

Morphoquantitative evaluation of the duodenal myenteric neuronal population in rats fed with hypoproteic ration

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ABSTRACT: The purpose of this work was to analyze the morphoquantitative behavior of the neurons of the myenteric plexus, as well as the morphometry of the duodenal wall, in adult rats fed with normoproteic (22%) and hypoproteic (8%) rations, killed at the age of 345 days. For neuronal assessments duodenal whole-mounts stained with the Giemsa method were used, and for the evaluation of the duodenal wall routine histological processing and staining with Hematoxilin-Eosin were employed. The means of the number of neurons in 80 microscopic fields (12.72 mm²) and of the size of the neuronal cell bodies did not reveal statistically significant differences between the groups, but there was a greater incidence of large neurons in the protein restriction group (RP). The duodenum was markedly smaller in the RP group and, although there was no difference in the thickness of its wall, the mucosa was larger and the muscular layer was smaller in group RP. It was concluded that the neuronal and non-neuronal components of the duodenum adjust themselves to the nutritional condition, assuring the maintenance of their functions.

Introduction

The reduction of the protein level in the formulation of rodents chow and its consequences to the intestinal morphology has been raising the interest of many researchers. Among the effects observed it is highlighted the reduction in the size of the organs and, depending on the time interval and the degree of restriction, significant reduction in the thickness of the intestinal wall (Viteri and Schneider, 1974; Natali *et al.*, 1995; Torrejais *et al.*, 1995; Natali *et al.*, 2000).

Morphometric variations were verified in the mucosa (Takano, 1964; Viteri and Schneider, 1974; Natali *et al.*, 1995), submucosa (Shrader and Zeman, 1969; Torrejais *et al.*, 1995) and outer muscular layer (Takano, 1964; Hill *et al.*, 1968; Natali *et al.*, 2000; Brandão *et al.*, 2003).

Evidence points to the muscular layer as the target tissue responsible for the maintenance, development and plasticity of the neurons of the Enteric Nervous System (ENS), more specifically the myenteric or Auerbach's plexus, located between the inner circular and the outer longitudinal layers of the muscular tunica. In opposition, the other neurons, certainly, depend on neurotrophic factors for their maintenance and development (Saffrey and Burnstock, 1994).

According to Gabella (1987), the variations that take place on the peripheral neurons are simultaneous to the occurrence of variations in the organs that they innervate, and this manifestation of neuronal plasticity is not

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restricted to the initial periods of body growth, but instead persists in the completely differentiated nervous tissue.

The association of studies in the fields of nutrition and neuronal plasticity has become a fruitful area of investigation, because while it is known that different diets have profound effects on the general morphology of the gastrointestinal tract, little is known on the interdependence of the neuronal and non-neuronal mechanisms that subside these changes (Furness and Costa, 1987).

Morphologic and quantitative evaluations of the neurons of the myenteric plexus of the small intestine, carried out in rats subjected to protein desnutrition during gestation and lactation, have revealed morphologic changes of the neuronal cell body and of the number of neurons in the disnurtured animals (Torrejais *et al.*, 1995; Natali and Miranda-Neto, 1996; Meilus *et al.*, 1998; Brandão *et al.*, 2003).

Myenteric neurons of adult rats (Natali *et al.*, 2000, 2003) also show significant reduction in the size of the cell body, and frequently the alterations were linked to morphometric changes of the intestinal wall.

This study has the purpose of analyzing morphoquantitatively the neurons of the myenteric plexus and the morphometry of the duodenal wall of adult Wistar rats, fed with a hypoproteic chow for a prolonged time.

Material and Methods

Animal treatment

All procedures in this study that involve the use of animals are in accordance to ethical principles and were approved by the Ethical Committee on Animal Experimentation of the State University of Maringá.

Samples of duodenum of 10 adult male Wistar rats (*Rattus norvegicus*) aging 345 days were used. These animals were kept in individual cages since the age of 210 days, at constant temperature and light/dark cycles of 12/12 hours.

