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...
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 in 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 norvegicus) 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 1000 mg/kg) was administered through the penile vein for the later analysis of cell proliferation. Two hours later, the animals were anesthetized intraperitoneally using 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 transluminatation.

Histological processing for intestinal sections

Other samples were embedded in historesin (Leica Historesin Kit), and 2.5-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 were measured in micrometers in 10 histologic sections per animal. Other samples were embedded in historessin (Leica Historessin 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 with a microscope equipped with a 40× objective and a Moticam 2500 camera. The total area analyzed per animal was 5.9 mm²/thickness. 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 were measured in micrometers in 10 histologic sections per animal.

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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 × 10² 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, Na2HPO4 2.302 g/L, and NaH2PO4 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

The whole-mount preparations were washed twice in 0.1 M PBS (pH 7.4) with 0.02% 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 binding of the antibodies. 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 were 1:500; 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 cover-slipped with glycerol.

Morpho–quantitative analysis

The quantification of the total HuC/D myenteric neuron population, nNOS-positive nitergic subpopulation, and glial cells (S100) was undertaken in 64 microscopic images per animal in the intermediate (32 images) and anti-neuronal (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 mm²/thickness. The area of 100 cell bodies per animal was measured using Image-Pro Plus 4.5 (Media Cybernetics, Silver Springs, MD, USA).

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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) 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 calciform cells to the number of enterocytes in each villus was defined as the goblet cell index. Therefore, the goblet cell index = (number of calciform 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.
Statistical analysis

The data were analyzed using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA). The Shapiro-Wilk test, one-way analysis of variance, and the Tukey post hoc test were used to analyze the data. The significance level was 5%, and the results are expressed as mean ± standard error.

Results

Animal treatment

The period of dietary restriction did not cause hair loss, diarrhea, or lethargic behavior in the animals. Body weight gain and decreased ration intake (P < 0.05) were observed in the C12 compared with the C7 group. The RA12 group exhibited decreased body weight and ration intake and increased water intake compared with the other groups (P < 0.05).

The Lee index, a parameter regarded as analogous to the body mass index, did not differ (P > 0.05) in the C7 and C12 groups. The RA12 group showed a significant decrease (P < 0.05) of the Lee index compared with controls.

A significant decrease also was observed in the weight of adipose tissues (peri-epididymal and retroperitoneal) isolated in rats from the RA12 group compared with the other groups. Although non-significant, the adipose tissue weight in the properly fed C12 group was greater than in the C7 group (Table 1).

Biochemical analysis of blood components

No effect of age (C7 and C12) was found on the levels of total cholesterol and triacylglycerol, the values of which were lower (P < 0.05) in the animals subjected to dietary restriction. Significant differences (P < 0.05) were found in the levels of total proteins and globulins among all the groups. This did not indicate protein malnutrition, but rather constant albumin levels. Glycemia was decreased the C12 group (P < 0.05) compared with the other groups, with no difference between the C7 and RA12 groups (Table 2).

Oxidative status

Erythrocyte and plasma TBARS levels increased in the C12 group during the aging process (P < 0.05). A positive effect of dietary restriction was reflected by a decrease in erythrocyte TBARS levels (P < 0.05) and plasma TBARS levels (P > 0.05) in the blood of the RA12 group (Fig. 1).

Morpho-quantitative analysis of myenteric plexus

Ganglionic morphology was preserved in all the groups. HuC/D-nNOS double staining showed that nitrergic neurons were located predominately in the periphery of the ganglion, whereas the other neurons were inside (Fig. 2A–C). HuC/D-S100 double staining indicated that glial cell bodies were smaller than neuronal bodies but were more numerous, with an approximate ratio of 1 neuron per 1.51, 1.47, and 1.46 glial cells in the C7, C12, and RA12 groups, respectively (Fig. 3A–C).

Age did not quantitatively affect the HuC/D population, positive nNOS subpopulation, or enteric glia when comparing the C7 and C12 groups. However, 50% dietary restriction decreased the HuC/D population and enteric glia (P < 0.05) without affecting the nitrergic population (Figs. 2D and 3D).

The morphometric analysis showed no differences in the intermediate area of the cell profile of the HuC/D neuron population, positive nNOS subpopulation, or glial cells between the C7 and C12 groups, indicating the absence of an effect of age. In the animals subjected to dietary restriction (RA12 group), a decrease was observed in the cell profile area of HuC/D neurons, with an increase in the area of glial cells (P < 0.05) and no effect on the nitrergic subpopulation (Table 3).

