 25 ml of phosphate buffer 0.1M pH 7.3; 50 ml of distilled water and 50 mg of β-NADH (Sigma, Steinheim, Germany). After incubation the segments were opened at the mesocolic border and immersed in 10% buffered formol solution[22].

The descending colon of four animals was washed and filled with phosphate buffer (PB; pH 7.4), fixed in 4% paraformaldehyde in PB 0.1M for 30 min, immersed in 0.3% Triton X-100 (Sigma, St. Louis, USA) in phosphate buffered saline (PBS pH7.4) for 10 min. and washed 10 times (10 min. each) in PBS. Next they were immersed in incubation medium for neuronal evidenciation of the NADPH-diaphorase enzyme for 2 hours. This medium contained in each 100 ml: 25 mg of NBT; 50 mg de b-NADPH (Sigma, Steinheim, Germany), 0.3% Triton X-100 in Tris-HCl buffer (GibcoBRL, New York, USA) 0.1M (pH 6.0). After incubation the segments were opened at the mesocolon and washed three times in PBS (5 min. each) and immersed in 5% paraformaldehyde solution[23].

The whole-mounts of the different techniques were prepared under stereomicroscope with trans-illumination through dissection of the mucosa and submucosa. Then they were dehydrated in ascending series of ethylic alcohol, cleared in xylene and mounted between slide and cover glass with synthetic resin Permount (Fischer Chemical, New Jersey, USA).

The quantitative analysis was carried out with all techniques in the antimesocolic (120°-240°) and intermediate regions (60°-120° and 240°-300°, with 0° being the insertion of the mesocolon) of the descending colon[14,17]. The neurons were counted in an Olympus BX40 microscope with 40X objective. In each whole-mount, 40 microscopic fields were counted in each region[14,17]. Half-seen neurons were counted in alternate fields. The area of each microscopic field was of 0.1735 mm^2.

The mean and standard deviation of the obtained data were calculated. To compare the neuronal incidence between the antimesocolic and intermediate regions in each technique, the unpired test t of Student was employed at the significance level of 5%. To compare the neuronal incidence with Giemsa, NADH-diaphorase and NADPH-diaphorase in each region, the test of Kruskal-Wallis was used at the significance level of 5%.

The photographic documentation was obtained with an Olympus BX50 photomicroscope and PM 10AK photographic equipment.

RESULTS

When we compared the intermediate and antimesocolic regions of the intestinal circumference with different techniques, we did not notice large differences in the morphology of the myenteric plexus between the regions of the descending colon. In addition, we notice that the neurons of this plexus

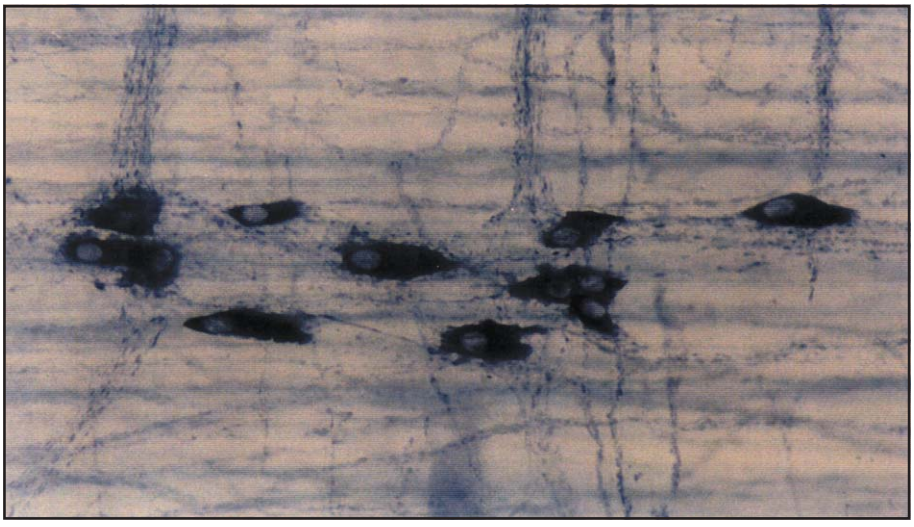

Fig 1. Whole-mount showing NADPH-diaphorase positive neurons in the myenteric plexus of the descending colon of adult rats. 408X.

