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Atrophy of the Nitrergic Myenteric Neurons in the Descending Colon Rats Submitted to Protein and Vitamin Deficiency

Atrofia en Neuronas Mientéricas Nitrérgicas en el Colon Descendente de Ratas Sometidas a Deficiencia de Proteínas y Vitaminas

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SUMMARY: Effects of protein and B-complex vitamin deficiency were assessed with respect to the morphometry of myenteric neurons in the descending colon of adult rats. Sixteen animals were divided into two groups: Control Group (CG, n=8) and Experimental Group (EG, n=8). The CG received 22% protein chow and the EG received 4% protein chow for 120 days. The descending colon was submitted to NADH- and NADPH-diaphorase technique in order to evidence nervous cells in the whole mounts preparations. In the EG, NADH-d positive neurons presented reduced nuclei, while NADPH-d positive neurons showed atrophy of the soma area (~41.7%) inducing an increase of the proportion occupied by the nucleus inside in the soma of these cells.

KEY WORDS: Malnutrition; Large intestine; Enteric nervous system; Myenteric plexus; Neural plasticity.

INTRODUCTION

The plasticity of the nervous system, a unique characteristic when compared with other systems, has been widely proved by neurobiology studies. Plastic changes are also observed in the Enteric Nervous System (ENS), with modifications in the number and size of myenteric neurons (Gabella, 1990). These changes may be related to alterations either in the target tissues (Gabella, 1987), or in the action of the immune system on these cells (Bauer, 2008) or in nutrient intake (Sant'Ana *et al.,* 2006).

There is a close association between the occurrence of severe protein-calorie malnutrition and impaired brain development, with consequent functional changes in this tissue (Monteiro *et al*., 2002). However, there is great concern on the effects of malnutrition on the ENS due to its role in the coordination of gastrointestinal functions, more specifically on the myenteric plexus, responsible for controlling intestinal motility (Furness & Costa, 2006).

Although functional changes in myenteric neurons may cause morphometric alterations, many authors have studied the effects of malnutrition on quantitative aspects of the myenteric plexus (Natali & Miranda-Neto, 1996; Meilus *et al*., 1998; Fiorine *et al.*, 1999; Araújo *et al.,* 2003, 2006; Sant'Ana *et al*., 2006; Muniz *et al*., 2007; Moreira *et al.,* 2008; Hermes *et al.*, 2008). There are only few studies involving the morphometric evaluation of these neurons (Sant'Ana *et al*., 2006; Muniz *et al*.; Hermes *et al*.).

This study analyzed the effects of protein and B-complex vitamin deficiency on the soma area and on the proportion occupied by the nucleus inside of the soma of myenteric neurons from descending colon of rats.

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MATERIAL AND METHOD

All procedures in this study were previously approved by the UNIPAR (Universidade Paranaense) Ethics Committee in Researches Involving Animal Experimentation.

Sixteen male, 90-days-old Wistar rats (Rattus norvegicus) (299.8±27.7g) were randomly placed in one of two groups: control (CG; n = 8) and experimental (EG; n = 8), housed in individual cages and kept in a room with temperature constant (25ºC) and with light/dark (12/12h) cycle. Animals had free access to water and feed. During 120 days, animals from the CG were fed NUVILAB® commercial chow (22% protein) and for GE animals were given prepared chow with 8% protein, obtained by adding corn starch, sucrose and a mineral salt mix, without vitamin supplementation (Araújo *et al.,* 2005). After 120 days, rats were submitted to euthanasia using an ethyl ether saturated chamber. Descending colons of all animals were removed by laparotomy using the ventral median line for the incision.

Descending colons of five animals of each group were submitted to NADH-diaphorase (NADH-d) technique. Segments were filled and washed twice (10 min) using Krebs solution (pH 7.3). After that, they were immersed in 0.3% Triton X-100 in Krebs solution (5 min), and washed (2x10 min, each) again in Krebs solution. They were then immersed for 45 min in an incubation medium containing in each 100 mL: 25 mL Nitro Blue Tetrazolium stock solution (NBT, Sigma, St. Lois, USA); 25 mL 0.1M phosphate buffer, pH 7.3; 50 mL distilled water and 50 mg b-NADH (Sigma, Steinheim, Germany). After incubation, segments were opened along the mesocolic border and immersed in 10% formaldehyde (Gabella, 1969).