The animals were distributed in two groups. The Control Group (C) had five animals fed with standard chow for rodents – NUVILAB-NUVITAL (recommended by the National Research Council & National Health Institute – USA), with protein level of 22%. The Group with Protein Restriction (RP) had five animals that during 135 days received chow with protein level of 8%, obtained through the addition of corn starch to the NUVILAB chow. This ration was supplemented with hydrosoluble vitamins of complex B and saline

mixture (American Institute of Nutrition, 1977; Natali and Miranda-Neto, 1996; Natali *et al.*, 2000; 2003). Chow and water were offered *ad libitum* to the animals of both groups.

The body weight of the animals was recorded each fifteen days, and the consumption of chow was controlled for a week (seven days) each month. This control consisted in offering 100 grams of ration daily to each animal and weighting the leftovers.

At the age of 345 days the animals were weighted and their blood collected for the essays of total protein (Biuret Method – LABTEST), albumin (Bromocresol Green – LABTEST), and globulins (taken as the difference between total protein and albumin). After inhalation of ethylic ether they were killed, laparotomized and the initial segment of the small intestine (sectioned immediately after the pylorus and proximal to the duodenojejunal plica) was removed, measured and weighted.

Morphoquantitative study of the myenteric neurons

Samples of duodenum (proximal or oral end) were washed with saline, filled and immersed in Giemsa fixative (modified from Barbosa, 1978) for 24 h. Next they were microdissected under stereomicroscope with trans-illumination, preserving the muscular tunica and the serosa. The whole-mounts were then subjected to staining with the methylene blue's based Giemsa stain, in Sorensen's phosphate buffer 0.1 N (pH 6.9) during 24 h at room temperature.

After dehydration in ascending series of alcohols, the material was diaphanized in xylene and mounted in slide with coverglass with Permunt synthetic resin.

The counts of the myenteric neurons were made through sampling under light microscope using 40X objective. The neurons observed in 80 microscopic fields of each whole-mount were counted, considering the intermediate and antimesenteric regions of the circumference. The area of the microscopic field, measured with the aid of a micrometered lens, was 0.159 mm², yielding a total area of 12.72 mm².

The areas of 100 neuronal cell bodies, totaling 500 cells per group, were measured in the whole-mounts stained with Giemsa with the help of a computerized image analysis system (Image-Pro Plus 5.0, Media Cybernetics), coupled to the microscope. Based on the mean \pm standard deviation of the areas for the control group, the neurons of both groups were labeled as small, medium and large.

Histological procedure

After being washed in saline, samples of duodenum (distal or aboral end) of five animals of each group were fixed in 10% formol solution, dehydrated, diaphanized and included in paraffin, then cut into 5- μ m thick histological sections which were stained with Hematoxilin-Eosin.

The sections were used in the morphometric evaluation of the mucosa and muscular layers and total intestinal wall with the computerized image analysis system.

Statistical Analysis

The quantitative data obtained were subjected to Student's t test. The significance level was 5%. Data are expressed as mean \pm standard deviation.

Results

Body weight and food consumption

The mean body weights of the rats, obtained each fifteen days during the 135 days of treatment, did not differ significantly in this period (Table 1).

Rats fed with hypoproteic chow (group RP) ate less than the rats fed with normoproteic ration (Table 2). These values correspond to the mean seven-day ingestion of each month during four months, from 210 days of age on.

Dosage of total protein and protein fractions

Total protein and globulin levels in the RP group were significantly lower than those observed in the control group. The albumin fraction from the RP group

TABLE 1.

Body weights (grams) of the control group (C) and the group with protein restriction (RP). Mean \pm standard deviation (n=5).

Groups	Days									
	210	225	240	255	270	285	300	315	330	345
C	437.9a \pm 16.89	446.1a \pm 19.24	451.6a \pm 20.43	460.4a \pm 19.20	461.0a \pm 16.78	469.2a \pm 24.00	471.2a \pm 21.08	473.6a \pm 26.18	469.2a \pm 30.64	459.4a \pm 27.04
RP	432.9a \pm 45.64	435.3a \pm 40.26	440.0a \pm 42.00	449.8a \pm 48.54	455.0a \pm 51.70	453.9a \pm 57.75	443.4a \pm 58.08	441.6a \pm 64.45	448.2a \pm 63.92	435.2a \pm 67.81

Means followed by the same letter do not differ statistically (Student's t test, $p < 0.05$).

