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Morphoquantitative Study of *Rattus norvegicus* Submucosal Plexus by Different Neuronal Evidentiation Histochemical Techniques

Estudio Morfocuantitativo del Plexo Submucoso de *Rattus norvegicus* por Medio de Diferentes Técnicas Histoquímicas para Verificar Características Neuronales

Marcelo Biondaro Góis[*]; Catchia Hermes-Uliana[**]; Alessandra Paltanin[***]; Willian Pontes[***]; Eduardo José De Almeida Araújo[****]; Marcilio Hubner Miranda Neto[*] & Débora Mello Gonçales Sant'Ana[*]

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SUMMARY: Enteric nervous plexuses have been the object of several studies, specially the myenteric plexus whose studies describe its organization, functions and alterations. On the other hand, the submucosal plexus has been less studied and still needs descriptive studies. To analyze morphologically and quantitatively submucosal neurons of the jejunum of 90-day-old healthy rats using different techniques for neuronal staining as a way to provide normality data to compare with future experimental studies. Whole mount preparations of the jejunum were submitted to Giemsa, NADH-diaphorase and NADPH-diaphorase techniques to stain the total neuronal population, more metabolically active subpopulation and subpopulation of nitrergic neurons, respectively. Neurons of the submucosal plexus of adult rats are mainly organized in ganglia with varied sized and shapes. Giemsa technique stained 243.93 ± 7.68 neurons per mm^2. Regarding the total population stained by Giemsa, NADH- diaphorase positive (139.09 ± 11.14/mm^2) neurons represented 57 % and NADPH-diaphorase positive (18.17 ± 0.28/mm^2) represented 7.5 %. The area of the cell body was bigger in nitrergic neurons (412.29 ± 150.22) than in the ones stained by Giemsa (254.71 ± 63.32) and NADH-diaphorase positive (243.98 ± 123.82).

KEY WORDS: Enteric nervous system, Meissner's plexus; Giemsa, NADH-diaphorase, NADPH-diaphorase.

INTRODUCTION

The enteric nervous system (ENS) is the third component of the Autonomous Nervous System (ANS) (Langley, 1921) because of its structure and function maintenance in the absence of central nervous system (CNS) efferent neurons (Furness & Costa, 1987; Furness, 2000; Furness, 2006). This independence is modulated by the signal exchange between ENS and CNS. ENS consists of segments from the upper esophagus until the internal anal sphincter, formed by interconnected meshes: plexuses containing neurons, axons and glial cells. In the intestines, most of the nervous cells form two ganglionated plexus: (Auerbach' s) myenteric plexus located between the longitudinal stratum and the circular muscle layer (Auerbach, 1862 apud Furness, 2006; Alves *et al*., 2011; Furness, 2012) and the submucosal plexus (Meissner, 1857 apud Furness, 2006; Billroth, 1858 apud Furness, 2006), formerly known as Meissner' s plexus, found in the tela submucosa of the large and small intestines (Mckeown *et al*., 2001; Wood, 2004; Furness, 2006; Grundy *et al*., 2006).

Detailed reports on the morphology, size and distribution of ganglia of the submucosal plexus are described in the studies by Dogiel (1899) apud Furness (2006), Irwin (1931), Schabadasch (1930) for different species of animals like sheep and cats. These animals were not considered experimental models, therefore, their study was based on the interest in the filogenetic description. Despite the similarity among mammals, it is suggested that the arrangement of the submucosal neurons are species specific (Hoyle & Burnstock, 1989; Timmermans *et al*.,

[*] Universidade Estadual de Maringá. Programa de Pós-graduação em Biologia Comparada. Av. Colombo, 5790, Zona 07, 87020-900, Maringá, PR, Brasil.
[**] Universidade Federal de Mato Grosso do Sul. Av. Marcio Lima Nantes, sn. Estrada do Pantanal, 79400-000, Coxim, MS, Brasil.
[***] Universidade Paranaense. Praça Mascarenhas de Moraes, 4282, Centro, 87502-210, Umuarama, PR, Brasil.
[****] Universidade Estadual de Londrina, Departamento de Histologia, Centro de Ciências Biológicas. Rodovia Celso Garcia Cid. 86057-970, Londrina, Paraná, Brazil.

2001) and their description in rodents broadens the possibility of studies on experimental models.

