Int. J. Morphol.,
28(2):497-502, 2010.

Intestinal Wall Atrophy and Increase of Sulphomucin Secretion in the Jejunal Epithelium of Rats Submitted to Severe Protein Malnutrition

Atrofia de la Pared Intestinal y Aumento de la Secreción de Sulfomucinas en el Epitélio Yeyunal de Ratas Sometidas a Intensa Desnutrición Protéica

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FRANCO, C. L. M.; SANT'ANA, D. M. G. & ARAÚJO, E. J. A. Intestinal wall atrophy and increase of sulphomucin secretion in the jejunal epithelium of rats submitted to severe protein malnutrition. ***Int. J. Morphol., 28(2)***:497-502, 2010.

SUMMARY: The effects of severe protein malnutrition on the morphometry of the intestinal wall in the jejunum of adult rats were evaluated in this study. Six 90-day-old Wistar rats (*Rattus norvegicus*) were assigned into two groups: CG (Control Group, n = 3) and MG (Malnourished Group, n = 3). CG animals were fed a 26%-protein chow whereas controls were fed a 4%-protein chow. After 90 days, part of the jejunum was collected and subjected to routine histology. HE-staining and histochemical techniques - Periodic Acid Schiff (PAS) and Alcian Blue (AB), pH 2.5/pH 1.0 - were used for the detection of glycoconjugates in 4µm -thick transverse cuts. Morphometric analysis of the HE-stained cuts revealed a decrease of the total thickness of the jejunal wall, mostly on the following layers: external muscle and mucosa - enterocyte height also decreased. Cuts stained by using histochemical techniques for the detection of glycoconjugates revealed maintenance of goblet cells reactive to PAS and AB pH 2.5, whereas the number of cells reactive to AB pH 1.0 increased. Protein malnutrition was concluded to provoke severe atrophy of the jejunal wall and an increase of sulphomucin secretion within the intestinal epithelium.

KEY WORDS: Protein-energetic malnutrition; Small intestine; Morphometry; Mucins.

INTRODUCTION

Malnutrition is still one of the major health issues faced by developing countries, either by the morbidity rate associated to such event or its high prevalence (Oliveira *et al*., 2007).

Morphologic changes caused by malnutrition have been extensively studied in rats due to their reduced size, reproductive behavior and adaptability to different diets (NRC, 1995). Thus, these studies can reflect what occurs with malnourished humans metabolism (Giacomelli & Natali, 1999).

Rats submitted to protein malnutrition are known to present lower growth (Sant'ana *et al*., 1997; Sant'ana *et al.*, 2001; Araújo *et al*., 2003; Araújo *et al.*, 2005; Hermes *et al.,* 2008; De Azevedo *et al*., 2007). Moreover, it is notorious that all organs are affected, differently indeed, being the tissues with higher cellular renovation rate the mostly compromised (Deo, 1978). Therefore, metabolic and morphologic changes which occur in every organ are different and deserve specific studies so that the mechanisms involved in this phenomenon can be understood. Thus, the gut has been the object of study regarding this issue, as changes in it are capable of aggravating the depletions which occur during malnutrition.

Although commercial chows for rats contain at least 22% of protein, rats are known to be capable to grow, without apparent impairments, with up to 12% of protein in their diet (NRC). Other studies have demonstrated that adult rats manage to gain weight for a long amount of time, even receiving only 8% of protein in their diets (Natali *et al*.,

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This study was fully financially supported by Universidade Paranaense - UNIPAR, Brasil.

2005; Schoffen *et al.*, 2005). Considering the effects of malnutrition for the gut and being aware of the nutritional demands of the rat, we conducted this study in order to evaluate the layers constituting the larger segment of the small intestine - the jejunum - in rats submitted to severe lack of protein (4%).

MATERIAL AND METHOD

The animal protocol was previously approved by the Ethics Committee Involving Animals Experimentation of the Universidade Paranaense.