TABLE 2.

Ration consumption (grams) of the control group (C) and the group with protein restriction (RP). Mean \pm standard deviation (n=5).

Groups	Month				
	1° month	2° month	3° month	4° month	X
C	28.5 \pm 2.98 a ⁽¹⁾	28.2 \pm 1.67 a	28.1 \pm 1.59 a	25.2 \pm 1.74 a	27.5 \pm 1.57 a
RP	21.3 \pm 2.16 b	20.2 \pm 1.73 b	19.7 \pm 1.14 b	18.9 \pm 1.97 b	20.0 \pm 0.97 b

Means followed by the same letter do not differ statistically (Student's t test, $p < 0.05$).

had a significant increase when compared to that of the control group (Table 3).

Morphoquantitative study of the myenteric neurons

The number of neurons counted on the Giemsa whole-mounts revealed a mean of 4,090 neurons \pm 456.0 (32.154 n/cm²) for the control group (C), and a mean of 3,472 neurons \pm 922.2 (27.295 n/cm²) for the group with protein restriction (RP). The statistical test showed that these differences did not attain significance.

As for the area of the cellular profile, it was verified a wide range of cell body sizes for these neurons, from 20.44 to 380.99 μ m². The mean obtained for the area of the cell bodies in the animals subjected to protein restriction was 159.6 \pm 16.77 μ m² and for the control group it was 141.8 \pm 13.65 μ m², so that the difference was not significant (Figs. 1A and 2B).

According to the values of the mean and standard deviation obtained in the control group it was possible to classify the neurons. In this way, small neurons were those having cellular areas \leq 128.15 μ m², large neurons were those with cellular areas \geq 155.46 μ m², and medium neurons had their areas between 128.16 μ m² and 155.45 μ m² (Table 4 and Fig. 2).

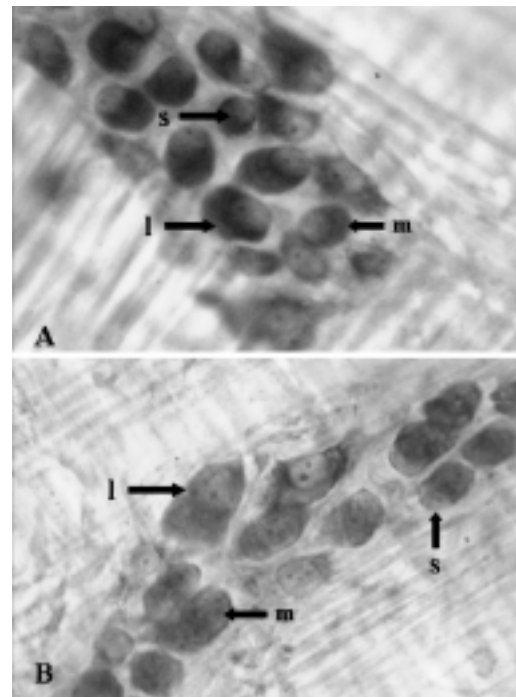


FIGURE 1. Whole-mount of the duodenum depicting small neurons (s), medium neurons (m) and large neurons (l). A. Control group (C), normoproteic ration (22%). B. Group with protein restriction (RP), hypoproteic ration (8%). Giemsa, X 612.

TABLE 3.

Total protein, albumin and globulins (g/dl) of the animals of the control group (C) and the group with protein restriction (RP). Mean \pm standard deviation (n=5).

Variable	Total protein	Albumin	Globulins
C	6.76 \pm 0.14a ⁽¹⁾	3.19 \pm 0.10a	3.57 \pm 0.12 ^a
RP	6.23 \pm 0.09b	4.12 \pm 0.17b	2.11 \pm 0.21b

(1) Means followed by the same letter do not differ statistically (Student's t test, $p < 0.05$).