The descending colons of the other three animals of each group were submitted to NADPH-diaphorase (NADPH-d) technique. These intestinal segments were washed and filled with phosphate buffer saline - PBS (pH 7.4), fixated for 30 minutes with 4% paraformaldehyde (Merk, Darmstad, Germany) prepared in 0.1M phosphate buffer (pH 7.4), and immersed in 0.3% Triton X-100 in 0.01M PBS pH 7.4. Then, they were washed (10x10 min each) in PBS and immersed for 60 minutes in incubation medium containing in each 100 mL: 25 mg NBT; 50 mg b-NADPH (Sigma, Steinheim, Germany); 0.3 mL Triton X-100 and 100 mL 0.1M tris-HCl buffer (GibcoBRIL, New York, USA) (pH 6.0). After incubation, segments were opened in the mesocolon insertion, washed three times in PBS (5 min each) and immersed in 4% paraformaldehyde solution (Scherer-Singler *et al.,* 1983).

The whole mounts containing myenteric plexuses were obtained by dissecting the intestinal segments using a stereomicroscope with transillumination (Motic SMZ-140) to remove the mucosa and the submucosa. After that, segments were dehydrated in ascending concentrations of ethanol, diaphanized in xylol, and mounted among slide coverslip using Permount™ synthetic resin (Fischer Chemical, New Jersey, USA).

Morphometric analysis of myenteric neurons was carried out using the software Motic Images Plus 2.0. Images were captured using a digital camera (Moticam 2000, 2.0 M Pixel) attached to a trinocular microscope (MOTIC B5) with a 40x objective. Soma and nucleus areas were measured in 300 neurons of each animal, in a total of 2,400 neurons per group. Based on these values, nucleus / soma area ratio was calculated in order to assess the proportion occupied by this organelle in the total area of the soma. Besides, the correlation coefficient between these two areas was determined, and neurons were divided into classes by considering the (100 μm^2 intervals) soma areas and the nucleus / soma ratio (0.10 intervals).

As for the statistical analysis, data were initially submitted to Kolmogorov-Smirnov test to assess the type of distribution. Data with normal distribution were expressed as means ± standard deviation. The comparison between the control and experimental groups was carried out by unpaired Student t test by considering $p<0.05$ significant values.

RESULTS

No significant difference was observed between the two groups in the comparison of mean soma areas of NADH-diaphorase positive neurons ($p>0.05$). Nucleus areas tended to decrease ($p<0.05$) without significant changes in nucleus / soma area ratio ($p>0.05$), in spite of the strong positive correlation between them (Table I). A strong correlation (over 90%, $p<0.05$) between soma and nucleus areas (Figs. 1 and 2) was observed in both groups and techniques. It was observed that the soma area, nucleus area and the ratio between these areas were reduced in malnourished animals ($p<0.05$) as described on Table I.

The analysis of the frequency histogram of the different neurons showed that the neuronal subpopulations evaluated are heterogeneous both for soma areas and for the proportional area occupied by the nucleus in the trophic region of these cells (Figs. 3 and 4).

Table I. Mean± standard deviation of soma and nucleus areas, and nucleus- soma area ratio of myenteric neurons in the descending colon of rats submitted to normal (control group, CG) and to hypoproteic diet without supplementation of B-complex vitamins (experimental group, EG). Means marked with asterisk (*) showed significant differences between the two groups (CG and EG) in the same technique, considering 5% as the significance level, as determined by the unparied t test.

