Histomorphometric Analysis of Duodenum of Rats Submitted to Food Stress

Análise Histomorfométrica do Duodeno de Ratos Submetidos ao Estresse Alimentar

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ABSTRACT
Stress was interpreted as a nonspecific reaction of the organism to a situation that would threaten its homeostasis. Several factors in the modern world can be related to this condition: the search for a perfect body, the labor market dispute or the pressure to conquer everything as fast as possible. Associated with these factors, one can add the intense, naturally stressful, routine in which man is inserted. At the experimental level, several effects are observed at a systemic and behavioral level in rats that have been submitted to food restriction models, finding that the gastrointestinal system is quite vulnerable to stress in general. In specific cases of food stress, it was observed that the manifestations vary according to the life stage of the animal and the applied model. In this sense, the present research aims to evaluate the influence of food restriction on the intestinal tunica morphology of rats submitted to a chronic food stress model. For this, 27 animals were divided into control (n = 11) and test (n = 16) groups. From the 60th day of life, the test group was submitted to four stages, each one being performed in one day: 1) palatable diet; 2) visual stimulation to the diet, but without access; 3) fasting; 4) standard diet of the biotery. The four steps were repeated until the animals completed 90 days. In the morphometry of the duodenum were analyzed: length, width and area of vill, as well as area of intestinal gland (Lieberkühn). Statistical inference of data showed that the applied stress model affected the morphology of the stressed group, since the intestinal villi appeared wider and with less area in this group. The villi length as well as the intestinal gland area did not undergo morphological changes. The alterations found reinforce that the fasting process acts as a stressor and a predisposing factor for morphological alterations, as observed in other studies in our laboratory. However, there are not many studies in the literature that allow the knowledge of the consequences of this type of stress. In this case, further research is needed on the relationship between the gastrointestinal tract and the experimental model applied to elucidate such findings.

Keywords: Food stress, Small intestine, Experimental study, Food restriction.

RESUMO
O estresse foi interpretado como uma reação não específica do organismo a uma situação que ameaçaria sua homeostase. Vários fatores no mundo moderno podem ser relacionados a esta condição: a busca de um corpo perfeito, a disputa do mercado de trabalho ou a pressão para conquistar tudo o mais rápido possível. Associado a estes fatores, pode-se acrescentar a intensa, naturalmente estressante, rotina em que o homem está inserido. No nível experimental, vários efeitos são observados a nível sistêmico e comportamental em ratos que foram submetidos a modelos de restrição alimentar, descobrindo que o sistema gastrointestinal é bastante vulnerável ao estresse em geral. Em casos específicos de estresse alimentar, foi observado que as manifestações variam de acordo com o estágio de vida do animal e o modelo aplicado. Neste sentido, a presente pesquisa visa avaliar a influência da restrição alimentar sobre a morfologia da túnica intestinal de ratos submetidos a um modelo de estresse alimentar crônico. Para isso, 27 animais foram divididos em grupos de controle (n = 11) e teste (n = 16). A partir do 60º dia de vida, o grupo de teste foi submetido a quatro estágios,
sendo cada um realizado em um dia: 1) dieta palatável; 2) estimulação visual da dieta, mas sem acesso; 3) jejum; 4) dieta padrão do biotério. As quatro etapas foram repetidas até que os animais completassem 90 dias. Na morfometria do duodeno foram analisados: comprimento, largura e área da vila, assim como área da glândula intestinal (Lieberkühn). A inferência estatística dos dados mostrou que o modelo de estresse aplicado afetou a morfologia do grupo estressado, já que as vilosidades intestinais pareciam mais largas e com menos área neste grupo. O comprimento das vilosidades assim como a área da glândula intestinal não sofreram alterações morfológicas. As alterações encontradas reforçam que o processo de jejun age como um fator de estresse e um fator predisponente para alterações morfológicas, como observado em outros estudos em nosso laboratório. Entretanto, não há muitos estudos na literatura que permitam o conhecimento das conseqüências deste tipo de estresse. Neste caso, são necessárias mais pesquisas sobre a relação entre o trato gastrointestinal e o modelo experimental aplicado para elucidar tais descobertas.

Palavras-chave: Estresse alimentar, Intestino delgado, Estudo experimental, Restrição alimentar.