TABLE 4.

Classification of the neurons of the control group (C) and the group with protein restriction (RP) according to size using the mean size of the neurons of the control group (C). n=500/group.

Groups	Small ($\leq 128.15 \mu$ m ²)	Medium (128.16 and 155.45 μ m ²)	Large ($\geq 155.46 \mu$ m ²)	Total
C	47.8% (239)	19.4% (97)	32.8% (164)	100% (500)
RP	37.0% (185)	16.2% (81)	46.8% (234)	100% (500)

Morphometry of the duodenal wall

When the animals were killed the duodenum was weighted and its length measured, revealing that in group RP this organ had a significant decrease in its size, both in its length ($8.5 \text{ cm} \pm 0.44$) and its weight ($0.842 \text{ g} \pm 0.03$) as compared to the control group ($10.4 \text{ cm} \pm 0.67$ and $1.007 \text{ g} \pm 0.05$, respectively).

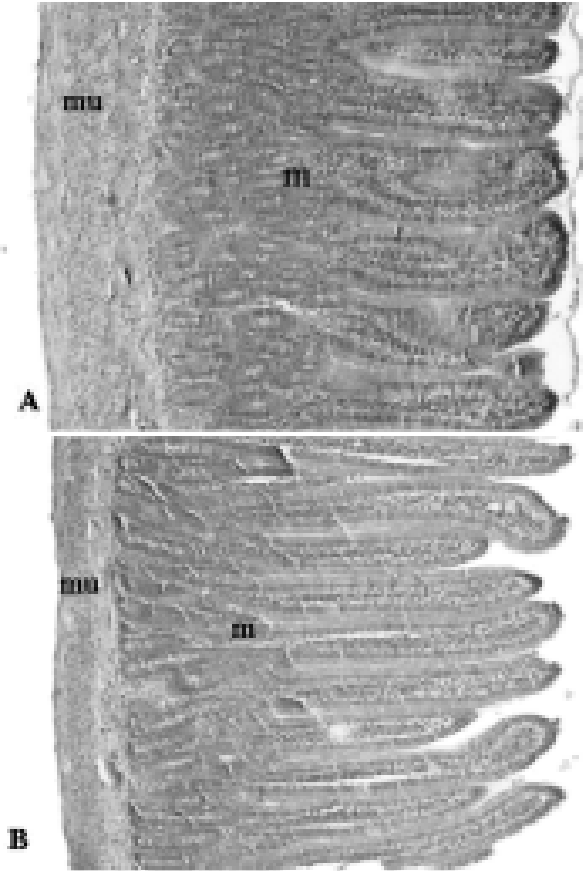


FIGURE 2. Classification of the myenteric neurons according to size.

The morphometric parameters of the histological sections of the duodenum showed significant differences between the groups for the mucosa and muscular layers, although the wall as a whole was similar in both groups (Table 5 and Figs. 3A and 3B).

Discussion

Nutritional aspects

The body weight of the animals subject to the hypoproteic diet (8% protein in the chow) had a behavior similar to that of the control group (22% protein in the chow), evidencing that the reduced protein level did not affect significantly this parameter. Nevertheless, the former group always had smaller fifteen-day weight gains than the normoproteic group.

FIGURE 3. Duodenum of rats. A. Control group (C), normoproteic ration (22%). B. Group with protein restriction (RP), hypoproteic ration (8%). Observe differences in the thickness of the mucosa (m) and muscle layer (um) between groups. H.E X 306.

TABLE 5.

Morphometry (μm) of the mucosa, muscular layer and total wall of the duodenum of rats of the control group (C) and group with protein restriction (RP). The results are expressed as mean \pm standard deviation (n= 5).

Groups	mucosa layer	muscular layer	intestinal wall
C	$635.5 \pm 103.4 \text{ a}^{(1)}$	$110.8 \pm 30.7 \text{ a}^{(1)}$	$806.8 \pm 137.1 \text{ a}^{(1)}$
RP	$673.3 \pm 123.9 \text{ b}$	$77.1 \pm 18.0 \text{ b}$	$804.8 \pm 141.9 \text{ a}$

(1) Means followed by the same letter do not differ statistically (Student's t test, $p < 0.05$).