	NADH diaphorase		**NADPH diaphorase**	
Parameter	CG	EG	CG	EG
Soma area (μm^2)	659.19 ± 1,392.80	456.23 ± 735.87	658.71* ± 1,303.70	383.73* ± 1,136.35
Nucleus area (μm^2)	300.18* ± 539.07	216.06* ± 303.68	217.24* ± 351.02	134.51* ± 275.52
Nucleus-soma area ratio	0.51 ± 0.12	0.51 ± 0.11	0.44* ± 0.16	0.47* ± 0.12

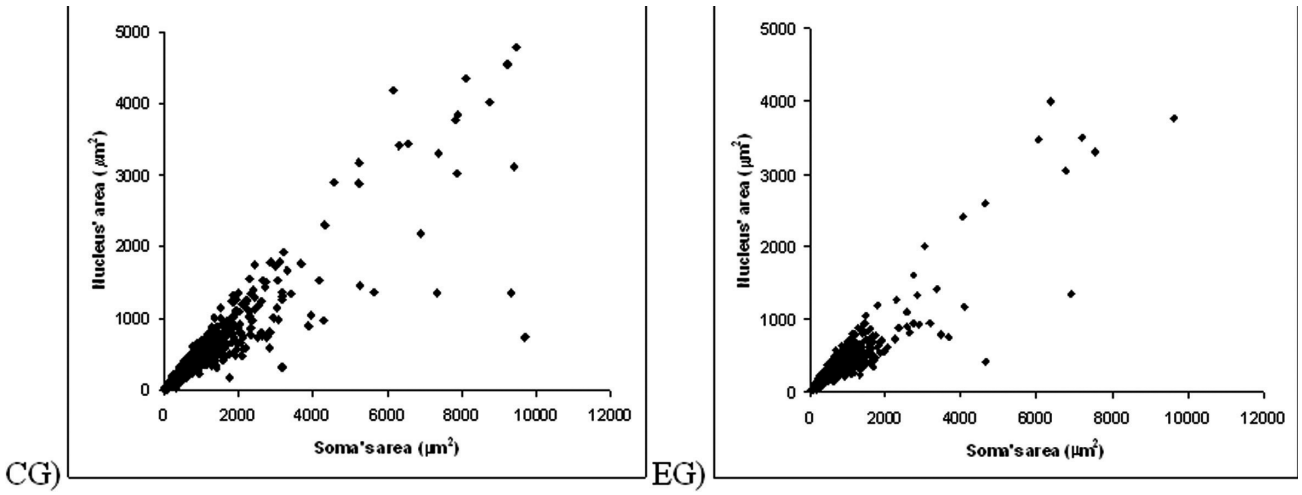

Fig. 1. Dispersion graph of soma and nucleus areas in NADH-diaphorase positive neurons of rats submitted to normal (CG, r = 0.9680, p<0.05) and hypoproteic diet without supplementation of B-complex vitamins (EG, r = 0.9713, p<0.05).

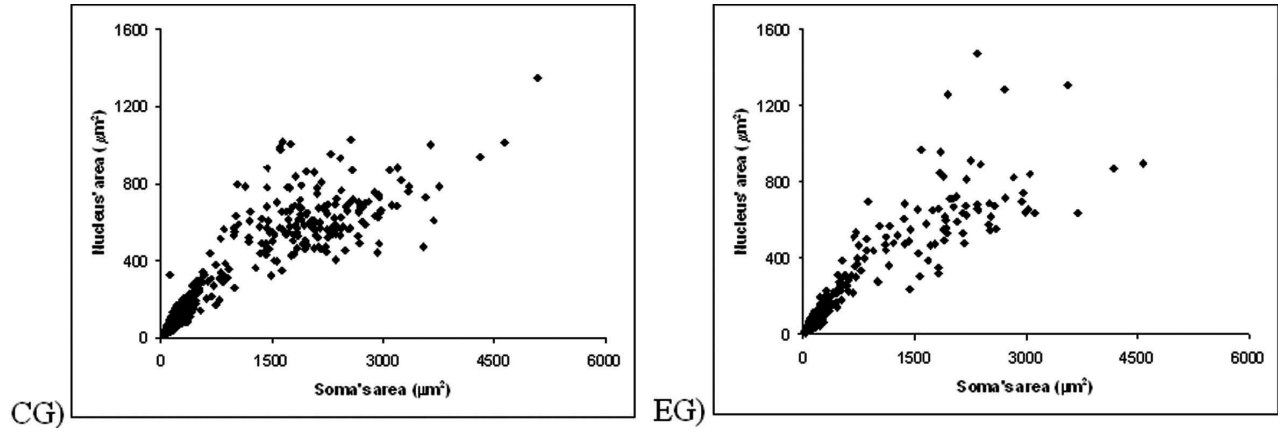

Fig. 2. Dispersion graph of the soma and nucleus areas in NADPH-diaphorase positive neurons of rats submitted to normal (CG, r = 0. 9500, p<0.05) and hypoproteic diet without supplementation of B-complex vitamins (EG, r = 0. 9311, p<0.05).