In large mammals, like pigs and humans, submucosal ganglia are interconnected and distributed in two or three levels (Schabadasch; Gunn, 1968; Hoyle & Burnstock; Timmermans *et al*.), and the ganglia at different depths have distinct neuronal populations. In small rodents, the submucosal plexus is unique, probably due to the thickness of the submucosal tela (Gunn; Furness & Costa; Brehmer *et al*., 2010).

Regarding their function, submucosal neurons are divided into motor neurons, interneurons, intrinsic primary afferent neurons (IPANs) and intrinsic fleeting afferent neurons (IFANs). As to their chemical code, several neurotransmitters have been described like adenosine triphosphate (ATP), nitric oxide (NO), P substance and the vasoactive intestinal peptide (VIP) (Furness, 2006). Through the production and release of neurotransmitters, these neurons coordinate the mucosal secretion, regulate the fluid motility through the intestinal epithelium, change the local blood flow and modulate the inflammatory response interacting with the local immune system (Weber *et al*., 2001; Neunlist *et al*., 2003; Toumi *et al*., 2003; Ekblad & Bauer, 2004; Furness, 2006; Savidge, 2007).

In the past decades, the myenteric plexus has received special attention and numerous studies have been done to understand its organization, functions and alterations in details. However, the submucosal plexus lacks descriptive studies because it has been less explored concerning its morphofunctional and pathological aspects. Thus, this study aimed to analyze the jejunum submucosal neurons of rats morphometrically and quantitatively with different techniques of neuronal evidentiation as a way to provide normality data for future studies with experimental models.

MATERIAL AND METHOD

The experimental protocol of this study was previously approved by the Ethics Committee in Research Involving Animal Experimentation (CEPEEA) from Universidade Paranaense (Protocol 12361/2008).

Experimental design. The study used fifteen 90 day-old male Wistar rats (*Rattus norvegicus*). Rats were submitted to euthanasia in chamber saturated with halothane (Vivas *et al*., 2007). Necropsia was performed immediately and the jejunum was removed, having the following anatomic limits as reference: duodenojejunal flexure and ileocecal fold. After microdissection, Whole mounts preparations with the submucosal plexus of each animal were obtained and submitted to the Giemsa (Barbosa, 1978), NADH-diaphorase (NADH-d) (Gabella, 1969) and NADPH-diaphorase (NADPH-d) (Scherer-Singler *et al*., 1983) histochemical techniques to stain the total neuronal population, more metabolically active subpopulation and subpopulation of nitrergic neurons, respectively.

Quantitative analysis of submucosal neurons. For each utilized histochemical technique, the number of ganglia and neurons present in 50 microscopic fields (0.14 mm^2/field) and the number of neurons per ganglion in 50 ganglia were counted. For ganglion counting, an optical microscope Motic BL with 40X lens was used, and the number of stained ganglia varied according to the utilized technique: ganglia with three or more neurons by Giemsa technique, and ganglia with more than two neurons by NADH-diaphorase positive (NADH-d^+) and NADPH-diaphorase positive (NADPH-d^+).

Morphometric analysis of submucosal neurons. For each animal, areas of the cell body and nucleus of 100 neurons were measured by all techniques. The cytoplasm area was calculated from the differences of these areas. Besides, the ratio between the nucleus area and the cell body area was determined to verify the rate occupied by this organelle inside the neuron. From the calculation of the cell body area of neurons stained by different techniques, it was possible to divide them in groups. The measurement was done using a photonic microscope with a 100X lens coupled to the image analysis system Motic Images Plus, version 2.0.

RESULTS

The submucosal plexus is a continuous structure along the intestinal circumference, organized in a single plan. In this plexus, numerous isolated neurons are found (Fig. 1 a, b, c) predominantly neurons grouped in ganglia of different shapes (Fig. 1 d, e, f).

The number of neurons and ganglia per mm^2, the number of neurons per ganglia, and the mean profile areas of cell bodies, cytoplasm and nuclei as well as the nucleus / cell body ratio of the neurons stained by each utilized technique can be verified in Table I.

Regarding morphology, neurons with rounded, oval and elliptical bodies were observed and most of them presented an eccentric nucleus.

Figure 2 shows the neuron distribution per group considering the profile areas of the cell body as basis.

Table I. Population density and morphometric analysis of the jejunum submucosal plexus neurons of rats stained with Giemsa, NADH-d+ and NADPH-d+.

