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QUANTITATIVE ANALYSIS OF THE NEURONS FROM THE MYENTERIC PLEXUS IN THE ILEUM OF RATS SUBMITTED TO SEVERE PROTEIN DEFICIENCY

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Abstract – The effects of protein malnutrition on the quantitative aspects of the myenteric plexus in the ileum of adult *Rattus norvegicus* were assessed. Thirty 90-day-old rats were divided into two groups: Control Group (CG, n=15) and Experimental Group (EG, n=15). The CG received 26% protein chow and the EG received 4% protein chow for 90 days. At the end of the experiment, the animals from the CG weighed 369.63±26.33, and the ones from the EG 215.34±56.31. The ileum was submitted to Giemsa, NADH- and NADPH-diaphorase technique in order to evidence nervous cells in the whole-mount preparations. Animals from the EG presented a 41.75% body weight loss in relation to the CG as well as 17.6% length reduction for the ileum-jejunum. Moreover, the organ was 41% lighter for the EG. Giemsa-stained neurons were 17.02% more concentrated in the EG ($p>0.05$). NADH-diaphorase-stained neurons were 26.6% more concentrated in the EG ($p<0.05$), while the NADPH-diaphorase were 26.28% more concentrated in this group ($p<0.05$).

KEY WORDS: protein malnutrition, rat ileum, enteric neurons.

Análise quantitativa dos neurônios do plexo mientérico do íleo de ratos submetidos a intensa carência de proteínas

Resumo – Avaliou-se o efeito da desnutrição protéica sobre o número de neurônios mientéricos do íleo de ratos adultos. Foram utilizados 30 animais (90 dias de idade), divididos em dois grupos: controle (GC, n=15) e experimental (GE, n=15), sendo oferecido ao GC ração com teor protéico de 26% e, para o GE, ração com 4% de proteína, durante 90 dias. Os animais do grupo controle pesaram 369,63±26,33g e o experimental 215,34±56,31g. Preparados de membrana do íleo foram submetidos à técnica de Giemsa, NADH- e NADPH-diaforase. Os animais do GE apresentaram perda de peso de 41,75%, em relação ao GC e redução do comprimento do jejuno-íleo de 17,6%, além disso, o órgão apresentou-se 41% mais leve no GE. Os neurônios corados com a técnica de Giemsa apresentaram-se 17,02% mais concentrados no GE ($p>0,05$). Os neurônios NADH-diaforase apresentaram-se 26,60% mais concentrados no GE ($p<0,05$). E os neurônios NADPH-diaforase apresentaram-se 26,28% mais concentrados neste grupo ($p<0,05$).

PALAVRAS-CHAVE: má nutrição protéica, íleo, rato, neurônio entérico.

The digestive tube presents its own nervous system named enteric nervous system (ENS), from the esophagus through the end of the anal canal. The ENS independently and integratively coordinates all the digestive processes (nutrient absorption, secretion, and intestinal motility) even though it differs from the sympathetic and parasympathetic nervous systems with respect to its structure[1,2]. The ENS is constituted by groups of neurons organized in ganglions and interconnected by nervous fiber bundles constituting the intramural plexuses. Among these, myenteric and submucous plexuses are the most important for the coordination of the digestive activities. Comprehending the functioning and alterations of the myenteric plexus is aimed in the scientific studies[1,2]. Morphofunctional alterations in the enteric plexuses may occur due to age and unbalanced diets. Thus, studies inducing nutritional deficiency may aid explaining common clinical signs on the malnourished such as abdominal pain, constipation, fecal incontinence, diarrhea, and malabsorption[3].

Malnutrition is a set of pathological conditions and

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may differently occur resulting in either reversible or irreversible organic alteration[4]. All normal metabolic processes demand protein participation and all tissues are affected by a state of protein malnutrition; however, it does not present the same speed and type of modification. The former tissues suffering alterations caused by protein deficiency are those which present high cell renovation rates such as the intestinal mucosa, as the latter are those presenting low cell renovation rates such as the nervous system[5]. A number of articles demonstrate that the small intestine in face of malnutrition situations suffer functional and morphological alterations. Hypoplasia and hypotrophy of the mucosa in malnourished rats[6,7] and the increase of the myenteric neuronal density while reducing the protein level on the chow to 8%[6,8-13].

As there is a gap in the literature with respect to the effects of severe malnutrition (4%) on the enteric neurons, this paper analyzed the neuronal density of the neurons from the myenteric plexus in the ileum of malnourished rats subjected to a hypoproteic diet (4%) for 90 days.

METHOD

The experimental protocol was previously approved by the UNIPAR (Universidade Paranaense) Ethics Committee on Animal Experimentation.