1 INTRODUCTION

Stress, in its first definition in health studies, was interpreted as a nonspecific reaction of the organism to a situation that would threaten its homeostasis (SELYE, 1936). This reaction generates individual adaptations and, according to the etiological factor, the organism responds positively or negatively, leading to eustress or distress, respectively. Distress, according to the World Health Organization, was cataloged and recognized as a disease in 1992 and, because of its high incidence and disastrous consequences, especially in the occupational environment, was known as the twentieth-century disease (DAMBROSKI; MOURA, 2014).

Several factors in the modern world may be related to stress: the search for a perfect body, the labor market dispute, the need to want more than we have and be the best, the pressure to conquer everything as fast as possible. Associated with these factors, one can add the intense, naturally stressful routine in which we are subjected. Because of this, stress is closely related to psychological disorders such as depression and anxiety, where it may be considered a risk factor for its development, or mental state may influence adaptive responses of the body, which characterizes stress (MARGIS et al. al, 2003).

Also the intense dissemination of the idea of a beauty pattern to be followed in the most diverse media is responsible for an incessant persecution of the individuals' self-esteem, leading to a low acceptance of the body image and leaving the individual subordinated to extreme situations to get what you want. This phenomenon can be understood as a stressor stimulus, which can lead to the development of a number of psychopathological disorders, such as anorexia, bulimia and vigorexia (PINHEIRO and FIGUEIREDO, 2012). Thus, if the change in the pattern of food behavior and stress can alter the organism leading to a disease, what would consequences be associated with the
gastrointestinal tract, the main actor in the process of nutrient uptake to maintain the body metabolism?

Several effects are observed at the systemic and level behavioral of rats submitted to the experimental models of food restriction (Giacomelli et al., 1999). Blundell et al. (1985) observed that alteration occurs in the alimentary behavior resulting from an adaptation process, when an animal is subjected to some limitation. In addition, it is known that the gastrointestinal system is quite vulnerable to stress in general. Its signs and symptoms can be: loss of appetite, vomit, diarrhea, irritation and perforation of the organ as in the case of gastric ulcers (CABRAL et al., 1997). In specific cases of food stress, it was observed that the manifestations vary according to the life stage of the animal and the experimental model applied.

In the presence of the stressor stimulus, it is known that the homeostatic balance is threatened and, if this is of a long duration, the organ function may be impaired. In this sense, the present research aims to evaluate the influence of food restriction on the intestinal tunica morphology of rats submitted to a chronic food stress model.

2 MATERIAL AND METHODS

2.1 ANIMALS AND EXPERIMENTAL GROUPS

This work was submitted to the Ethics Committee on Animal Use - CEUA of the Federal University of Pernambuco - UFPE and approved (under the number of Protocol 23076.020781 / 2011-31). It was used the small intestine of Wistar rats from the laboratory of the Nutrition Department of the Federal University of Pernambuco. These animals were kept in the Biotery of the Academic Center of Vitória, in adequate conditions of temperature and humidity, with water and ration ad libitum. After, they were mated in the ratio of one male to two females. One day after birth, the offspring of several females were randomized, and sexing was performed, with 8 male pups remaining per maternity. At 60 days of age, the experimental groups were divided in control group (N = 11) and stress group (N = 16).

2.2 STRESS

The animals were exposed to food stress from 60 days of age to 90 days, after the rats were sacrificed. On the 60th day, the animals received a palatable and calorie diet based on labina (ration) and nutella (hazelnut chocolate cream) for 3 consecutive days to adapt to diet. After one day of rest the stress sessions were started. On the first day the animals received the same palatable diet for 1 hour. On the second day, the animals received the same food stimulus, but the diet was in a container.
that just allowed a visual and olfactory stimulation, without the animals having access to diet. On the third day, the animals were fasted for 24 hours. The rest was performed on the fourth day, they received the standard diet of the biotery. The four steps were repeated until the animals completed 90 days.

2.3 EUTANASIA AND BIOLOGICAL PROCESSING

On the 91st day of life, the animals were anesthetized and then perfused with 10% neutral buffered formalin (10% NBF) and necropsied. A longitudinal abdominal incision was performed in the midline to expose the internal organs. The small intestine was removed and placed in 10% NBF solution for 48 hours. After this time, the material was stored in 70% ethanol and 5% glycerol. The collected material was dehydrated by ethanol (70% to 100%), diaphanized and included in paraffin. The obtained blocks were cut to 4μm thickness and stained with hematoxylin-eosin.