We consider that the 8% level was sufficient for the maintenance of the body weight, and that the 22% level could be considered excessive, once these animals are aged (345 days) and have lower metabolic rates.

In a previous experiment, Natali *et al.* (2000) supplied the same kind of protein-restricted chow to rats from 90 to 210 days of age. It was also observed the maintenance of the body weight in the group fed with the hypoproteic ration. However, control animals (22% protein level) had a body weight gain of about 45% at the end of the experiment.

As for the organ under investigation, there was a significant decrease in the weight and length of the duodenum in the animals of group RP when compared to the control group (C), thus revealing the smaller growth of this organ.

Aging involves a sequence of physiologic changes with cellular loss and organic decline, decrease of the glomerular filtration rate, intestinal constipation, reduction of glucose tolerance and decrease of the cellular immunity (Stump, 1999).

In this way, the smaller size of the organs could be considered as an adaptive response to the decreased food volume or to the reduced metabolic rate.

This fact could explain the smaller duodenum observed in the animals subject to the protein restriction, despite the steady body weight. A similar result was observed by several authors working with protein restriction models (Younoszai and Ranshaw, 1973; Torrejais *et al.*, 1995; Natali and Miranda-Neto, 1996).

As for the food consumption, assessed through the record of the chow ingestion during seven days each month, we verified that the animals of group RP ingested fewer ration, this reduction being significant compared to group C, and this condition was maintained throughout the experiment. These animals supposedly handled their nutrients better than the controls because, despite the smaller ingestion and the decreased protein level in the chow, their body weight was not decreased and their conditions were similar to those of the control group. The content of raw fibers in the chow is also a factor that could be taken into account. In the control group this level was 4.81% and in the group with protein restriction it was 1.59%; this could have led to a slower intestinal transit and an increased nutrient absorption in the protein-restricted group.

It is worth noting that the smaller ingestion observed during the whole experiment is taken, together with other symptoms, as an indicative of experimental protein deficiency (Lima *et al.*, 1993).

The levels of total protein, albumin and globulins were significantly different in the group with protein

restriction when compared to the control group. The changes in the levels of plasma protein are one of the most important biochemical aspects in models of protein restriction. Our results are indicative that, despite the protein-restricted animals having a body weight similar to the control group, they did suffer with the protein restriction.

In experiments with adult rats fed with chow of smaller protein level during 84 and 120 days, the levels of total protein and globulins were also reduced (Amorim, 1984; Natali *et al.*, 2000).

The albumin fraction, however, did not decrease; indeed, it had a significant increase in the animals of group RP. This was probably caused by a compensatory mechanism aimed at assuring good hemodynamic conditions, steady functioning of the circulatory and renal systems, and at preventing edema.

This compensatory mechanism was summarized by Marcondes (1978) in the following basic sequence: low protein ingestion determines an immediate decrease in albumin synthesis & small decrease in the plasma levels of albumin & compensatory mechanisms represented by albumin translocation from the extravascular to the intravascular compartment and decrease of albumin catabolism.

Our results demonstrate that in aged animals subjected to hypoproteic diet these systems of shunting and/or catabolism reduction were active, as demonstrated in the significant increase in the albumin level of these animals when compared to the controls.

Quantitative and morphometric analysis of the myenteric neurons

The neuronal count was carried out in the intermediate and antimesenteric regions, because the mesenteric region of the circumference is more vascularized and does not show a good definition of its structural elements after staining, rendering the counting of neurons more difficult (Gabella, 1971; Santer, 1994; Natali *et al.*, 2000).

The greater neuronal density in the duodenum of animals with protein restriction is a divergent result in the literature that links the increase of the neuronal density to reduced body weight, intestinal size and thickness of the muscular layer (Torrejais *et al.*, 1995; Natali and Miranda-Neto, 1996; Brandão *et al.*, 2003). It is considered that the smaller body growth leads to the smaller spread of the nerve cells and thus their greater concentration per area.