Measures	GIEMSA	NADH-d$^+$	NADPH-d$^+$
Neurons/mm^2	243.93 ± 7.68	139.09 ± 11.14	18.17 ± 0.28
Ganglia/mm^2	19.21 ± 0.99	25.54 ± 3.03	6.48 ± 0.33
Neurons/ganglion	11.40 ± 0.22	5.36 ± 0.63	2.80 ± 0.10
Cell body (μm2)	254.71 ± 63.32	243.98 ± 123.82	412.29 ± 150.22
Cytoplasm (μm^3)	138.53 ± 21.83	189.31± 26.25	302.20 ± 101.46
Nucleus (μm3)	110.44 ± 11.41	54.77 ± 12.36	110.14 ± 35.52
Nucleus/Cell body	0.44 ± 0.01	0.24 ± 0.06	0.40 ± 0.16

Values were expressed by mean ± standard error

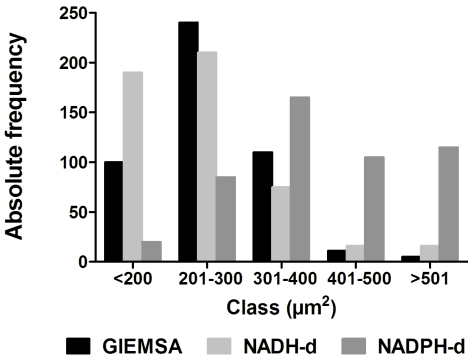

Fig. 2. Distribution of neurons stained by Giemsa, NADH-d+ and NADPH-d+ techniques in different groups according to the cell body area.

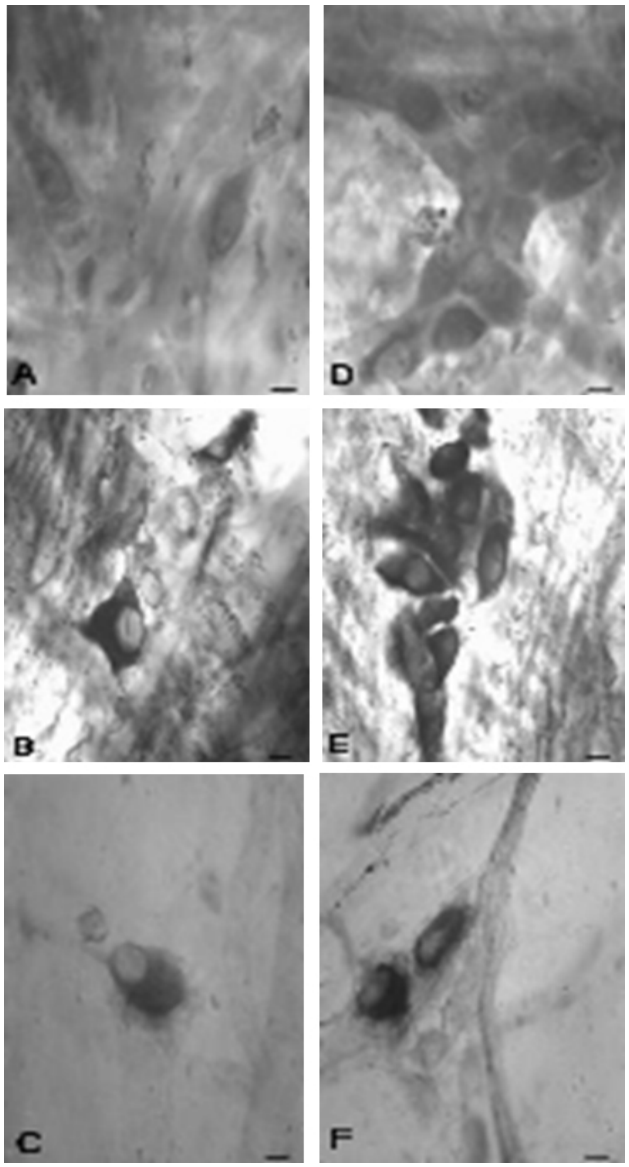

Fig. 1. Photomicrographs of isolated submucosal neurons (a, b and c) and in jejunum submucosal plexus ganglia (d, e and f) of adult rat stained by Giemsa (a and d), NADH-d$^+$ (b and e) and NADPH-d$^+$ (c and f) techniques. Fifty µm bar.