2.4 HISTOMORPHOMETRIC ANALYSIS

The histological images were captured by the "Moticam 2300 3.0M Pixel USB 2.0" digital camera coupled to the "Olympus CX22" optical microscope with a magnification of 400X. Photomicrographs were evaluated using ImageJ software version 1.44 (Research Services Branch, U.S. National Institutes of Health, Bethesda, MD, USA), where length, width and area of intestinal microvilli, and intestinal gland area were analyzed. For the study were chosen the structures intact of the slides, being measured in such a way:

2.5 VILLI LENGTH

Ten uninjured villi of each lamina (no folds or out of the expected pattern), with continuous upper, middle and lower portions, a straight line drawn with the computer cursor from the center of the lower portion to the apex of the villus;

2.6 VILLI WIDTH

Ten uninjured villi of each slide (no folds or out of the expected pattern), a straight line being drawn with the computer cursor in the middle portion of each villi;

2.7 VILLI AREA

Ten uninjured villi of each slide (no folds or out of the expected pattern), a line being drawn around the entire structure with the computer cursor;
2.8 AREA OF THE INTESTINAL GLAND (IN LIEBERKÜHN)

Ten uninjured glands of each slide (no folds or out of the expected pattern), a line being drawn around the entire structure with the computer cursor.

2.9 STATISTICAL ANALYSIS

The analyses of data were performed using SPSS (Statistical Package Social Sciences) software 15.0. Initially, the Kolmogorov-Smirnov test was done, indicating that the data did not show a normal distribution. Thus the Mann-Whitney test was performed, with significant difference to \( p < 0.05 \).

3 RESULTS

The Mann-Whitney test did not show a significant difference (\( p = 0.998 \)) between the intestinal glands area (Lieberkühn) (\( \mu \text{m}^2 \)) of the control animals (2427.49 ± 1515.81) and the animals of the stress group (2287.92 ± 1143.35). However, there was a statistically significant difference (\( p = 0.002 \)) between the intestinal villi area (\( \mu \text{m}^2 \)) of two groups, as shown in Figure 1-B. The animals of the stress group (9901.77 ± 5810.86) had villi with a smaller area than the control group (13099.85 ± 7968.56).

In relation to the villi length, the control group (251.03 ± 95.40) did not present statistical difference (\( p = 0.091 \)) when compared to the stress group (237.94 ± 97.70). Regarding the villi width, there was a significant difference (\( p < 0.001 \)) between the values of the stressed group (93.82 ± 53.67) and the control group (77.58 ± 41.60), with the intestinal villi of the stressed rats being wider than the structures of the control group. These results can be seen in Figure 1-C and 1-D.
Figure 1. Effect of chronic food stress on the intestinal glands (1-A) and intestinal villi (1-B) in μm², in the control and stress groups. Mean length of intestinal villi (1-C) and width of intestinal villi (1-D) in μm, of the control and stress groups. The animals were submitted to a dietary stress protocol from the 60th day of life. For the first three days, the animals initially received a palatable and caloric diet based on labina and nutella (hazelnut chocolate cream) for diet adaptation. After one day of rest the stress sessions were started. On the first day the animals received the palatable diet for 1 hour. On the second day, the animals received the palatable diet, but without access to the consumption, receiving only visual and olfactory stimulation. On the third day, the animals were fasted for 24 hours. On the fourth day, they received the standard diet of the biotery, being considered the day of rest. The four steps were repeated until the animals completed 90 days. Data are expressed as mean ± standard error. (*) significant difference between the stress group and the control group (Mann-Whitney U test with significance level p < 0.05).