**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>C7 group</th>
<th>C12 group</th>
<th>RA12 group</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>458.30 ± 23.63*</td>
<td>497.10 ± 23.63*</td>
<td>359.60 ± 26.81*</td>
</tr>
<tr>
<td>Ration intake (g)</td>
<td>27.43 ± 0.966*</td>
<td>24.56 ± 2.53b</td>
<td>15.60 ± 1.34b</td>
</tr>
<tr>
<td>Water intake (mL)</td>
<td>43.7 ± 3.76a</td>
<td>40.11 ± 5.30a</td>
<td>65.41 ± 11.44a</td>
</tr>
<tr>
<td>Lee index</td>
<td>295.6 ± 2.86a</td>
<td>301.2 ± 3.60a</td>
<td>270.2 ± 6.39b</td>
</tr>
<tr>
<td>PER (g)</td>
<td>7.80 ± 1.15a</td>
<td>9.49 ± 0.35a</td>
<td>3.25 ± 0.24a</td>
</tr>
<tr>
<td>RET (g)</td>
<td>7.88 ± 1.14a</td>
<td>10.04 ± 1.25a</td>
<td>1.42 ± 0.23a</td>
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</table>

C7, properly fed rats at 7 mo of age; C12, properly fed rats at 12 mo of age; PER, peri-epididymal adipose tissue; RA12, 12-mo-old rats subjected to dietary restriction; RET, retroperitoneal adipose tissue.

Values are expressed as mean ± SE (n = 5). Means followed by different letters in the same row differ significantly (P < 0.05).

* Body weight = n/aso-anal length × 1000.

**Table 2**

<table>
<thead>
<tr>
<th></th>
<th>C7 group</th>
<th>C12 group</th>
<th>RA12 group</th>
</tr>
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<tbody>
<tr>
<td>Total cholesterol</td>
<td>98.07 ± 0.08a</td>
<td>112.90 ± 7.51a</td>
<td>76.64 ± 1.38b</td>
</tr>
<tr>
<td>(mg/dL)</td>
<td>130.90 ± 9.88a</td>
<td>153.70 ± 11.40a</td>
<td>91.55 ± 5.46b</td>
</tr>
<tr>
<td>Triacylglycerols</td>
<td>6.03 ± 0.26a</td>
<td>7.05 ± 0.21b</td>
<td>6.52 ± 0.19f</td>
</tr>
<tr>
<td>(mg/dL)</td>
<td>2.25 ± 0.10a</td>
<td>2.50 ± 0.07f</td>
<td>2.32 ± 0.05f</td>
</tr>
<tr>
<td>Total proteins</td>
<td>3.78 ± 1.04a</td>
<td>4.55 ± 0.09f</td>
<td>4.20 ± 0.08f</td>
</tr>
<tr>
<td>(g/dL)</td>
<td>127.10 ± 3.40a</td>
<td>109.10 ± 4.47b</td>
<td>114.40 ± 3.34b</td>
</tr>
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</table>

C7, properly fed rats at 7 mo of age; C12, properly fed rats at 12 mo of age; RA12, 12-mo-old rats subjected to dietary restriction.

Values are expressed as mean ± SE (n = 5). Means followed by different letters in the same row differ significantly (P < 0.05).

**Fig. 1.** Erythrocyte and plasma TBARS levels in groups C7, C12, and RA12. * Significant difference compared with the C7 group (P < 0.05). † Significant difference compared with the C7 and C12 groups (P < 0.05). # C7, properly fed rats at 7 mo of age; C12, properly fed rats at 12 mo of age; RA12, 12-mo-old rats subjected to dietary restriction; TBARS, thiobarbituric acid-reactive substances.
Intestinal analysis

No effect of age was found on the small intestinal area, total wall and mucosa tunic thicknesses, villus height, crypt depth, and villus/crypt ratio when comparing the C7 and C12 groups. However, the muscular coat decreased in the C12 group compared with the C7 group ($P < 0.05$; Table 4).

In the RA12 group, the intestinal area was unchanged, with significant decreases ($P < 0.05$) in the total ileum wall, mucosa tunic, villus height, crypt depth, and villus/crypt ratio compared with the C7 and C12 groups. The muscular coat was smaller in the C12 and RA12 groups than in the C7 group, but the difference was significant only in the C12 group.

The cell proliferation analysis showed a decrease in the metaphase index in the C12 and RA12 groups compared with the C7 group ($P < 0.05$). The rate of caliciform cells did not vary among the groups ($P > 0.05$).

Discussion

Experimental model and nutritional status of animals

The present study used 7- and 12-mo-old rats [19,20]. The dietary restriction model has been one of the most widely discussed forms of nutritional intervention used to extend the life span of different animal species. Studies that feature the term caloric restriction and decrease the amount of food without manipulating the quantity and quality of calories are frequent [3, 19]. The present work considered the term dietary restriction as more appropriate than caloric restriction because we imposed a decrease of the supply of caloric content, in addition to a decrease of other dietary components, such as proteins, amino acids, and minerals.

In the aged control animals (C12 group), there was an increase in body weight despite a decrease of ration intake compared with adult controls in the C7 group, and no change in water intake. Greater fat accumulation in the body occurs with aging [21], confirmed by the increased weight of peri-epididymal and retroperitoneal adipose tissues [22]. A decrease in ration intake with aging was found in the control animals. The increase in body fat may have occurred because of several different factors, including decreased spontaneous physical activity, decreased basal energy requirements of the cells [23], and a lower lean mass—particularly muscle mass—that occurs during the aging process [24].