We believe that the smaller neuronal density verified here in the animals with protein restriction could

be explained by the association of two factors: protein restriction by itself, that would be a less relevant factor, and the aging of the animals leading to neuronal loss, once aging alters the adaptive ability of cells and tissues. The reduction in the number of myenteric neurons is a fairly common result in experimental models of aging (Santer and Baker, 1988; Gabella, 1989; Santer, 1994; Johnson *et al.*, 1998).

The mean value of the neuronal cell body in the control group was $141.8 \mu\text{m}^2$, obtained through the measure of 500 neurons of the control group. Similar values were observed in the duodenum of mice (Bor-Seng-Shu *et al.*, 1994), adult guinea-pigs (Liberti *et al.*, 1994) and adult rats (Natali *et al.*, 2003).

When the neuronal population was classified according to size, we verified that there were differences in the proportions of these neurons in the groups. The control group had 47.8% of small neurons, 19.4% of medium neurons and 32.8% of large neurons, while the group with protein restriction had 37% of small neurons, 16.2% of medium neurons and 46.8% of large neurons.

The mean area of the cell bodies in the animals subjected to protein restriction was $159.6 \mu\text{m}^2$, and was not different when compared to that of the control group.

This result is opposite to those of several authors that subjected their animals to protein restriction, such as Torrejais *et al.* (1995) and Meilus *et al.* (1998) in the ileum of 60-days old rats disnurtured during gestation and/or lactation; and Sant'Ana *et al.* (1997) in the ascending colon of 90-days old rats, and Natali *et al.* (2000) in the duodenum of 210-days old rats with protein restriction during adulthood. In these investigations it was verified a decrease in the mean size of the cell body and an increase in the population of small neurons.

Neuronal hypotrophy is considered a basic response mechanism to situations where the cells are injured, such as nutrient reduction. Also, according to Bogliolo (1981), the neurons adapt themselves with decreased metabolism, diminishing the synthetic events necessary for structural turnover and decreasing their size. Despite these arguments, we believe that the absence of difference in the neuronal size in our investigation could be related to the reduced number of neurons and the increased proportion of large neurons found in the animals with protein restriction.

The non-reduced mean area of the cell body and the possible increase of this area in myenteric neurons together with the decreased neuronal density was also observed by Gomes *et al.* (1997) when they compared adult humans aging 20-35 years with those aging 65. The authors suggest that the increased population of large neurons could be interpreted as a compensatory

growth of the remaining neurons in an attempt to keep an efficient innervation and assure an adequate intestinal motility.

Morphometry of the duodenal wall

The mucosa of the small intestine is characterized by an intense cellular proliferation, which is most sensitive to nutrient reduction (McNurlan *et al.*, 1979) and consequently may lead to mucosal atrophy. The maintenance of a suitable intestinal structure and function depends on the ability of the intestine to adapt to the variations imposed, but this ability can be changed in an age-dependent manner.

Our results indicated a significant reduction in the thickness of the muscular layer and a significant increase of the mucosa in the animals fed with 8% protein, indicating that there was a morphometric variation in face of the protein restriction, although the thickness of the total wall was preserved.

In opposition to most of the works, which observe atrophy of the intestinal mucosa in rats under protein restriction (Takano, 1964; Hill *et al.*, 1968; Viteri and Schneider, 1974; Natali *et al.*, 1995); in this experiment the mucosa had a greater thickness.

The greater thickness of the mucosa in the group RP and the similarity of the thickness of the total intestinal wall in both groups could suggest an important intestinal adaptive mechanism. This mechanism is reinforced by the fact that the hypoproteic-fed group ingested less food, had a smaller duodenum and yet kept a steady body weight throughout the 135 days.

The significant reduction of the muscular layer observed in this experiment is also seen by Takano (1964), Hill *et al.* (1968), Natali *et al.* (2000) and Brandão *et al.* (2003) in experiments of protein restriction. This could be the result of the lack of storage of proteic material in the smooth muscle fibers of the intestinal wall, resulting in smaller thickness when compared to the groups fed with normoproteic chow.

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