

# Quantitative Study of the Myenteric Plexus of the Descending Colon of Young Rats Subjected to Intense Protein Deficiency

Estudio Cuantitativo del Plexo Mientérico del Colon Descendente de Ratas Jóvenes Sometidas a Intensa Deficiencia Proteica

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**SUMMARY:** We studied the effects of a severely hypoproteic diet on the quantitative aspects of the myenteric plexus of the descending colon of young rats. Eighteen rats were divided into two groups, one of them being fed with a chow having 26% protein (control) and the other with a chow having 4% protein, balanced for minerals and vitamins, during 12 weeks. The whole-mounts of the descending colon had their myenteric neurons stained either with Giemsa or NADPH diaphorase. The rats from the experimental group had deficits of body weight (54.23%) and area of the descending colon (48.14%); additionally, we observed that there was no alteration in the total number of neurons of the colon, but a decrease in the number of NADPH-diaphorase positive neurons (37.80%). The implications of these results concerning the priority that some cellular types may have when nutrients are less available are discussed.

**KEY WORDS:** Myenteric plexus; Protein-energy; Malnutrition (PEM); Descending colon.

## INTRODUCTION

Malnutrition still is an important question of public health, encompassing sub-developed and developing countries and loci of poverty in developed countries (Mallikharjuna *et al.*, 2002; Menno *et al.*, 2002; Yuko *et al.*, 2002). The replacement of a balanced diet for another having more energy than proteins is a reality in these countries, once the foods with greater protein levels are often expensive and, frequently, unavailable. In this way, we can consider that the study of the energy-protein malnutrition (PEM) is both important and contemporary.

For many years, studies have been made with the purpose of shedding some light on the effects of PEM on the structure and functioning of several metazoan species. The use of animal models in investigations of food deficiency employing known and controlled diets has the advantage of increasing the knowledge about several aspects of human malnutrition (Enwonwu & Sreebny, 1970). Among these models, the rat has been used the most (Mello *et al.*, 1995; Torrejais *et al.*, 1995; Natali and Miranda-Neto, 1996; Sant'Ana *et al.*, 1997; Meilus *et al.*, 1998; Miranda-Neto *et al.*, 1999; Natali *et al.*, 2000; Sant'Ana *et al.*, 2001; Castelucci *et al.*, 2002; Araújo *et al.*, 2003; Brandão *et al.*, 2003; Natali *et al.*, 2003; Mello *et al.*, 2004). It is considered a good model for nutritional research due to its reduced size, reproductive behavior and adaptability to several diets (NRC, 1995).

As for the repercussions of malnutrition on the functioning of the gastrointestinal system, some findings show that generally there is poor absorption and alteration of the intestinal motility (Douglas, 2004). It is known that the regulation of this motility is made primarily by the enteric nervous system, especially the myenteric plexus, and this is the reason why the study of experimental malnutrition on the enteric neurons is considered to represent a fruitful area for the investigation of neuronal plasticity (Furness and Costa, 1987).

Considering the quantitative studies evaluating the consequences of PEM on the myenteric plexus of rats, it is observed a greater concentration of neurons per unit area, ascribed to a delayed intestinal growth (Mello *et al.*, 1995;

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Torrejais *et al.*; Natali & Miranda-Neto; Recinos, 1996; Sant'Ana *et al.*, 1997; Sant'Ana *et al.*, 2001; Araújo *et al.*, 2003). These studies focus, most of the times, on the duodenum (Santer & Baker, 1988; Natali and Miranda-Neto; Natali *et al.*; Brandão *et al.*, 2003; Mello *et al.*, 2004) and jejunum-ileum (Torrejais *et al.*; Meilus *et al.*; Miranda-Neto *et al.*). Few are those concerned with the large intestine (Mello *et al.*, 1995; Sant'Ana *et al.*, 1997; Sant'Ana *et al.*, 2001; Castelucci *et al.*, 2002), especially the descending colon (Recinos; Araújo *et al.*). Because of this, in this investigation we approached the effect of a hypoproteic diet on the myenteric plexus of the descending colon of young adult rats.

## MATERIAL AND METHOD

We used 18 male Wistar rats (*Rattus norvegicus*) aging 90 days (305.72±35.66g), which were placed in individual metabolic cages and kept in a room with controlled temperature (±25°C) and light/dark cycles (12/12 h). During the whole period, water and chow were offered ad libitum. Protocols for animal handling and killing were approved by the Committee on Ethics in Research Involving Animal Experimentation of the Paranaense University (UNIPAR), Brazil.

