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Basic nutritional investigation

Dietary restriction interferes with oxidative status and intrinsic intestinal innervation in aging rats

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ABSTRACT

Objectives: To evaluate the effects of dietary restriction on oxidative status, the HuC/D–neuronal nitric oxide synthase (nNOS) myenteric neuron population, HuC/D-S100 glial cells, and the morphometry of the small intestine in rats at various ages.
Methods: Fifteen Wistar rats were divided into 7-and 12-mo-old control groups and a 12-mo-old experimental group subjected to dietary restrictions (50% of normal ration) for 5 mo. At 7 and 12 mo of age, the animals were anesthetized, and blood was collected to assess the biochemical components and oxidative status. Ileum samples were subjected to double-marker (HuC/D-nNOS and HuC/D-S100) immunostaining and histologic processing to morphometrically analyze intestinal wall elements and determine the metaphase index and rate of caliciform cells. The data were subjected to analysis of variance and the Tukey post hoc test with a 5% significance level.
Results: Age affected the oxidative status by increasing lipid peroxidation, with no effect on blood components, intrinsic innervation, and intestinal wall elements. The animals subjected to dietary restriction showed improved levels of total cholesterol, triacylglycerols, and oxidative status, with no changes in the nNOS neuron population. However, the dietary restriction dramatically decreased the glial and HuC/D myenteric populations, led to atrophy of the neuronal cell body, induced glial hypertrophy, and decreased the thickness of the intestinal wall.
Conclusion: The high oxidative status of the aging animals was reversed by dietary restriction, which also lowered cholesterol and triacylglycerol levels. The present dietary restriction elicited morpho-quantitative changes in the myenteric plexus and histology of the ileum, with likely effects on intestinal functions.

Introduction

The enteric nervous system is composed of a group of neuronal and non-neuronal cells (enteric glia) that modulate complex functions, such as bowel movements, secretion, and blood flow in the gastrointestinal tract. Enteric neurons represent a complex and varied population, the identity and function of which must be known to better understand the mechanisms involved in digestive physiology [1], whereas glial cells produce neurotrophic factors and are involved in regulating the intestinal epithelial barrier [2].

Aging causes a decrease in the number of enteric neurons, the loss of which may be linked to an increase in free radicals from the diet [3] and attributable to a decrease of the neurotrophic factors produced by glial cells. The analysis of neuron subpopulations is important because some neurons may be more susceptible than others to aging [4].

Variations in myenteric plexus organization and the size and morphology of the intestine can be observed during aging, including increased small intestine length and muscular coat thickness [5], reorganization of the remaining neurons [6], and increased cell body size [4,5,7–10]. These variations are attributable to stress and the functional demand on certain types of

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the remaining neurons from gradual neuronal loss to allow the survival and adaptation of the neurons that remain to guarantee the preservation of intestinal function [9,10].

Experimental dietary restriction has been linked to lower rates of tumor formation and increased immune system efficiency [11], decreased cellular metabolism [12], improved neurotrophic signaling in myenteric neurons [3], increased myenteric neuronal plasticity, longevity, and survival [4], and the attenuated effects of oxidative stress generated during the cellular respiration process [13]. The presence of nutrients in the intestinal lumen stimulates the growth of villi and crypts [14], thereby decreasing food availability, and can lead to a decrease in digestion and intestinal absorption.

The objective of the present work was to evaluate the effects of dietary restriction on the morpho-quantitative aspects of the myenteric plexus and intestinal wall and the oxidative status of aging animals.

Materials and methods

Animals and treatment

Fifteen male Wistar rats (*Rattus novergicus*) were used. They were housed in polypropylene cages with a 12-h light/12-h dark cycle and a temperature of 22 ± 2°C. The animals were distributed into 7-mo-old (C7) and 12-mo-old (C12) control groups that received a standard NUVILAB-NUVITAL (Curitiba, PR, Brazil) rodent chow ad libitum and an experimental group subjected to dietary restriction from 7 to 12 mo of age (RA12) that received 50% of the mean daily ration of the control rats fed ad libitum. Body weight, ration intake, and water intake were monitored. All the procedures were approved by the committee for ethics in animal experiments of the State University of Maringá.

Tissue collection

At 7 and 12 mo of age, the mitotic fuse blocker vincristine sulfate (0.5 mg/kg) was administered through the penile vein for the later analysis of cell proliferation. Two hours later, the animals were anesthetized intraperitoneally with sodium thiopental (Thionembutal 40 mg/kg) (São Paulo, SP, Brazil), and the naso–anal length was measured to determine the Lee index ([body weight$^{1/3}$ {g}]/naso–anal length [cm] × 1000). Blood was collected by cardiac puncture to determine the levels of biochemical components and evaluate the oxidative status of the animals.

A laparotomy was performed to collect and measure the small intestine and weigh peri-epididymal and retroperitoneal adipose tissues. Ileum segment samples (10 cm) were removed from the ileocecal junction, measured lengthwise, and designated for double-marker HuC/D (pan-neuronal marker)–neuronal nitric oxide synthase (nNOS) and HuC/D-S100 (glial cell marker) immunostaining and histologic processing for intestinal analysis.

Biochemical analysis of blood components

To analyze the levels of total proteins, albumin, globulins, triacylglycerols, total cholesterol, and glucose, blood was collected, and the samples were centrifuged at 3000 rotations/min for 15 min using Analisa kits (Gold Analisa Diagnóstica, Minas Gerais, Brazil).

Evaluation of oxidative status

The collected blood was placed in tubes that contained ethylenediaminetetraacetic acid as an anticoagulant at a concentration of 3 mmol/L and centrifuged at 1000 × *g* for 10 min. Plasma was isolated and frozen at −80°C. The residual sediment was subjected to two rinsing cycles by resuspension and recentrifugation of the sediment at 1000 × *g* using 0.9% NaCl. The resulting cell mush was used to evaluate oxidative status in erythrocytes. All the procedures were performed at temperatures lower than 4°C.