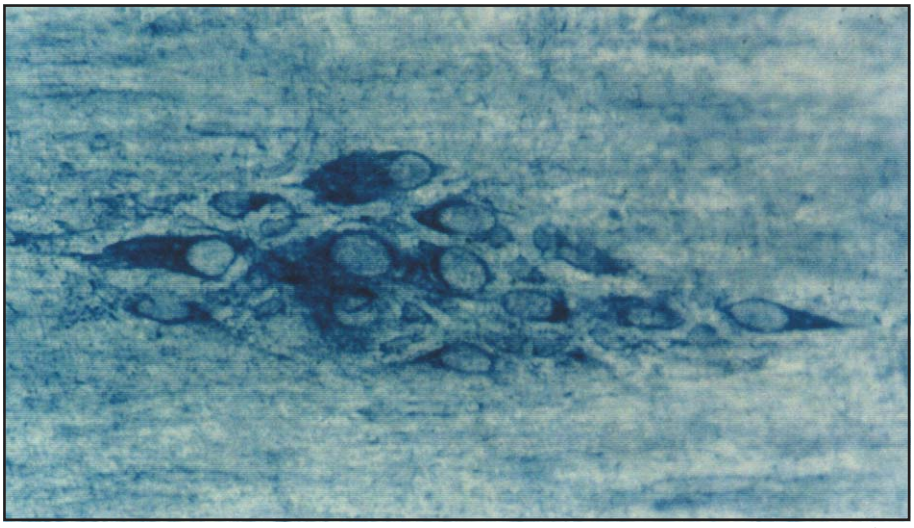

Fig 2. Whole-mount showing NADH-diaphorase positive neurons in the myenteric plexus of the descending colon of adult rats. 408X.

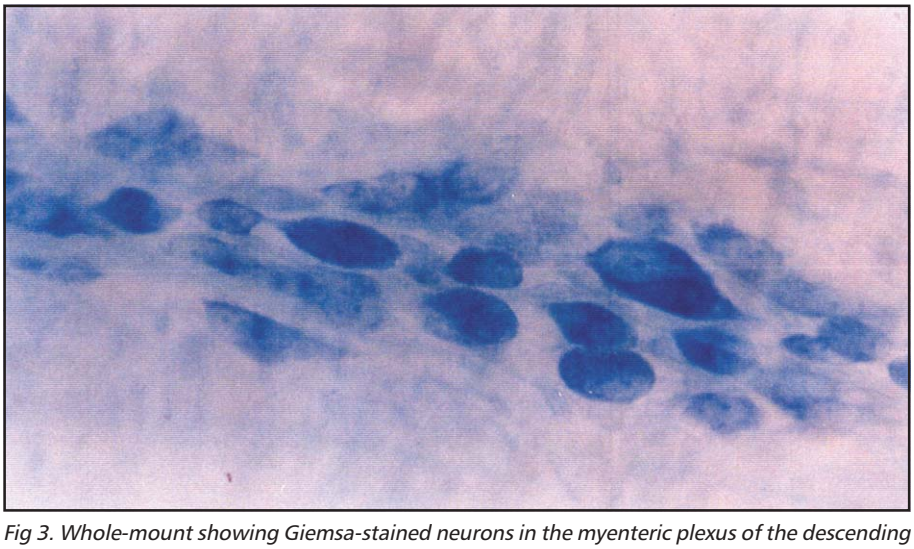

Fig 3. Whole-mount showing Giemsa-stained neurons in the myenteric plexus of the descending colon of adult rats. 408X.

Table 1. Incidence of neurons of the myenteric plexus found in 6.64 mm² of the intermediate and antimesocolic regions of the descending colon of seven month-old rats, with different techniques of neuronal staining.

Technique	Number of animals	Region	
		Intermediate	Antimesocolic
Giemsa	5	1271[a1] ± 227.54	1234[b1] ± 225.92
NADH-d	5	530[a1] ± 92.97	539[b1] ± 146.72
NADPH-d	4	417[a1] ± 34.42	547[a1] ± 84.01

Intermediate region, between 60º-120º and 240º-300º; Antimesocolic region: between 120º-240º, considering 0º as the mesocolon insertion; Means followed by the same letter in the same row differ statistically at the level of 5%; Means followed by the same number in the same column differ statistically at the level of 5%.

are found in ganglia which are often elongated with the major axis oriented longitudinally in relation to the circular layer of the smooth muscle tunica (Figs 1-3).