The animals were randomly allotted to two groups: control (n=9) and experimental (n=9). The control group (CG) received NUVILAB<sup>®</sup> commercial chow. This was bromatologically analyzed and, from the results, it was calculated the components to be added to reduce the protein level to 4% while keeping the vitamin and mineral balance. This prepared chow was offered to the experimental group (EG). Thus, for each kg of experimental chow it was used 153.85g of ground commercial chow, 53.10g of a mixture of mineral salts, 172.87g of sucrose as table sugar, 391.41g of commercial corn starch, 140.7g of vegetal oil as lipid source and 18g of complex B vitamins as ground pills. The other vitamins were in excess in the commercial formulation and their supplementation was not necessary.

Water was added to allow mixture and pelletization of the chow. The pellets were dried in stove at 55 °C and had their percentual composition assessed (Table I).

Both the CG and the EG were kept for 12 weeks and during this period, we monitored the animals' well being and weighted each rat weekly. At the end of the experiment, the rats from both groups were fasted for 12 hours and anesthetized with the following mixture, administered intramuscularly: Acepran (1.26 ml/Kg) + 10% Ketamine (1.26 ml/Kg) + 2% Xilazine (0.42 ml/Kg) and 1% Atropine (0.22 ml/Kg) (Pachaly *et al.*, 2003). We carried out the laparotomy, removed the descending colon and measured its length and width with a millimeter ruler.

The descending colons of five animals of each group were washed in 0.9% saline, filled and immersed in acetic formol for 48 hours. Then they were dissected and stained with Giemsa solution (methylene blue) in Sorensen's sodium phosphate buffer pH 7.0 (Barbosa, 1978).

The descending colons of the four remaining animals of each group were washed and filled with phosphate buffer pH 7.4. They were fixed for 30 minutes with 4% paraformaldehyde (Merck, Darmstad, Germany) dissolved in phosphate buffer 0.1M pH 7.4, immersed for 10 minutes in 0.3% Triton X-100 (Sigma, St. Louis, EUA) dissolved in saline phosphate buffer (PBS) pH 7.4 and then washed 10 times (10 min each) in PBS. They were then transferred to the incubation medium for neuronal staining through NADPH diaphorase activity for two hours. This medium had, in each 100 ml, 25 mg NBT, 50 mg b-NADPH (Sigma, Steinheim, Germany), and 0.3% Triton X-100 in Tris-HCl buffer (GibcoBRL, New York, EUA) 0.1M pH 6.0. After incubation, the segments were opened at the mesocolic insertion, washed three times in PBS for five minutes and immersed in 5% paraformaldehyde solution (Scherer-Singler *et al.*, 1983).

The whole-mounts were made under stereomicroscope with transillumination through removal of the mucosa and submucosa. Next, they were dehydrated in

Table I. Percentual composition of the chow offered to the control group (NUVILAB<sup>®</sup>) and of the hypoproteic chow prepared for the experimental group.

Component	Control Chow - NUVILAB <sup>®</sup> (%)	Experimental Chow (%)
Humidity	8.58	8.9
Proteins (% N x 6.25)	26.02	4.07
Lipids	6.45	14.8
Ashes	9.05	8.97
Fibers	7.24	2.34
Carbohydrates (by ≠)	42.66	60.92
Energy Value	332.77 Kcal/100g	402.60 Kcal/100g

Source: Percentual composition carried out by the Laboratory of Physic-Chemistries Analyses of the Paranaense University.

ascending series of ethylic alcohol, cleared in xilene and mounted in slide with Permout synthetic resin (Fischer Chemical, New Jersey, EUA).

The whole mounts stained with these two techniques were used to determine the population density of the stained neurons. For that, we used an Olympus microscope and counted the number of neurons seen in 40 fields magnified 400 times, which had an area of 0.1735 mm<sup>2</sup> each. This analysis was made in the intermediate region of the intestinal circumference (60° to 120° and 240° to 300°, considering the mesocolic insertion as 0°).

To analyze possible significant differences on the neuronal densities between the two techniques described, we used the Mann-Whitney test, at a significance level of 5%. To assess a possible significant difference between the mean weights of the two groups we employed the test t at the significance level of 1%. The results are presented as mean ± standard deviation.