DISCUSSION

In this experiment, the submucosal plexus was observed as a continuous structure along the intestinal circumference, organized in a single plan. These characteristics were also observed in small mammals whose submucosal plexus is unique due to the smaller thickness of the submucosal tela (Gunn; Furness, 2006; Brehmer *et al*.). Authors that have studied large mammals like apes, swine and human beings found the submucosal plexus divided in two or more smaller plexuses (Gunn; Hoyle & Burnstock; Furness and Costa; Brehmer *et al*.), mainly an internal submucosal plexus and an external one. Generally one is found in the basis of the mucosal tunica towards the structures located there and the other is seen inside the submucosal tela, enervating glands (Gunn; Balemba *et al*., 1998; Furness, 2006; Brehmer *et al*.).

From the neuronal evidentiation techniques utilized in this study, it can be noticed that the neurons of the submucosal plexus organize themselves into interconnected ganglia through fiber bundles. However, isolated neurons were observed in smaller number. The predominance of neurons organized in ganglia was also observed in submucosal tela of rats' ileum (Buttow *et al*., 2004) and other species (Sousa, 1994; Zanesco & Souza, 2011).

When the number and the size of stained ganglia were evaluated, it was verified that the ganglia stained by Giemsa were visibly bigger, gathering an average of twice the number of neurons than in ganglia stained by NADH-d$^+$ and about five times more than nitrergic neurons. However, when quantitative data were considered together, it was observed that most NADH-d+ and NADPH-d+

neurons were isolated. These findings may represent really isolated neurons in nervous fiber bundles and also neurons present in ganglia where most of the neurons were not stained by these techniques, except by Giemsa technique, because of their functional and biochemical characteristics.

Using Giemsa technique, it was verified that in the jejunum submucosal plexus of rats, ganglia had from 4 to 20 neurons (an average of 11.4 per ganglion). Our findings are similar to the ones by Zanesco & Souza that described submucosal ganglia stained by Giemsa technique and that were formed by one to 30 neurons, an average of nine neurons per ganglion, in guinea pigs' "jejunum-ileum". On the other hand, the number of neurons/ganglion observed by myosin V staining in the ileum submucosal tela of rats has an average of 4.79 (Buttow *et al*.). The number of neurons stained by Giemsa technique in this study (243 neurons/mm^2) is significantly greater than the one found by immune histochemical technique for myosin V (94.12 neurons/mm^2) in the ileum submucosal tela of rats (Buttow *et al*.). These differences can be related to the differences of neuronal density between the segments of each study, and also to the fact that not all myenteric neurons are myosin V positive while polyribosomes of all neurons are stained by Giemsa technique. Marese *et al*. (2007), when comparing the average number of duodenum myenteric neurons of rats at 21 days and at 428 days using non-histochemical Giemsa technique and immune histochemical technique for myosin V, verified a neuronal reduction of 85.4 % and 82.7 %, respectively, for both techniques. However, the comparison of ganglion number/mm^2 did not show a difference between this study and the one by Buttow *et al*. Souza (1994) demonstrated that in the submucosal plexus of the wild rodent Calomys callosus there are ganglia of several sizes formed by NADH-d^+ neurons. They also report observing isolated cells or paired cells among the ganglia.

A greater density of submucosal neurons was verified by Giemsa technique that can be considered a pan-neuronal technique because it stains neurons due to the affinity of the stain with structures formed by acid proteins, especially polyribosomes. Based on the staining of enteric neurons by Giemsa technique (Barbosa), they classify neurons according to the cytoplasm basophilia and state that this technique allows to visualize polyribosome disintegration (Nissl' corpuscles) in the neuronal chromatolysis processes (Sant`Ana *et al*., 1997).

NADH-d^+ neurons represented 57 % of the total population. This technique must be chosen when it is intended to evidentiate more active neurons metabolically because of the formation of formazan granules as a result of the reaction of H+ ions from the respiratory chain with an artificial electron acceptor - Nitroblue Tetrazolium (NBT) catalyzed by the mitochondrial enzyme NADH-d. It is essential to establish an experimental protocol in which the exposure time (reaction) and the ambient temperature are controlled and standardized. Our study group adopts 45 minutes as standard time (Sant'Ana *et al*., 1997) because longer exposure times may stain lower metabolism neurons and other cells.