Thirty male 90-day-old Wistar rats (303.8±29.73g) were divided into two groups: Control Group (CG; n=15) and Experimental Group (EG; n=15), housed in individual cages with constant temperature in dark/bright (12/12hr) cycle, receiving water and chow *ad libitum*. The CG was maintained on 26%-protein-commercial NUVILAB® (Paraná, Brazil) chow, the GE was maintained on 4%-protein- prepared chow[14].

Animals from both groups were weighed and monitored regarding their water and chow intake weekly.

After 90 days, the animals were anesthetized after a 12-hr fasting according to the following protocol: Acepran (1.26 mL/Kg) + Ketamine 10% (1.26 mL/Kg) + Xilazine 2% (0.42 mL/Kg) e Atropine 1% (0.22 mL/Kg) intramuscularly administered[15]. Laparotomy was carried out and the ileum-jejunum from each animal was removed, weighed, and measured with a millimeter ruler.

The terminal ileums from 5 other rats were washed with a 0.9% NaCl solution and immersed in a formal acetic fixation solution for 48 hour, then dissected and stained with Giemsa solution[16].

The ileums from other 5 animals from each group were submitted to the NADH-diaphorase histochemistry. Segments were first filled and washed twice (10 min) with Krebs solution (pH 7.3), second immersed in 0.3% Triton X-100 for 5 min in Krebs and washed (2x10 min, each) , then immersed for 45 min in an incubation medium containing in every 100 mL: 25 mL of stock solution of the Nitro Blue Tetrazolium (NBT, Sigma, St. Louis, USA); 25 mL 0.1M phosphate buffer, pH 7.3; 50 mL of distilled water, and 5 mg of β-NADH (Sigma, Steinheim, Germany)[17].

The ileums of other 5 animals from each group were washed and filled with phosphate buffer (pH 7.4) fixed with 4% paraformaldehyde (Merck, Darmstad, Germany) in 0.1M PBS (pH 7.4) for 30 min, immersed in Triton X-100 (Sigma, St. Louis, USA) in 0.3% 0.01M PBS (pH 7.4), then washed (10 x 10 min, each) in PBS and submersed for 60 min in an incubation medium containing in every 200 ml.: 200 ml tris-HCl (GibcoBRL, New York, EUA); 0.05g NBT (Sigma, Steinheim, Germany); 0.1 g β-NADPH (Sigma, Steinheim, Germany), and 0.6 mL Triton X-100[18]. Reactions were monitored in a stereoscopic microscope.

The whole-mount preparations from the different techniques were dissected under a transillumination stereomicroscope by removing the mucosa and the submucosa. Then, they were dehydrated in ascending series of ethanol, diaphanized in xilol and mounted among slides and coverslips with Permount® synthetic resin (Fischer Chemical, New Jersey, USA).

Sampling was carried out in order to quantify the myenteric neurons. Forty microscopic fields were counted on the following regions: mesenteric, intermediary, and antimesenteric on all animals and techniques on both groups. A MOTIC B1 microscope (40x objective lens) was used for counting. All the neurons from each field were counted considering the half-neurons from each alternate field. The area on each microscopic field was 0.21 mm^2.

All data were first submitted to the Kolmogorov-Smirnov test in order to verify the distribution type. Thus, data from the normal distribution were expressed as mean±standard deviation. *T*-test for independent samples was used to compare information between the CG and the EG. p values lower than 0.05 were considered statistically significant.

RESULTS

The weight from the experimental rats decreased (215.34±56.31g) in comparison with the control group (369.63±26.33g) ($p<0.05$) in the end of the experiment.

Length and body weight of the ileum-jejunum was significantly low for the EG as described on Table 1.

Results from the quantitative analysis of the neurons by three different techniques are described on Table 2.

DISCUSSION

The comparison of the final body weight between the CG and the EG demonstrated a difference of 41.75% ($p<0.05$). Studies with different experimental models of malnutrition carried out with rats with different ages also demonstrated retarded body weight gain for the animals while fed with a 8% hypoprotein diet[6,8,9,10,13]. Body weight reduction was probably a result of the adaptation to the protein malnutrition due to lower nutrient availability, as well as lower development of fat and lean mass in these animals. Such adaptation possibly demonstrates a mean of maintaining the metabolism of the noble tissues already constituted (such as the nervous tissue) in order to keep them alive longer so that the animal is able to find a new source of food fulfilling the demand of its organism[19].

Table 1. Length and weight of the ileum-jejunum of rats normally fed (Control Group – CG) and subjected to protein desnutrition (Experimental Group – EG).