## RESULTS

At the end of the experimental period, the animals from the control group had a mean body weight of 385.51 ± 43.26 g and the experimental group 249.96 ± 43.93 g. The

difference between these means attained significance (p<0.01).

Table II presents the length, width and area of the total colon of both groups. Table III depicts the result of the quantitative analysis on the density of myenteric neurons in the whole mounts of the descending colon of both groups. Table IV demonstrates the projection of the total number of neurons and number of NADPH-diaphorase positive neurons for the total area of the colon of the control and experimental animals.

## DISCUSSION

We verified that the reduction of the protein level from 26% to 4% in the chow offered to the animals from the experimental group caused, during the experiment, body weight loss, loss of fur, cutaneous rashes, and diarrhea. These data and observations, together with the smaller growth of the colon, led us to consider that this diet was efficient in inducing malnutrition.

The protein level offered to the EG rats (4%) was not sufficient to allow the body weight evolution of these animals, because they not only did not gain weight but also lost 18.01% of their initial weight after 12 weeks of treatment (p<0.05).

Table II. Mean length, width and area of the total colon of young rats from the control and experimental groups.

Group	Length (mm)	Width (mm)	Area (mm <sup>2</sup> )
Control (n = 9)	151.0 ± 12.9 <sup>a</sup>	15.0 ± 0.5 <sup>a</sup>	2,265.0 ± 208.6 <sup>a</sup>
Experimental (n = 9)	120.0 ± 10.6 <sup>b</sup>	12.8 ± 1.4 <sup>b</sup>	1,529.0 ± 143.9 <sup>b</sup>

Means followed by different letters in the same column are significantly different (p<0.05). The colonic area was calculated as the product of the length and width. Mean ± standard deviation.

Table III Neuronal density of the myenteric plexus of the descending colon of young rats of the control and experimental groups, evidenced through the techniques of Giemsa and NADPH-diaphorase, in an area of 6.94 mm<sup>2</sup> (40 microscopic fields).

Technique	Number of animals	Control	Experimental
Giemsa	5	1,406.4 ± 137.8 <sup>a</sup>	2,217.0 ± 341.9 <sup>b</sup>
NADPH-diaphorase	4	779.5 ± 123.2 <sup>a</sup>	761.0 ± 159.2 <sup>a</sup>

Means followed by different letters in the same line are significantly different (p<0.05). Mean ± standard deviation.

Table IV. Projection of the number of neurons, stained with Giemsa and NADPH-diaphorase, for the total area of the colon of the animals from the control and experimental groups.

Technique	Control	Experimental
Giemsa	456,036.02 ± 17,491.70 <sup>a</sup>	487,883.29 ± 77,809.77 <sup>a</sup>
NADPH-diaphorase	237,610.32 ± 43,897.78 <sup>a</sup>	172,432.28 ± 56,102.34 <sup>b</sup>

Means followed by different letters in the same line are significantly different (p<0.05). Mean ± standard deviation.

Other experimental studies using rats observed retarded body weight gain when the animals were fed with hypoproteic diet containing protein levels equal to or greater than 5% (Enwonwu *et al.*, 1973; Natali *et al.*; Sant'Ana *et al.*, 2001; Araújo *et al.*; Mello *et al.*, 2004); diets with protein levels lower than 5% demonstrated absence of body weight gain and even body weight loss (Enwonwu & Sreebny; Enwonwu *et al.*; Anthony & Edozien, 1975; Castelucci *et al.*, 2002). The reduction of the body weight probably is due to a smaller development of the fat and lean masses of these animals, which may represent an adaptive mechanism in face of the short supply of food. If this is so, the available resources are shifted to keep the metabolism of the "noble" tissues (such as the nervous system) already formed, so as to keep them in a steady-state for a longer time until the animal can find a new food source to meet the demands of the whole organism.