With the histoenzymelogic technique of the NADPH-diaphorase we verified that in both regions of the intestinal circumference there are thick bundles of nerve fibers (primary bundles) forming a network linking the ganglia both transversally and longitudinally. Thinner bundles (secondary bundles) connecting the primary bundles to each other, and several fine bundles and isolated nerve fibers (tertiary bundles) connecting different elements of the myenteric plexus (Fig 1) were also observed.

The incidence of neurons stained with the different techniques is presented in Table 1.

The Giemsa stain revealed a distribution of about 18,314 neurons/cm² in the intermediate region and 17,780 neurons/cm² in the antimesocolic region. With the NADH-diaphorase technique we verified some 7,636 neurons/cm² in the intermediate region and 7,766 neurons/cm² in the antimesocolic region. As for the NADPH-diaphorase positive neurons, we counted about 6,008 neurons/cm² in the intermediate region and 7,636 neurons/cm² in the antimesocolic region.

DISCUSSION

The apparently regular distribution of the myenteric plexus in the intestinal circumference of the descending colon of rats differs from that in guinea pigs, where there is a sparse plexus in the region corresponding to the antimesocolon and a dense arrangement in the regions corresponding to the intermediate region[8]. In our study, the analysis of the plexus was made with the NADPH-diaphorase technique, while in guinea pigs it was made with the NADH-diaphorase, thus revealing different neuronal subpopulations. It must be stated that the NADPH-diaphorase clearly stains neuronal cell bodies and nerve fibers and has been used to describe the arrangement of the plexus by other authors[9].

In all the techniques employed here we observed the location of the cell bodies in ganglia, which are predominantly elongated with the major axis longitudinal to the circular smooth muscle. The location of the neurons in ganglia is a common finding in the colon[13,24,25,8]. As for the ganglion shape, in the guinea pig colon they were found with irregular sizes[13,24], and polygonal, oval[25] and elongated shapes[26], the largest most often in the mesocolic region[8]. In the descending colon of mice, the ganglia are also elongated[27] and in the ascending colon of rats ganglia of varied shapes were found[12,14]. As for the ganglionar orientation, a similar distribution was found in other instances: in the jejunum-ileum of mice, guinea pigs and sheeps[8]; jejunum[18] and ileum[16] of rats; and ascending and descending colon of mice[27]. However, our results disagree with the perpendicular orientation relative to the circular smooth muscle described in the colon of guinea pigs[26].

When comparing the neuronal density around the circumference of the descending colon, we verified with Giemsa that the nerve cells are evenly distributed, similarly to the pattern described in the ascending colon[14]. On the other hand, in the ileum of rats there is a greater density of neurons in the mesenteric and intermediate regions than in the antimesenteric[16].

The comparison of the neuronal density in the ascending and descending colon with the Giemsa technique shows that there are 30,968 neurons/cm² in the intermediate region and 29,046 neurons/cm² in the antimesocolic region of the ascending colon, while there are 18.314 neurons/cm² and 17,780 neu-

rons/cm^2 in these regions, respectively, in the descending colon. As the Giemsa stain reveals all the neurons[14,16], we verified that the populations of myenteric neurons of the descending colon are 40.9% (intermediate region) and 38.8% (antimesocolic region) smaller than those of the ascending colon. A smaller neuronal density in the final colon in comparison to the ascending colon was also observed in a quantitative analysis in transverse sections of the rat intestine[10]. In whole-mounts of guinea pigs[13], mice[27] and humans[28] stained with methylene blue, a denser plexus was seen in the proximal colon.

The NADH-diaphorase positive neurons are evenly distributed in the descending colon circumference, contrary to early findings in the ascending colon with this technique, in which a larger number of neurons was found in the antimesocolic region[14]. Differences in the distribution of the neuronal subpopulation were also observed in the small intestine circumference. In the rat ileum it was observed a greater density in the intermediate than in the antimesenteric region[16]. In the jejunum the comparison of the mesenteric and antimesenteric regions showed a greater density on the first[18].