Experiments were conducted using six male Wistar (*Rattus norvegicus*) rats (285.0 ± 29.0 g), 90 days of age, housed singly under standard controlled conditions (~25°C). They were randomly assigned into two groups: Control Group (CG), three animals fed with a 26%-protein commercial chow for rats, NUVILAB®, and Malnourished Group (MG), three rats receiving a 4%-protein chow elaborated by Araújo et al. (2005). After 90 days, rats were fasted for 12 hours and anesthetized with intramuscular injections of Acepran 1.26 mL/kg, Ketamine (10%) 1.26 mL/kg, Xylazine (2%) 0.42 mL/kg and Atropine (1%) 0.22 mL/kg (Pachaly et al., 2003). Ventral midline laparotomy was performed and the jejunum of each animal was removed. Jejunal length and width were measured by using a millimeter ruler. Duodenal-jejunal flexure and ileocecal junction were the anatomic limits. The animals were submitted to euthanasia by deepening the anesthesia until cardiorespiratory arrest was verified.

The jejunum of the animals from each group was washed with 0.9% NaCl solution, filled and immersed in 10% formaldehyde solution for 48 h. Jejunal segments were dehydrated in ascending alcohols, diaphanized in xylol and included in paraffin for further making of transverse cuts, which were stained with hematoxylin-eosin (HE), Periodic Acid Schiff (PAS), and Alcian Blue (AB, pH 2.5 and 1.0). Morphometric analysis of the intestinal wall was performed on images of HE-stained cuts captured by using a Motic B5 professional microscope and a Moticam 2000 camera. Total thickness of the wall was measured by using images captured with a 4X objective. Thickness of the external muscle, mucosa and muscle mucosa were measured by using images captured with a 20X objective. Enterocytes and the largest diameter of their nuclei were measured by using images captured with a 100X objective.

Intestinal circumference was divided into four equal parts for these measurements. Twenty measurements from each part, totalizing 80 parts, were carried out.

The number of goblet cells in the mucosa of each animal was also quantified. Such quantification was performed in cuts stained with techniques to visualize glycoconjugates: PAS and AB (pH, 2.5 and 1.0). Thus, the counting was performed from images captured by using a 40X objective, in which the total number of goblet cells was counted and the area of the mucosa was measured so that the population density of this type of cell could be calculated.

For the statistical analysis, the type of data distribution was first verified through D'Agostino-Pearson test. T-test for independent samples was used to compare normal distribution data between CG and MG. Mann-Whitney test was used to compare free distribution data. P values less than 0.05 were considered significant.

RESULTS

Controls weighed 274.7 ± 37.0 g in the beginning of the experiment and 348.3 ± 6.5 g at the end, what demonstrates an increase of 26.8% ($p = 0.0273$), whereas MG animals weighed 295.0 ± 21.1 g in the beginning and 216.0 ± 13.1g at the end. They not only did not gain weight but also lost 26.7% of it ($p = 0.0053$), however, while evaluating the length of the jejunum, we realized that this measure was not altered by malnutrition as this organ was 95.3 ± 5.9 cm for the controls and 87.3 ± 16.0 cm for the MG ($p = 0.4605$).

Although significant differences in the jejunum were not investigated macroscopically, the morphometric analysis demonstrated intense atrophy of its wall, mostly in the mucosa in the malnourished animals (Table I). Quantifications of goblet cells showed no alterations in the population visualized with PAS and AB, pH 2.5, whereas there was hyperplasia of the cells reactive to AB, pH 1.0 (Table II).

DISCUSSION

Protein malnutrition is known to cause several metabolic alterations culminating in the decrease of the growth of the animals (Araújo et al., 2005), fact also observed in the animals of the malnourished group in this study. Thus, comprehending such metabolic alterations enabled the understanding that the tissues with higher mitotic index are the ones which suffer when the intake of amino acids is lower that the demand of the organism (Deo). This explains that although protein-malnourished animals are usually smaller than the well-nourished from the same species, their body

Table I. Total thickness of wall, external muscle, submucosa, mucosa, enterocyte height, and largest nuclear diameter of the jejunum of normally nourished rats (CG) and of malnourished rats (MG). Intestinal wall, mucosa, external muscle, submucosa and enterocyte nuclei are expressed as median (P25;P75). Thickness of external muscle and enterocyte height are expressed as mean ± standard deviation. Means and medians marked with asterisk differ significantly for the malnourished group in relation to their respective values for the controls (*p<0.05; **p<0.0001). ns: non-significant variance.