When we compared the area of the total colon of the animals from EG with that of CG, we noticed that the former represented 67.51% of the latter, thus we can say that the 22% reduction of the protein in the diet of growing rats leads to a deficit of 48.14% in the expected growth of the colon. This can be linked to the reduced synthesis of essential proteins needed for cellular hyperplasia and hypertrophy, probably due to the smaller availability of exogenous aminoacids, as well as by the decreased plasma levels of insulin, an important growth-promoting factor (Guyton & Hall, 2002). The scientific literature shows that the smaller growth of the viscera is a common feature of disnurtured children (Waterlow, 1996). A study assessing the effect of a 14%-protein deficiency demonstrated a growth deficit of 57.60% in the growth of the rat colon (Recinos). Although this author supplied more protein than we did, this greater retardation of growth was probably due to the prolonged duration of the experiment (120 days); this is in agreement with prior studied that demonstrate that the cellular response to malnutrition is time-dependent (Winick & Noble, 1966). The retarded growth of the colon is more severe when protein deficiency is associated to the lack of supplementation of complex B vitamins, as demonstrated in a 120-day experiment, in which they observed a development deficit of 92.31% (Araújo *et al.*).

The whole mounts of this study stained with Giemsa would have indicated that the neuronal density of the descending colon of the EG animals was greater than that of CG, but when we consider the projection of these cells for the total colon we verify that there is no significant difference in the number of myenteric neurons between these groups. We infer that the reduced area of the colon of the EG animals is the result of a greater damaging effect of the hypoproteic (4%) diet on the epithelial, connective and muscular tissues, which are those having greater mitotic indices and thus being affected earlier than those having a low turnover index (Deo, 1978); in

this case, the myenteric neurons would be preferably preserved. Castelucci *et al.* (2002) subjected pregnant rats to protein privation and observed that, soon after birth, the colon of the newborn was 40% smaller than that of the controls, but the total number of neurons of the organs had not been diminished. A different result was reached when young rats were subjected to a 8%-protein diet for 120 days (Recinos), and in a similar investigation but without protein supplementation (Araújo *et al.*); in both of them there was no significant alteration in the neuronal density of the descending colon, but on the other hand a number about 48.40% smaller of myenteric neurons was obtained, when considering the projection of these cells for the whole colon. In studies evaluating the ascending colon, it was also noticed that a 8%-protein diet offered for 120 days, either with or without complex B vitamins supplementation, caused an increase in the neuronal density and a decrease of about 36.68% in the total number of neurons (Sant'Ana *et al.*, 1997; Sant'Ana *et al.*, 2001). This agrees with our discussion concerning the time-dependent repercussions of malnutrition, but indicates that there is a limit for the preservation of the tissues of lower turnover.

As the digestory tract is exposed to a variety of potential injuries, Hanani *et al.* (2003) suggest that the myenteric plexus is endowed with a considerable ability of regeneration and plasticity, as observed in the study of Castelucci *et al.* (2002); they analyzed the litters of female rats disnurtured during gestation and lactation, and observed less reactive NADH-diaphorase positive neurons, but when the standard chow was offered to the weaned young rats the normal appearance of the neurons was reestablished after only 21 days. These authors also suggested that, during protein privation, the enteric neurons are protected when there is a marked deficit of body weight gain and of growth of the gastrointestinal segment, which is in agreement with our results, because we observed a severe deficit of both body weight gain (72.01%) and colon growth (48.14%) and no neuronal loss. On the other hand, the studies reporting neuronal loss during the experiment showed body weight gain or deficits not superior to 20% (Recinos; Meilus *et al.*; Sant'Ana *et al.*, 2001; Araújo *et al.*). This indicates that more studies are needed to evaluate the possible correlation between the deficit of body weight gain and the prevention of myenteric neuron loss, as well as to clarify the possible mechanisms involved in this process. Nevertheless, it should be taken into account that the experimental period, the protein level of the diet and the age of the animals are remarkable variables, much as a presumptive replacement of neurons from undifferentiated (Filagamo & Cracco, 1995) or glial cells (Rühl *et al.*, 2004).

As there was a smaller colonic growth of about 48.14%, it would be expected that the neurons stained with NADPH-diaphorase were more concentrated in a similar