When comparing the two groups, the data show that the experimental model of dietary stress affected the morphology of the stressed group, making it the largest in relation to the group in consumption of the standard diet. Since the villi of the control group had greater length and smaller width, implicating directly in the villi area which was higher (Figure 2) than the test group (Figure 3). The area of the intestinal glands did not undergo morphological changes.
4 DISCUSSION

The present work analyzed, through an experimental protocol of chronic stress, the microscopic alteration of the intestinal mucosa promoted by food stress. The rats submitted to the study were exposed to a stress model that provided the visual and olfactory stimulus, but without access to a palatable diet for 30 days, alternating to periods of fasting and periods with little access time to the palatable diet. Such model promoted changes in the width and area of the intestinal villi, with no change in their length and intestinal glands. The morphometric analysis used to examine the effects of restriction on the intestinal mucosa is of extreme importance for an efficient evaluation of quantitative parameters and not only qualitative (TRAMONTE et al., 2004).

The methodology used sought to represent the current pattern of society, where thinness is recognized as an ideal to be achieved. In the search by thinness, without the accompaniment of a qualified professional, many people undergo severe dietary restrictions and also they receive daily stimuli through situations characterized as stressful. Such situation can also be observed in individuals who are not able to adequately nourish each day. The difficulty in following the food restriction causes the individual to not resist the stimuli, ingesting a product of high caloric content and then returning to the state of deprivation, characterizing a stressful cycle. Dietary regimens that combine periods of food deprivation and ad libitum feedback can also be identified in intermittent fasting models (SANTOS, 2017).

In previous studies of our laboratory, which used the experimental stress model identical to
this study, significant alterations were observed in several organs: in the cerebellum, an increase in
the number of neurons in its granular layer was verified (SANTOS, 2015), being the same condition
observed in the granular cells of the hippocampus gyrus of the animals submitted to the experiment
(MIGUEL, 2013). In the study about the cortex of the adrenal glands, Xavier (2015) observed
significant changes in the three layers: the glomerulosa and fasciculate zones presented hyperplasia,
unlike the reticular zone which showed a considerable decrease in the number of cells.

The understanding of the main complications caused by malnutrition has been possible
through experimental studies, however most of the studies induce the model of protein malnutrition
in the animals and not a specific food stress, as the one used in the present research. However, it is
known that the stress caused by malnutrition in the various stages of life is a relevant factor for the
alteration of the intestinal morphology, as evidenced in several studies with malnourished rats
(AZEVEDO et al., 2007; GURMINI, 2005; HERMES, 2008; MADI; CAMPOS, 1975; MELLO,
2004).

It is known that hypoproteic diets may lead to decreased villi length and epithelial atrophy,
and a large decrease in leaf cells in the ileum (MADI, CAMPOS, 1975). The significant decrease
observed in the area of the villi, which culminated in the expressive change in their width, making
them smaller and wider than the control group, can be observed in studies aimed at this approach
(seen as a threat to homeostasis, a stressful factor). Azevedo et al. (2007) identified macroscopic
effects and microscopes in the small intestine of rats submitted to intense protein deficiency,
suggesting that nutrient deficiency may alter intestinal morphology, as observed in our study. Also
Azevedo et al. (2007) have noted a reduction in the body weight of the studied animals, as well as in
the intestinal wall of the ileum and the structures that compose it. In malnourished mother pups, it
was verified that those with low birth weight had morphometric measures lower than normal for their
age, besides showing underdevelopment after lactation (GURMINI et al., 2005).

It is known that the digestive capacity of the intestine is threatened when changes occur in its
mucosal layer - as it occurs in situations of nutritional deficiencies -, especially in the morphology of
its cells (Giacomelli et al., 1999). In animals that underwent fasting, weight loss of the small intestine
(53%) was greater than the body loss (23%), as well as a decrease in RNA, protein and total water in
the cells (STEINER et al., 1968). The fasting also caused a decrease in the levels of cholecystokinin
in the plasma and duodenum, and the levels were reestablished after the feedback process (KOOP et
al., 1987). Thus, these findings support the idea that animal physiology, especially intestinal, was
strongly altered in animals submitted to the experiment.
Experimental models similar to the present study, which used a combination of the fasting and feedback process to promote chronic stress and evaluated their respective effects on the small intestine, found that dietary deprivation induced increased apoptosis and reduced cell proliferation in rats completely fasted. In the animals that were fed back, the opposite condition was observed, that is, there was an increase in cell proliferation and a decrease in apoptosis (MARTINS; HIPÓLITO-REIS; AZEVEDO, 2001).