Parameter (µm)	CG	MG	Variance between CG and MG
Intestinal wall	327.7 (277.5; 369.6)	267.2* (245.8; 297.3)	↓ 18.5%
External muscle	74.9 ± 19.9	60.1** ± 18.6	↓ 19.8%
Submucosa	21.4 (17.0; 26.9)	21.2 (16.5; 30.3)	ns
Mucosa	213.2 (173.7; 264.8)	153.4** (134.8; 178.6)	↓ 28.0%
Enterocyte height	28.9 ± 5.9	27.5* ± 7.9	↓ 4.8%
Enterocyte nuclei	7.5 (6.3; 8.8)	7.6 (6.1; 9.0)	ns

Table II. Mean ± standard deviation of the number of goblet cells by visualizing glycoconjugates in the jejunum in the animals in the Control Group (CG) and Malnourished Group (MG) in an area of 0.5 mm2. PAS: Periodic acid Schiff. AB: Alcian blue. Means and medians marked with asterisk differ significantly for the malnourished group in relation to their respective values for the controls (*p<0.0001). ns: non-significant variance.

Technique	CG	MG	Variance between CG and MG
PAS	709.9 ± 49.0	756.1 ± 281.4	ns
AB, pH 2.5	653.6 ± 70.4	560.6 ± 164.3	ns
AB, pH 1.0	390.8 ± 64.1*	582.3 ± 140.2*	↑ 49.0%

composition is not always proportionally smaller (Waterlow, 1996). In this sense, we observed that the controls gained weight, as it was already expected since they were in their growing phase, whereas rats fed with a 4%-protein diet (MG) not only gained no weight, but lost it (p<0.05). This indicates that MG rats suffered alterations for both fat and lean mass, but such modifications did not affect the length of the jejunum in this animals. In parallel investigations of the same animals in this study, their duodenum was observed not to suffer any alterations in its length (Pilegi *et al.*, 2004) even though the opposite occurred for both ileum (De Azevedo et al.) and colon (Hermes *et al.*). These findings indicate that when rats are submitted to severe lack of protein the anatomy of the proximal two-thirds of the small intestine tends to be preserved, as this is the site in which intense digestion and absorption of nutrients occur. Other studies in malnourished rats demonstrated decrease in the small intestine length (da Costa Ribeiro et al., 1987; Firmansyah *et al.*, 1989; Meilus *et al.*, 1998; Torrejais *et al.*, 1995; Natali *et al.*, 2000; Brandão *et al.*, 2003; Natali *et al*., 2005).

On the other hand, morphometric analysis of the jejunal wall in the MG animals demonstrated intense atrophy in most of its layers, compromising significantly its total thickness, which became 18.5% smaller in relation to controls. Parallelly, atrophy was also observed in the ileum of the MG animals (De Azevedo *et al*.), even though the general structure of the colon was little altered (Hermes *et al*.). Malnourished rats fed with a 8%-protein diet also demonstrated atrophy of the duodenal wall (Natali *et al*., 2005) and ileum (Torrejais *et al*.). These results demonstrate that although an 8%-protein diet provokes no alterations in the body weight of adult rats (Natali *et al*., 2005; Schoffen *et al.*), it can generate atrophy of the small intestine wall; moreover, this study demonstrated that rats submitted to a more severe lack of protein (4%) not only lost body weight but also suffered atrophy of the small intestine wall.