NADPH-d^+ neurons stained in this experiment correspond to nitrergic neurons because NADPH-diaphorase and NOS (Nitric Oxide Synthase) neurons are co-located, according to Belai *et al*. (1992). Several authors showed this correspondence between them, (Scherer-Singler *et al*.; Santer *et al*., 1994) to name a few, indicating that NADPH-d^+ histochemical can be used as a nitrergic neuron marker.

In this study with 90-day-old rats, a small rate of nitrergic neurons was found (7.5 % of the total population). In adult mice, Young & Ciampoli (1998) estimated that the nitrergic neuron population was 3 %. However, studies on jejunum (Hermes-Uliana *et al*., 2014) and duodenum (Lopes *et al*., 2012) of 210-day-old rats found 26 % and 23 % of nitrergic neurons in the submucosal plexus, respectively. A greater density of NADPH-d^+ neurons and a greater expression of NOS in aging process rats was verified by several authors that have related this finding to a greater resistance of these neurons to nutritional deficiencies (Sant'Ana *et al*., 2012a), degenerative processes caused by aging and by chronic degenerative diseases (Fregonesi *et al*., 2004), and NOS expression by neurons that did not use to express it but start doing it when initiating the cell death process (Fregonesi *et al*.). A greater density of nitrergic neurons was also related to a greater relaxation of smooth musculature (Hermes-Uliana *et al*.).

Enteric neurons can also be evidentiated by silver staining (Irwin), acetylcholinesterase (Gunn), immune staining of substance P (SP), tachykinin (NK), calcitonin gene related peptide (CGRP) (Mitsui, 2010), vasoactive intestinal peptide (VIP) (Hernandes *et al*., 2004; Sant'Ana *et al*., 2012b), nitric oxide synthase (NOS), Calretinin (Sayegh & Ritter, 2003), myosin V (Buttow *et al*.; Marese *et al*.) among others. It is important to point out that when adopting a staining technique, researchers have to clearly remember which variables they want to study and not only worry about verifying if the technique stains all neurons; for example, neurons that were immune reactive to SP represented 11 % and NK 73 % of total submucosal population of rats' colon (Mitsui).

The submucosal neurons described in this study have presented varied sizes and distinct shapes like triangular, oval, polygonal and elongated, similar to the ones found in rodents like guinea pigs (Zanesco & Souza) and Calomys callosus (Sousa). The contraction of the intestinal smooth musculature causes alterations in the ganglion shape and, therefore, when evaluating the shape and density, the contractile status of the intestinal wall should be taken into consideration (Gabella, 1971). Mitsui verified that neurons of the colon submucosal plexus of rats also presented round and oval cell bodies. In general, when compared to what Gunn observed in the submucosal plexus of different species of mammals stained by silver, there was homogeneity in the shapes of the neuron cell bodies.

Through histochemical of NADPH-d, it was also possible to verify the beginning of cytoplasm extensions of neurons which emerges from the opposing pole to the nucleus with characteristics of unipolar neurons. However, new studies are necessary utilizing techniques that better stain the extensions so that they can be classified according to the different types of Dogiel.

When observing the average cell body area of submucosal neurons, it can be noticed that NADPH-d$^+$ neurons are the largest cells with larger cytoplasm areas than the others. On the other hand, NADH-d$^+$ and the total population of neurons is composed by 250 μm^2 cell body neurons, similar to what was observed in the ileum submucosal neurons of rats, stained by immune histochemical of myosin V (Buttow *et al*.). Utilizing Giemsa and myosin V techniques, Marese *et al*. described two patterns of neuronal body profiles. Neurons with areas smaller than 200 μm^2 were predominant in young rats (21 to 60 days of age) whereas neurons with area of 200-600 μm^2 were predominant in adult animals. Similar results were described by Gabella (1971), who analyzed the relationship between the size of the neuronal cell body of the small intestine myenteric plexus by means of NADPH-d$^+$ histochemical and development period of albino neonatal rats and adult rats (*Epimys norvegicus*). The author concluded that while growing there was an augmentation of the neuronal size, and the predominant population in neonatal animals was small neurons. In wild rodents Calomys callosus Sousa found that the average size of NADH-d$^+$ neurons in the jejunum was 156 μm^2.