However, when the effects of the diet were analyzed in relation to this distribution, changes were only visible in NADPH-d positive neurons. This analysis showed more these neurons in classes of smaller soma area; that is, in classes where the nucleus occupied a greater proportion of the soma (Fig. 4).

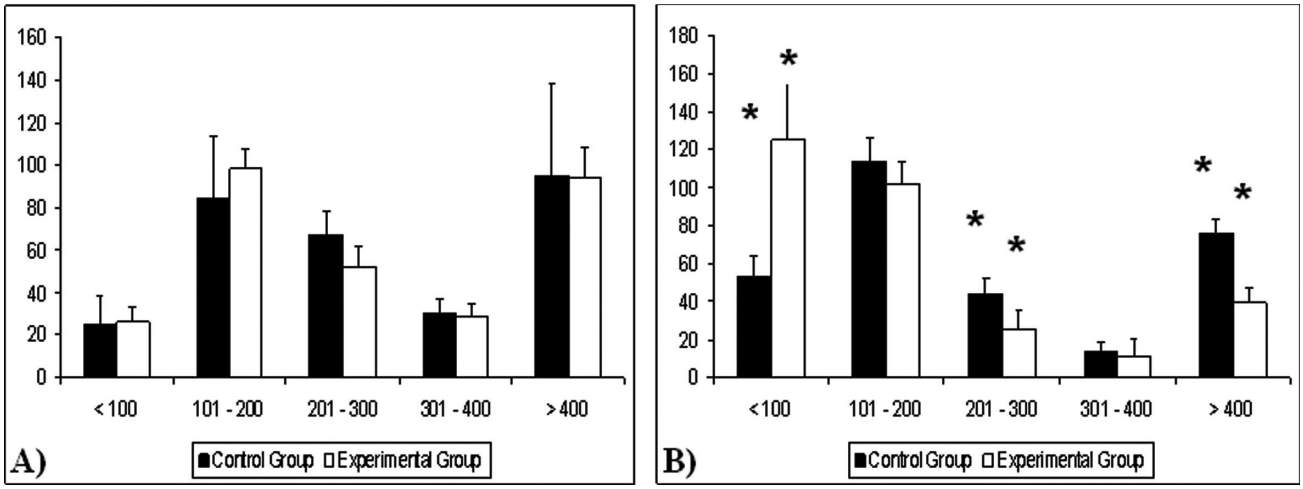

Fig. 3. Histogram of absolute frequency of NADH-diaphorase positive (A) and NADPH-diaphorase positive (B) neurons in the descending colon of rats submitted to normal (Control Group) and hypoproteic diet without supplementation of B-complex vitamins (Experimental Group) distributed in classes in relation to soma area. Columns of the same class marked with an asterisk (*) are significantly different at a 5% significance level.