Lara-Padilla et al. (2015) combined intermittent fasting with a forced swimming (stressful source), identifying that the combination of these factors was responsible for the decrease of Immunoglobulin A (IgA) in the duodenum and ileum, in addition to modulating transcription of pro-inflammatory and producing IgA in the ileum. In animals that have gone through a short period of fasting, the main consequence was a decrease in the mass of the intestinal segments, and it was noticed that the small intestine is extremely sensitive to food withdrawal (DOU et al., 2001). In that same study, the lost mass was regained during the period of feedback (with great rapidity in the duodenum and jejunum), so as the villi height, the crypt depth and the mucosa thickness were also recovered.

In animals that underwent an induced process of psychological stress, it was observed that stress was responsible for the promotion of anxiety in the rats, while the offer of palatable diet canceled the effects of stress, acting as anxiolytic (PAULA, 2010). Therefore, the ingestion of palatable foods is responsible for lowering the glucocorticoid level, consequently decreasing the responses to stress, but increasing the propensity to consume such foods as a reward measure (MORRIS et al., 2015). Thus, it is understood that the food restriction induced and combined with periods of visual and olfactory stimuli to the palatable diet functioned as a responsible factor in the alteration of the intestinal villi of the animals studied. However, access to the hypercaloric and palatable diet, known as comfort foods, during one of the phases of the experiment, suggests attenuation of the endocrine effects of stress, as pointed out by Ortolani (2014), in addition to softening the anorectic effect in rats stressed by shock on paws (ORTOLANI, 2010).

Feedback is a decisive factor in preventing chronicity of the damage caused by fasting. However, the ingestion of palatable foods in this process can generate metabolic alterations, making the animals more susceptible to obesity and developing insulin resistance (ORTOLANI, 2010). In addition, the presence of stress in the initial life of rodents seems to increase predilection for comfort foods in adult life (CUNHA, 2015).

In view of the essential function of increasing the absorption surface, the reduction observed in the area and width of the villi of the animals in this study may imply a lower absorptive area in organisms that are being harmed by food deprivation. Short-term malnutrition leads to intestinal
tissue damage that induces a compensatory mechanism due to poor nutritional utilization (Giacomelli et al., 1999). If the period of malnutrition is prolonged, the main damage is loss of organ function, as seen in malabsorption syndromes. However, there has been a shortage of studies in the literature that allow greater comparisons and investigations regarding the effects identified in rodent mammals.

Thus, it is understood that the changes observed in the microscopy of the intestinal mucosa could have been more pronounced in the absence of comfort food. But the withdrawal of such food would not reliably represent the cycle of food deprivation and food intake hypercaloric that characterize the consumption pattern of humans who are adherents to highly restrictive diets. Still, the predilection for palatable foods that function as anxiolytics and help prevent chronicity of damage is not at all a beneficial alternative, since such habit tends to cause metabolic consequences in animals.

5 CONCLUSION

The performance of this research, by simulating a dietary pattern similar to that adopted by individuals who adherent to overly restrictive diets, was important to investigate the main complications caused by this habit. Changes were observed in the morphometry of intestinal villi, especially in their width and total area. No statistically significant changes were found in the Lieberkuhn's gland as well as at villi height.

Findings from the literature show that the gastrointestinal tract is strongly influenced by stress in general, from motivating the preference for foods with higher caloric content to morphophysiological changes in the organ. Induced malnutrition and fasting, the provision of palatable diet and feedback are factors that when combined can promote significant modifications. Such factors on a scale of importance can be classified as maleficent, intermediate, and beneficial, respectively.

The alterations found reinforce that the fasting process and malnutrition act as stressors and predisposing factors for morphological changes, as observed in this study and in other studies of our laboratory. The feedback process has proved to be extremely important in order to avoid that the consequences become chronic, however if this process is done with the offer of palatable diet such option may act as a reason for the appearance of metabolic alterations, although it attenuates the effects of stress and acts as anxiolytic.

In research that sought to investigate stress in rodent and its relationship to the intestine were observed decreased digestive capacity, threat to physiology, and increased apoptosis, suggesting that the same effect or similar effects occurred in the animals in the test group. It is understood that the feedback phase was essential to avoid that the alterations found were even more serious. However,
there are not many studies in the literature that allow the knowledge of the consequences of the specific type of stress applied in this research. In this case, further research is needed on the relationship between the gastrointestinal tract and the experimental model applied to elucidate such findings.

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