Atrophy of 19.8% of the external muscle in the jejunum of the MG animals (p<0.0001) was observed in this investigation. This morphometric alteration enables us to infer that the motility of the intestinal segment probably suffered some adjustments, as a result of alterations in the control via nervous system and/or via humoral system; however, neither diarrhea nor constipation was observed in these animals. With respect to the control via nervous system, it is mostly performed by a large group of ganglionated and interconnected neurons – the myenteric plexus (Auerbach) – between the two layers of smooth muscle cells which constitute the external muscle (Furness, 2006). Therein, it should be remarked that in a parallel study the nitrergic neurons of this plexus in the jejunum of the MG animals were

observed to be more grouped (Reis, 2005), possibly as a result of the atrophy of the wall of this organ. Other studies on the jejunal myenteric neurons in the MG animals are currently being conducted by our research group. With respect to the humoral control, it is known that along the external muscle of the gut of rodents there is a more dense macrophage network and other few leukocytes, which are the first to manage and respond to the inflammatory events after being in touch with the endotoxins secreted by the microorganisms, activating the smooth musculature (Bauer, 2008). Thus, we suggest further studies to understand whether the relation among the macrophages in the external muscle and smooth muscle cells is altered in animals with lack of protein, mainly considering the fact that malnutrition in humans is often associated to conditions of exposition to environments without basic sanitation. In other parallel investigations in the same animals of this study, we observed that external muscle of the ileum decreased 58.9% (De Azevedo et al.), whereas no alterations were observed in that layer of the colon (Hermes *et al*.). Moreover, atrophy of the external muscle was also observed in the duodenum (Natali *et al*., 2005) and in the ileum (Torrejais *et al*.) of rats fed with an 8%-protein diet, what indicates that this layer of the small intestine wall is very susceptible to protein malnutrition, probably due to a mechanism similar to the one which occurs with the skeletal musculature to provide endogenous amino acids to compensate the lower intake of those molecules through the diet (Waterlow; Araújo *et al*., 2003).

The submucosa, in this study, was the only layer not to suffer atrophy as a result of malnutrition ($p = 0.4873$). This could be attributed to the unmodeled dense connective tissue constitution, mainly the collagen-and-elastic-rich fibers (Junqueira & Carneiro, 2008). Moreover, the fact that no alterations have been observed in the thickness of this layer of the jejunal wall in the MG animals suggests that, possibly, the area occupied by these fibers was not altered, as in a qualitative analysis of the histological cuts increase of the number of cells in that layer was not observed, thus indicating that the extracellular matrix continued to be its major component. Consequently, in a severe lack-of-protein condition, the protein constituting the connective tissue fibers in the submucosa within the jejunal wall are preserved, opposite to what occurs to the fibers of this tissue within the skin of malnourished animals and humans (Waterlow). Commonly, the submucosa is not measured in morphometric evaluations in the gut of malnourished animals (Natali *et al.,* 2000; De Azevedo *et al*.; Hermes *et al*.). The only study evaluating the thickness of this layer was performed in the ileum of rats fed with an 8%-protein diet, in which decrease of the submucosa is reported; however, the authors based their conclusions merely on one observational study (Torrejais *et al*.).

Thus, most of the studies evaluating the gut of malnourished animals demonstrate that the mucosa is the major layer suffering alterations (Viteri & Schneider, 1974; Rodrigues *et al.*, 1985; Ribeiro *et al.*; Torrejais *et al*.; Natali *et al.*, 2005; Schoffen *et al*.; De Azevedo *et al*.; Hermes *et al*.), what also meets the findings of the present study, which demonstrated decrease of 28.0% in the thickness in this layer in the MG ($p<0.0001$). This phenomenon can be explained by the fact that the mucosa is mainly constituted of high cellular renovation tissues, what requires a higher demand of amino acids (Deo). Among these tissues, the epithelial stands out as it is completely renovated in malnourished humans within 7 days (Junqueira & Carneiro). Concerning the gut epithelium, the enterocytes and goblet cells were evaluated in this study. Thus, for the enterocytes, we observed that their height decreased 4.8% ($p<0.05$), although there were no alterations in the largest diameter of their nuclei. Such decrease can correspond to a compensatory mechanism aiming at approximating the nutrients of the intestinal lumen of the blood vases within the lamina propria, what could contribute for the increase of their diffusion and, consequently, absorption. On the other hand, this mechanism also approximates the microbiota to the lamina propria, making the organism susceptible to the translocation of microorganisms. Therefore, to prevent this from happening, it is necessary that the epithelial cells are well united by means of its lateral membrane, what should probably occur since an immunohistochemical study demonstrated higher positivity in presence of cadherins (normally in the desmosomes) among cells in the intestinal epithelium of malnourished rats during the gestational period (Dalçik *et al.,* 2003). Therefore, alterations in the morphometry of the enterocytes in the MG animals indicate that these cells had to re-organize their ultra-structure in order to save amino acid consumption. Thus, analysis of electron micrographs could help understanding the alterations occurred within these cells. It should also be remarked that enterocytes have microvilli containing enzymes at its apical surface, finishing the hydrolysis of protein and carbohydrates, which decrease their activity in animals submitted to protein malnutrition (Collares-Buzato, 2005). In parallel studies in the MG animals, mucosa, enterocyte height and largest diameter of their nuclei were observed to have decreased in both the ileum (De Azevedo *et al.*) and the ascending colon (Hermes et al.) – reinforcing that atrophy of the mucosa in malnourished animals occurs in the whole gut. Besides, the goblet cells were evaluated in this study with respect to the chemical nature of what they secret – the mucins – and it was verified that in the MG animals there was an increase of around 50% of the subpopulation reactive to AB, pH 1.0, that is, the protein malnutrition induced in this experiment provoked increase of sulphomucin secretion, what probably made the mucus on the intestinal jejunal epithelium more intense and fluid. On the other hand, in a parallel study in the colon of the MG animals, decrease of the number of the