When observing Figure 2, it can be noticed that NADH-d+ neurons are concentrated in smaller classes (< 200 and 201-300 μm^2), therefore, when compared to others, they are smaller. On the other hand, NADPH-d$^+$ neurons are bigger because their distribution slides to the right in Figure 2. These cells are concentrated in classes over 301 μm^2. When evaluating the total neuronal population, stained by Giemsa, the tendency decreases and the area occupies a more central position with intermediary cell body sizes.

It was verified that NADH-d$^+$ neurons had smaller nuclear areas in which the nucleus occupied only 24 % of the cell body area. It is believed that these neurons possibly have a more developed synthesis mechanism which would contribute to a larger cytoplasm area, because these cells have more active metabolism. Studies on the ultrastructure are necessary to stain the subcellular components of this neuronal population.

Knowing the shape and area of the cell body, nucleus position and area, as well as the proportion of the cell body and the nucleus is important because they reflect the functional state of the neurons. These parameters may undergo alterations caused by intra and intercellular disorders, for example, in the apoptosis processes in which the neuron nucleus shows several condensation and aggregation stages of chromatin and disproportionally becomes small "pyknotic nuclei" (Danial & Korsmeyer, 2004; Kumar *et al*., 2008). On the other hand, it is important to point out that the peripheral positioning of the nucleus is normal in ENS, while it indicates axonal lesion in CNS and is generally followed by central chromatolysis (Kumar *et al*.).

Studies involving neurons and fibers of the submucosal plexus are promising because of their involvement in the maintenance of the intestinal mucosal structure. These neurons detect variations of the intestinal lumen and in the mucosal homeostasis, presenting specific receptors for serotonin (5-HT) secreted by enterochromaphin cells of the mucosa, and performing an important role in ENS afference (Michel *et al*., 2005).

CONCLUSION

Thus, it is concluded that in the jejunum submucosal plexus of adult rats:

1. a lot of isolated neurons are found, but the predominant organization is ganglia with various sizes and shapes.
2 . Giemsa technique stains the total neuronal population, (243.93±7.68 neurons/mm^2) where NADH-d$^+$ neurons represent 57 % and NADPH-d$^+$ neurons represent 7.5 % of total population.
3. The average cellular body areas of NADPH-d+ neurons are bigger than the average od the cellular bodies stained by Giemsa and NADH-d$^+$.

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RESUMEN: El plexo nervioso entérico ha sido objeto de varios estudios, especialmente el plexo mientérico, cuyos estudios consisten en describir su organización, funciones y alteraciones. Por otro lado, el plexo submucoso ha sido menos investigado y todavía necesita estudios descriptivos. Para analizar morfológica y cuantitativamente las neuronas de la submucosa del yeyuno de ratas de 90 días de edad, se realizaron diferentes técnicas de tinción neuronales, en animales sanos, como una forma de proporcionar datos de normalidad y compararlo con futuros estudios experimentales. Se realizaron montajes con preparados enteros del yeyuno que fueron sometidos a las técnicas de Giemsa, de NADPH-diaforasa y NADH-diaforasa para teñir la población total neuronal, subpoblación más activa metabólicamente y subpoblación de neuronas nitrérgicas, respectivamente. Las neuronas del plexo submucoso de ratas adultas se organizan principalmente en los ganglios con variaciones de tamaño y formas. Con la técnica de Giemsa se tiñeron 243.93±7.68 neuronas por mm^2. Con respecto a la población total teñida con Giemsa, fueron positivas para NADH- diaforasa en 139.09 ±11.14 / mm^2 neuronas, representando el 57% y fueron positivas para NADPH-diaforasa en 18,17 ± 0,28 / mm^2 neuronas, lo que representó el 7,5%. El área del cuerpo celular fue mayor en neuronas nitrérgicas (412,29 ± 150.22) que en las teñidas con Giemsa (254,71 ± 63,32) y NADH-diaforasa positivas (243,98 ± 123,82).

PALABRAS CLAVE: Sistema nervioso entérico; Plexo de Meissner; Giemsa; NADH-diaforasa; NADPH-diaforasa.

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Corresponding author:
Marcelo Biondaro Góis
Department Morphological Sciences
State University of Maringa
Av. Colombo, 5790
Zona 07, Zip code 87020-900 Maringá - PR,
BRAZIL

E-mail: marcelobiondaro@gmail.com

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