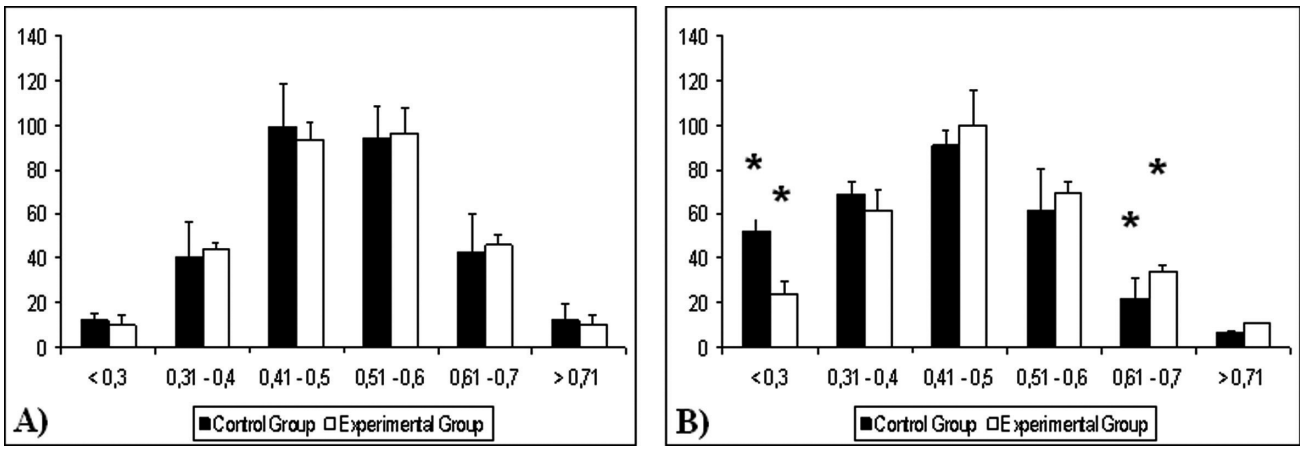

Fig. 4. Histogram of absolute frequency of NADH-diaphorase positive (A) and NADPH-diaphorase positive (B) neurons in the descending colon of rats submitted to normal (Control Group) and hypoproteic diet without supplementation of B-complex vitamins (Experimental Group) distributed in classes in relation to nucleus / soma area ratio. Columns of the same class marked with an asterisk (*) are significantly different at a 5% significance level.

DISCUSSION

Morphometric analysis of NADH-diaphorase positive neurons showed no loss in soma area. However, nucleus area decreased in 28.2% when the hypoproteic diet was used. Considering that the size of the nucleus is directly related to cell metabolism (Andrade & Jordão, 2005), it may be inferred that these cells have been inhibited in a moment of deprivation of exogenous amino acids, mainly because they represent a more active group. This inference is reasonable, considering that rats in the malnourished group needed to spare nutrients.

Previous quantitative studies of this same research group showed that a diet containing 8% protein caused a 0.9% reduction in the number of neurons evidenced by NADH-diaphorase in the ascending colon (Sant'Ana *et al.*, 1997), and a 28.3% reduction in the descending colon (Araújo *et al*., 2003). A diet containing 4% protein led to a 27.2% reduction in this neuron population in the ascending colon (Sant'Ana *et al.*, 2006). A pan-neuronal marking in the descending colon showed that the number of neurons was constant (Araújo et al., 2006). These data

demonstrate that diets with 8% protein were able to preserve the metabolic activity of NADH-diaphorase positive neurons, keeping their numbers constant and, as demonstrated in this study, maintaining their soma area.

NADPH-diaphorase is an enzyme colocalized with nitric oxide synthase (NOS). The histochemical technique that evidences this enzyme shows a neuronal subpopulation involved in the nitrergic route, which potentially inhibits muscle contractions (Scherer-Singler *et al.*; Young *et al.*, 1992). Therefore, reduction in nucleus and soma areas, as observed in this study, shows that the expression of NADPH-diaphorase was reduced, probably due to the decreased availability of amino acids. This may have induced myenteric neurons that produced this enzyme to prioritize the synthesis of other proteins essential for their survival. Studies demonstrated that population density of this neuron class is reduced from 25 to 50% in the colon of rats submitted to hypoproteic diets (Sant'Ana *et al*., 2001; Araújo *et al.*, 2003; 2006). It should be emphasized that protein deficiency associated with lack of supplementation with B-complex vitamins is more severe, leading to a deficit of 92.31% in colonic development (Araújo *et al*., 2003).

Altered intestinal motility is one of the characteristics of malnutrition, leading to longer intestinal transit time or to diarrhea (Viteri & Schneider, 1974). Results obtained in this study suggest that malnutrition may cause intestinal transit to be unbalanced, once atrophy of nitrergic neurons responsible for non-adrenergic and non-cholinergic relaxation of smooth muscles (Takeuchi *et al*., 1998) may indicate reduction in their function.