subpopulation of goblet cells which secret either neutral mucins or acid mucins was observed (Hermes *et al.*). Moreover, in face of an intense protein malnutrition condition, the organism, in order to maintain intestinal homeostasis, induces the colon to refrain from secreting mucins so that the jejunum can maintain the mucus covering the epithelium more intense and fluid. Considering that the jejunum is the longest part of the small intestine, thus responsible for most of the nutrient absorption, the alterations observed in the dynamics of mucin secretion within the gut should probably contribute for a higher absorption of the small amount of amino acids provided in the diet. However, few are the studies evaluating this phenomenon (Montagne *et al.,* 2004).

We concluded that intense lack of protein in the diet of adult rats provokes atrophy of almost all layers of the jejunal wall, mostly the mucosa. Moreover, the number of goblet cells reactive to Alcian Blue, pH 1.0, in the jejunal epithelium increases indicating increase of the secretion of sulphomucin covering the internal surface of this organ.

FRANCO, C. L. M.; Sant'Ana, D. M. G. & ARAÚJO, E. J. A. Atrofia de la pared intestinal y aumento de la secreción de sulfomucinas en el epitélio yeyunal de ratas sometidas a intensa desnutrición protéica. ***Int. J. Morphol., 28(2)***:497-502, 2010.

RESUMEN: Fueron evaluados los efectos de la desnutrición proteica severa sobre la morfometría de la pared intestinal del yeyuno de ratas adultas . Para esto, se utilizaron seis ratas (*Rattus norvegicus*) Wistar, con 90 días de edad, distribuidas en dos grupos: GC (grupo control, n=3) y el GD (grupo desnutrido, n=3). Los animales del GC recibieron ración con un contenido proteico de 26% y los del GD ración con 4% de proteínas. Después de 90 días de experimentación, parte del yeyuno fue retirado y sometido a un procesamiento histológico de rutina. Cortes transversales de 4 µm fueron teñidos con HE y técnicas histoquímicas (Periodic Acid Schifff (PAS) y Alcian Blue (AB) pH 2,5 y pH 1,0.) para evidenciar glicoconjugados. El análisis morfométrico teñidos con HE demostró reducción en el grosor total de la pared del yeyuno, especialmente de las túnicas muscular y mucosa, además se observó una disminución en la altura de los entericitos. Los cortes teñidos con técnicas histoquímicas revelaron que el número de células caliciformes reactivas al PAS y al AB pH 2,5 se mantuvo; por otro lado, hubo un aumento en el número de células reactivas al AB pH 1,0. Se concluye que la desnutrición proteica severa provoca atrofia de la pared yeyunal y aumento de la secreción de sulfomucinas en el epitelio intestinal.

PALABRAS CLAVE: Desnutrición proteico-energética; Intestino delgado; Morfometría; Mucinas.

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Received: 07-12-2009
Accepted: 23-03-2010