proportion. However, we noticed that there was no alteration in the populational density of these neurons. On the other hand, when we observe the projection of the neuronal subpopulation for the whole colon we verified that 37.80% of the neurons from EG rats were not reactive to the technique when compared to CG. This technique consists in the incubation of the tissues in a medium saturated with NADPH and tetrazolium so that the cells containing the NADPH-enzyme reduce the tetrazolium to formazan using the electrons from NADPH (Scherer-Singler *et al.*, 1983). In this way, the neurons having this enzyme are highlighted from the others by the blue color of the formed salt. Neurons stained with this technique possess the enzyme nitric oxide synthase (NOS) co-localized with the NADPH-diaphorase (Young *et al.*, 1992; Toole *et al.*, 1998). NOS converts the aminoacid arginine to nitric oxide (NO). This is an important neurotransmitter triggering non-adrenergic, non-cholinergic (NANC) relaxation of the smooth muscle tissue (Takeuchi *et al.*, 1998), including that of the colon (Borjesson *et al.*, 1997; Takahashi & Owyang, 1998). Our results allow us to infer that there was a smaller expression of the NADPH-diaphorase enzyme, possibly due to the smaller availability of aminoacids, and this may have induced the neurons having this enzyme to give priority to the synthesis of other, more essential, proteins. However, we cannot assure that in the pathologic situation of this study the co-existence of the NADPH-diaphorase with the NOS is kept in the same proportion; immunohistochemical studies for NOS are needed to test this hypothesis with this experimental model. Yet, if we consider that the lower number of NADPH-diaphorase positive neurons observed in EG is indicative of a lower production of NO, the absence of neuronal loss in disnurtured rats can also be explained by a decreased apoptosis: many neurons release NO soon before dying, which can even lead to the death of neighboring cells because NO is toxic for the cells that do not produced it (Wang *et al.*, 2003). The observation that this neuronal subpopulation tends to be preserved during the natural losses of aging sustains this (Phillips-Robert *et al.*, 2003). In studies carried out with young and aged rats subjected to restricted offer and ingestion of food, it was demonstrated that the population of NADPH-diaphorase positive neurons was normally reduced (about 50%) during aging when the animals were normally fed (control). However, when the animals

received half the normal amount of food, the number of neurons did not decrease, showing that there was no loss in aging; even animals fed with a quarter of the normal food supply did not have neuronal loss at the age of 30 months and an increase with aging was predicted (Cowen *et al.*, 2000).

We can suggest that the effects of malnutrition on the population of nitregic neurons is proportional to the proteic deficit of the diet, if we compare the results of this study with a previous one, in which we assessed the effect of a less severe (8%) hypoproteic diet and verified that there was no significant difference in the projection of NADPH-diaphorase positive neurons for the total colon (Araújo *et al.*). This is an indicative that there is a positive correlation between protein deficiency in the diet and a smaller NANC relaxation of the descending colon that can unbalance the mechanism of fecal propulsion in animals subjected to a severe protein restriction; excitatory neurons could predominate over the inhibitory ones, and this could be an explanation for the diarrhea in some EG rats during the experiment.

In this way, we can suggest that when a 4%-protein is offered in association with a large energy supply to young rats, there are evidence of metabolic alterations, especially in what concerns protein synthesis, with marked damage to several tissues. Nevertheless, the use of exogenous aminoacids seems to be priority for some cellular types, in this case the neurons of the myenteric plexus, which do not show significant reduction of their total number, but did undergo metabolic modulations such as reduction in the expression of the NADPH-diaphorase enzyme. This prompt us to agree with Furness & Costa, that put forward that the study of malnutrition is a means of understanding neuronal plasticity.

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ARAÚJO, A. E. J.; SANT'ANA, G. D. M.; MOLINARI, S. L. & MIRANDA NETO, M. H. Estudio cuantitativo del plexo mientérico del colon descendente de ratas jóvenes sometidas a intensa deficiencia proteica. *Int. J. Morphol.*, 24(4):591-597, 2006.

**RESUMEN:** Estudiamos los efectos crónicos de una dieta severamente hipoproteica sobre los aspectos cuantitativos del plexo mientérico del colon descendente de ratones jóvenes. 18 ratones fueron divididos en dos grupos, a uno de estos grupos se le dió ración con contenido proteico del 26% (control) y al otro, ración con contenido proteico del 4%. Se mantuvo el balance vitamínico y mineral, durante 12 semanas. Elaboramos los preparados de membrana del colon descendente y marcamos las neuronas del plexo mientérico con Giemsa y NADPH-diaforasa. Los ratones del grupo experimental presentaron déficit de peso corporal (54,23%) y del área del colon descendente (48,14%); además, observamos que no hubo alteración en el número total de neuronas en todo el colon; sin embargo, hubo una disminución en la marcación de neuronas NADPH-diaforasa positivas (37,80%). Los resultados son discutidos, respecto a la prioridad que ciertos tipos celulares pudiesen tener, con la menor disponibilidad de nutrientes.

**PALABRAS CLAVE:** Plexo mientérico, Mala nutrición proteico-energética (MPE); Colon descendente.

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