It is important to emphasize that nitric oxide (NO) has a role in the preservation of myenteric neurons (Cowen *et al.,* 2000), and that NO in neurons of the submucous plexus - possibly together with Vasoactive Intestinal Peptide (VIP) – is important for neuronal adaptation, maintenance and survival (Lin *et al.*, 2004). There are descriptions demonstrating that the numbers of nitrergic neurons may be increased, showing that this subpopulation is less vulnerable to cell death (Cowen *et al*.), possibly because they have improved their defense mechanisms against damage caused by free radicals. Thus, reduction in the area of NADPH-diaphorase positive neurons observed in this study may be considered to be a compensation mechanism, preventing their death. On the other hand, the role of intracellular NO may be different. It may have a cytoprotection role in some instances, and in other situations, it may be cytotoxic. Thus, it has been considered to be both anti- and proapoptotic, depending on the conditions and on the type of cell (Wiley, 2007).

Myenteric neuron atrophy as a response to malnutrition was also observed in other studies involving the large (Sant'Ana *et al*., 1997, 2006; Castelucci *et al.,* 2002) and the small intestines (Torrejais *et al*., 1995; Natali *et al.*, 2003). However, some studies showed that a diet containing 4% protein cause hypertrophy of myenteric neurons in the ascending (Hermes et al.) and descending colon (Fontes *et al.*, 2007), results that were different from those obtained in the present study.

The strong correlation observed in both groups and techniques demonstrated that changes in the nuclei of myenteric neurons may accompany (as causes or consequences) the changes observed in their somas.

The comparison of the number of neurons distributed in classes according to their nucleus / soma arearates showed a predominance of neurons whose nucleus occupied from 41 to 60% of the soma in both technique used in this study. However, comparing the control and experimental group of each NADPH-diaphorase class, it was observed that there was a reduction in the number of neurons whose nucleus occupied less than 30% of the soma. These neurons probably moved up into the class where the nucleus occupied 61-70% of the soma.

The analysis of the number of neurons according to soma area showed no difference between the control and experimental groups for neurons evidenced by NADH-diaphorase. However, many neurons larger than 400 μm^2 – considered to be big – were observed. This may be due to the technique used, once NADH-diaphorase marks those neurons that have a more active metabolism. The NADPH-diaphorase technique, on the other hand, showed an increase in neurons smaller than 100 μm^2 in the experimental group, what was probably counterbalanced by the reduction in the number of neurons between 201 and 300 μm^2 and larger than 400 μm^2. There are similar descriptions in the literature; some authors found a lower incidence of large neurons (Meilus *et al*.; Sant'Ana *et al*., 1997, 2006; Torrejais *et al*.).

It was concluded that protein and complex vitamin deficiency caused a reduction both in the soma and nucleus areas of NADPH-d positive myenteric neurons and only in the nucleus areas of NADH-d positive myenteric neurons in the descending colon of adults rats.

ARAÚJO, E. J. A.; HERMES,C.; NETO, M. H. M.; ALMEIDA, E. C. & SANT'ANA, D. M. G. Atrofia en neuronas mientéricas nitrérgicas en el colon descendente de ratas sometidas a deficiencia de proteínas y vitaminas. ***Int. J. Morphol., 27(3)***:939-945, 2009.

RESUMEN: Esta investigación buscó evaluar los efectos

de la desnutrición proteica y vitamínica delcomplexo B sobre aspectos morfométricos del plexo mientérico del colon descendente de ratones adultos. Dieciséis animales fueron distribuidos en dos grupos: control que recibieron ración comercial con 22% de proteína y experimental alimentados con ración de tenor proteico reducido para 8%, durante 120 días. Neuronas del plexo mientérico presentes en preparados totales fueron evidenciados a través de la técnica histoquímica de la NADH-diaforasa y de la NADPH-diaforasa. En el grupo experimental, las neuronas NADH-d positivos sufrieron reducción del núcleo celular, ya las neuronas NADPH-d sufrieron atrofia de 41,7% de la superficie de su pericarion, lo que hizo con que el núcleo celular pasase a ocupar una mayor proporción de la región trófica de las neuronas.

PALABRAS CLAVE: Desnutrición; Intestino grueso; Sistema nervioso entérico; Plexo mientérico; Plasticidad neuronal.

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