Parameter	GC (n=15)	EG (n=15)
Length (cm)	105.00±5.58[a]	86.55±13.84[a]
Weight (g)	9.82±1.75[b]	5.79±1.14[b]

Data presented as mean±standard deviation. Means followed by the same letter on the same row showed significant difference ([a]p=0.001; [b]p<0.0001.

Table 2. Populational density of myenteric neurons of the ileum of rats normally fed (Control Group – CG) and subjected to protein desnutrition (Experimental Group – EG) in an area of 25.2 mm^2.

Technique	CG (n=5)	EG (n=5)
Giemsa	6,648.6±790.2	8,012.0±1,368.4
NADH-diaphorase	2,383.4±721.84a	3,247.0±337.15a
NADPH-diaphorase	1,083.4±170.37b	1,469.6±231.92b

Data presented as mean±standard deviation. Means followed by the same letter on the same row showed significant difference ([a]p=0.0416; [b]p=0.0170.

Protein level reduction not only commits body development but also organs and system[6]. They respond differently to malnutrition effects and the digestive tube is usually committed by the amino-acid deficiency[5].

The ileum-jejunum was 17.6% smaller and 41% lighter for the EG which demonstrates that the protein level reduction committed the normal development of the organ (p<0.05). While studying the protein availability reduction to 8% for rats during pregnancy, lactation, and weaning until their 60-day-old, it was observed a 45.21% reduction on the ileum length and wall thickness[6]. The small size of the organ may be considered as an adaptive response either to the decreased amount of chow or the reduced metabolic rate[19]. The reduction of the organs is believed to be a reflex of its own tissue alterations as they may present different degrees of commitment according to their own structural and cellular organization[6].

By comparing the total population density of the stained neurons with Giemsa technique, neurons were expected to be 17.60% more concentrated as the organ was 17.60% smaller; however, the concentration was 17.02% - a very close value not statistically significant – indicating that there were not any reductions on the total population of neurons. Previous studies carried out on malnourished animals have also showed the preservation of total neuronal population in the ileum of rats[6,9,10]. Other experiments on different segments in the small intestine did not evidence any myenteric neuron losses[12,20] as well when rats were submitted to different protein level malnutrition. The protein level was probably sufficient to ensure neuron survival in this study reinforcing the hypothesis that the reduction of chow during aging has demonstrated to be a neuronal population protection factor providing a longevity increase[21,22].

Positive NADH-diaphorase neurons from the EG were 26.60% more concentrated. Malnutrition does not increase the number of total neurons as it is determined since the embryonic life, therefore, the neurons which were not from this class started to do it after malnutrition[13]. Subpopulation of positive NADH-d neurons on CG was 35.85% of the total neuronal population and 40.53% on the EG. As the neurons present the highest metabolic activity[23], protein reduction seemed to have activated neuronal metabolism in this study. Rats submitted to 8% protein malnutrition for 120 days have also presented an increase with respect to this neuronal subpopulation in the duodenum[12] and 96.7% higher in the jejunum[13]. On the other hand, long-term diet restriction demonstrated that positive NADH-diaphorase subpopulation in the ileum decreased for the 24-month-old animals without reducing total population. The authors associate such reduction to lower enzymatic activity relating to NADH-diaphorase as though it is not associated with neuronal death[22].

NADPH-diaphorase neurons were 26.28% more concentrated than those in the CG. Although NADPH-d histochemistry does not evidence neurons the overall neuronal population, it has been largely used for evidencing neurons presenting the NO synthase responsible for the production of nitric oxide[24] – an important mediator of the intestinal relaxing. The nitrergic subpopulation on the CG was 16.3% and 18.34% on the EG, demonstrating amplification. As the studied organ was 17.60% smaller, the natural concentration increase of the subpopulation would be 17.60%; however, 26.8% was verified suggesting a 9.2% neuron increase expressing the NOS. It has been studied that the positive NADPH-d neurons are invulnerable to cellular death in animals under controlled diet[22]. Studies carried out on young and old rats submitted to chow intake restrictions showed that the NADPH-diaphorase neurons reduced in average of 50% during the aging period when they were normofed in the CG. Nevertheless, when they received a 50% normal diet, there were not any neuronal reductions showing that there were not any losses due to aging. In the same study, animals fed with just a 25% normal diet did not present any neuronal losses at 30 months of age as well[22].

In this study, we observed that 4% protein malnutrition for 90 days of age resulted in the decrease of the body weight, as well as the organ size and weight. When comparing the neuronal density, we observed that malnutrition did not cause in any losses with respect to the

overall neuronal population, and resulted in an increase of the positive NADH-diaphorase and NADPH-diaphorase neuron subpopulations possibly reflecting the organ functional adaptation to protein deficiency.

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