Lipid peroxidation levels in plasma were determined using the thiobarbituric acid-reactive substances (TBARS) method [15]. This colorimetric method quantifies low-mass molecules, particularly malondialdehyde, that react with thiobarbituric acid in acidic medium, forming a complex absorbed at 532 nm. The lipoperoxide concentration was determined using a 1.56×10^5 M/cm molar extinction coefficient, and the values are expressed as nanomoles of TBARS per milliliter of plasma.

Lipid peroxidation levels in erythrocytes were determined using the TBARS method [16]. Aliquots of the washed erythrocyte suspension were resuspended with phosphate buffered saline (PBS; NaCl 8.1 g/L, $Na_2HPO_4^{-2}$ 2.302 g/L, and $NaH_2PO_4^{-}$ 0.194 g/L, pH 7.4) and deproteinized with 30% trichloroacetic acid. After sitting in an ice bath for 2 h, the material was centrifuged at 2000 × *g* for 10 min, and the TBARS levels were determined in the clear supernatant similarly to plasma. The values are expressed as nanomoles of TBARS per gram of hemoglobin. Hemoglobin content was determined using the cyanmethemoglobin method.

Immunohistochemistry of myenteric neurons and enteric glia

Ileum samples were rinsed in 0.1 M PBS (pH 7.4), tied off at both ends, filled in, and distended with 4% paraformaldehyde fixer (pH 7.4) for 2 h. The samples were later opened and washed in 0.1 M PBS (pH 7.4) for 24 h at 4°C and stored in 0.1 M PBS (pH 7.4) with 0.08% sodium azide at 4°C. Whole-mount muscle membrane preparations were obtained by dissection under a stereomicroscope with transillumination.

HuC/D-nNOS and HuC/D-S100 double staining

The whole-mount preparations were washed twice in 0.1 M PBS (pH 7.4) with 0.05% Triton X-100 for 10 min and blocked for 1 h in a solution that contained 0.1 M PBS (pH 7.4), 0.05% Triton X-100, 2% bovine serum albumin, and 10% goat serum to prevent non-specific bonding. The tissues were then incubated for 48 h in a solution that contained 0.1 M PBS (pH 7.4) with 0.05%Triton X-100, 2% bovine serum albumin, 2% goat serum, and primary antibodies. For HuC/D-nNOS double staining, anti-HuC/HuD antibodies produced in mice (Invitrogen, Eugene, OR, USA), and anti-nNOS antibodies produced in rabbits (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. For HuC/D-S100 double staining, anti-HuC/HuD antibodies produced in mice and anti-S100 antibodies produced in rabbits (Sigma, St. Louis, MO, USA) were used. The dilutions of the anti-HuC/HuD, anti-NOS, and anti-S100 antibodies were1:500; 1:500, and 1:200, respectively.

After incubation, the membranes were washed three times in 0.1 M PBS (pH 7.4) with 0.05% Triton X-100 for 5 min and incubated with secondary Alexa Fluor 488 anti-mouse antibodies (Invitrogen) and Alexa Fluor anti-rabbit 546 (Invitrogen) for 2 h at room temperature, both at a 1:500 concentration. The preparations were washed with PBS, mounted on microscope slides, and coverslipped with glycerol.

Morpho-quantitative analysis

The quantification of the total HuC/D myenteric neuron population, nNOS-positive nitrergic subpopulation, and glial cells (S100) was undertaken in 64 microscopic images per animal in the intermediate (32 images) and antimesenteric (32 images) regions of the intestinal circumference using an Olympus BX40 light microscope (Olympus America, Burnaby, BC, Canada) equipped with filters for immunofluorescence and coupled to a Moticam 2500 camera (Moticam, Hong Kong, China) that featured a 20× lens. The total area analyzed per animal was 5.9 mm^2/technics. The area of 100 cell bodies per animal was measured using Image-Pro Plus 4.5 (Media Cybernetics, Silver Springs, MD, USA).

Histologic analysis

Ileum samples were fixed in Bouin fixative for 6 h, embedded in paraffin, and subjected to microtomy (Leica RM 2145 microtome) (Wetzlar, Germany), resulting in 6-μm-thick semi-serial sections. The sections were stained with hematoxylin and eosin, and 100 random points of the intestinal wall, muscular coat, and mucosa tunic were measured in micrometers in 10 histologic sections per animal.

Other samples were embedded in historesin (Leica Historesin Kit), and 2.5-μm-thick semi-serial sections were made and stained with hematoxylin and eosin. The heights of 90 villi and 90 crypts were measured from images captured using an Olympus BX41 optical microscope equipped with a 10× lens and coupled to a Q Color 3 high-resolution camera (Olympus America) and analyzed using Image-Pro Plus 4.5 (Media Cybernetics).

The metaphase index was obtained in longitudinal crypt sections with visible lumen. In total, 2500 cells were counted per animal [17]. The obtained value for cells in metaphase was multiplied by the Tannok constant (Kt = 0.57) to correct the tissue geometry and avoid overestimating the number of cells in metaphase [18]. Therefore, metaphase index = number of cells in metaphase × 100 × Kt/total number of cells in the crypts.

To evaluate the rate of goblet cells reflected by the periodic acid-Schiff histochemical method, 2500 cells were quantified per animal in intact villi. The ratio of the number of caliciform cells to the number of enterocytes in each villus was defined as the goblet cell index. Therefore, the goblet cell index = (number of caliciform cells/total number of cells in the villus) × 100. An Olympus BX41 binocular optical microscope with a 40× lens was used for metaphase index and goblet cell index analyses.