When comparing the density of NADH-diaphorase positive neurons between the ascending and descending colon, we found on the former first 8,798 neurons/cm^2 in the intermediate region and 12,308 neurons/cm^2 in the antimesocolic region; and on the latter 7,636 neurons/cm^2 and 7,766 neurons/cm^2 in the intermediate and antimesocolic regions, respectively. As with the total neuronal population, this subpopulation is less evident in the descending than in the ascending colon, with a neuronal density 13.2% smaller in the intermediate region and 36.9% smaller in the antimesocolic region.

The NADH-diaphorase positive neurons of the descending colon, when compared to those stained with Giemsa, represent 43.7% of the neuronal population in the intermediate and 41.7% in the antimesocolic region. In the ascending colon, this subpopulation represents 42.4% and 28.2% of the total neuronal population of the antimesocolic and intermediate regions, respectively. It was observed that the proportion of myenteric neurons which is NADH-diaphorase positive é greater in the descending than in the ascending colon of rats, despite being less numerous. Our data agree with those authors which state that the NADH-diaphorase technique does not stain all the neurons[14,16], but only those with greater metabolic activity[14,17]. The greater proportion of NADH-diaphorase positive neurons in the descending colon could be linked to distinct functional demands, reflecting the difference of action of these two organs. In spite of the total number of neurons being smaller, there is evidence that the incidence of muscle contractions is greater in the descending colon[5], which could explain the greater proportion of metabolically active neurons in this organ. The fact that we did not encounter differences in this subpopulation in the intestinal circumference, as well as in the general neuronal population, allow us to suggest that the fecal propulsion in the descending colon is made in a circularly uniform way, as evidenced by fluoroscopy in the descending colon of humans[4].

When we compared the density of the NADPH-diaphorase positive neurons in the descending colon circumference, we noticed a greater concentration in the antimesocolic region relative to the intermediate region. The opposite was observed in the ileum, that is, a greater density in the intermediate region[16].

In the ascending colon the NADPH-diaphorase positive neuronal density was of 8,970 neurons/cm^2, while in the descending colon it was of 6,822 neurons/cm^2. Similar to what occurred with the total neuronal population, the incidence of this subpopulation was 23.9% smaller in the descending that in the ascending colon. Another investigation, also in rats, showed agreement with our results[2].

In proportion to the neurons stained with Giemsa, the NADPH-diaphorase positive neurons represented 29.9% in the ascending and 37.8% in the descending colon. Thus, the proportion of NADPH-diaphorase positive myenteric neurons is greater in the descending colon of rats. A greater proportion of these neurons was also observed in the descending colon of guinea pigs, compared to oral segments of the intestine[11].

The studies have been demonstrating that the neurons stained with the NADPH-diaphorase technique are nitric oxide-producers through the nitric oxide synthase[29]. Nitric oxide is one of the inhibitory neurotransmitters of the intestinal muscle[30], e therefore the NADPH-diaphorase positive neurons are inhibitory motor neurons. Despite the greater proportion of these neurons in the descending than in the ascending colon, physiological studies comparing these segments have shown that there is a greater propulsive activity of the luminal content in the descending colon, once the dehydrated feces are denser and harder to propel[2-5]. Possibly the greater proportion of NADPH-diaphorase positive neurons is related to the need of a greater relaxation in the segment distal to that where strong propulsive movements

are taking place, so as to warrant fecal propulsion. In this way this intestinal segment must have a well-developed musculature, subjected to an efficient neural control to promote intense contractions spaced by equally intense relaxations.

The balance of these contractions and relaxations would be related not only to the density of subpopulations, but also to the capacity of producing and/or respond to specific neurotransmitters. In the descending colon, although there is a large population of NADPH-diaphorase positive neurons, studies revealed that the catalytic activity of the nitric oxide synthase can be smaller in this organ[2], and this, to an extent, reduces the inhibitory ability. On the other hand, studies in guinea pigs showed a greater contraction response in the descending colon relative to the ascending colon, due to an increase in the cholinergic neuronal response and in the muscle sensitivity to acetilcholine[5].

In situations in which the defecation reflex must be inhibited, even when the colon is plenty, the inhibitory neurons are possibly put into action and, as this situation may last for relatively long periods, there is enough time to produce and store nitric oxide precursors, making the action of these neurons more efficient. The return of the reflex of propulsive movements would represent the reactivation of the intrinsic and extrinsic cholinergic neuronal responses.

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