Dietary restriction led to decreased body weight and adipose tissue. This is attributable to an increase in lipid metabolism, directly reflecting body composition [25]. The ration intake in RA12 animals should not be considered a parameter for comparison because it was an imposed condition. The significant increase in water intake can be attributable to a compensatory mechanism that reflects lower ration availability [25].

The Lee index [26] is used in models that affect metabolism, such as obesity [27,28] and dietary restriction [29], and it did not differ between the two control groups. However, the Lee index was lower in the animals subjected to dietary restriction, confirming the efficacy of our model, especially when linked to adipose tissue weight.

The levels of total cholesterol and triacylglycerol remained constant with age, with a decrease in the animals subjected to dietary restriction. This suggests a positive effect when the amount of ingested food is decreased because any increase has
been linked to the onset of chronic degenerative diseases, such as atherosclerosis [30].

The lower glycemic value observed in the C12 group compared with the C7 and RA12 groups does not indicate an effect of age or dietary restriction. In the literature, despite some disagreements, the reference values for glycemia in adult rats are 84.7 to 131.9 mg/dL [31], indicating that the values obtained in the present study were within normal limits. Values for total proteins, albumin, and globulins also were within the reference values for Wistar rats [32,33]. Therefore, dietary restriction did not cause a state of malnutrition.

Oxidative status

An increase in lipid peroxidation was observed with age, resulting in higher plasma and erythrocyte TBARS levels. In animals subjected to dietary restriction, lipid peroxidation was lower than in the control groups. These results demonstrate a positive effect of dietary restriction on oxidative status. The formation of free radicals is considered one of the main factors in the etiology of aging. Antioxidant substances are decreased during the aging process, further favoring the occurrence of oxidative stress [34] and justifying the increase in TBARS levels during aging. Dietary restriction may attenuate the effects of oxidative stress during the cellular respiration process, thus lowering the levels of free radicals [13,35].

Morpho-quantitative analysis of myenteric plexus

No quantitative changes were observed in HuC/D neurons, nitrergic neurons (nNOS), or glial cells (S100) in the C7 and C12 animals. A progressive decrease in the number of enteric neurons with aging frequently has been reported in the

Table 3

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<thead>
<tr>
<th>Cell profile (area) of HuC/D neurons, nNOS neurons, and enteric glia (square micrometers) in groups C7, C12, and RA12</th>
</tr>
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<tbody>
<tr>
<td>C7 group</td>
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<tr>
<td>-----------</td>
</tr>
<tr>
<td>HuC/D neurons</td>
</tr>
<tr>
<td>nNOS neurons</td>
</tr>
<tr>
<td>Glial cells (S100)</td>
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</table>

C7, properly fed rats at 7 mo of age; C12, properly fed rats at 12 mo of age; nNOS, neuronal nitric oxide synthase; RA12, 12-mo-old rats subjected to dietary restriction

Values are expressed as mean ± SE (n = 5). Means followed by different letters in the same row differ significantly (P < 0.05).

Table 4

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<tr>
<th>Intestinal morphometry, including SI area, thickness of total wall, mucous tunic, muscular coat, villus height, crypt depth, villus/crypt ratio, metaplasia index, and rate of caliciform cells in groups C7, C12, and RA12</th>
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<tbody>
<tr>
<td>C7 group</td>
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<tr>
<td>-----------</td>
</tr>
<tr>
<td>SI area (cm²)</td>
</tr>
<tr>
<td>Total wall (µm)</td>
</tr>
<tr>
<td>Mucosa tunic (µm)</td>
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<tr>
<td>Muscular coat (µm)</td>
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<tr>
<td>Villus height (µm)</td>
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<tr>
<td>Crypt depth (µm)</td>
</tr>
<tr>
<td>Villus/crypt ratio</td>
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<tr>
<td>Metaplasia index (%)</td>
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<td>Goblet cell index (%)</td>
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</table>

C7, properly fed rats at 7 mo of age; C12, properly fed rats at 12 mo of age; RA12, 12-mo-old rats subjected to dietary restriction; SI, small intestinal

Values are expressed as mean ± SE (n = 5). Means followed by different letters in the same row differ significantly (P < 0.05).
literature for myenteric neurons in the small [9,20] and large [7,19,36] intestines in different species, with various types of responses according to neuronal population and subpopulation.

The number of nitrergic neurons, which inhibit different gastrointestinal segments in rodents, remained constant with age [4,7]. This indicates that not all enteric neurons are similarly affected by aging.

No decreases were observed in the HuC/D and nitrergic neuronal populations, which may be attributable to the ages under analysis (7 and 12 mo). In rats, a significant decrease in the number of neurons in the small and large intestines has been reported beginning at 12 mo of age [9,36] and continuing throughout the life span. Another factor that supports this hypothesis is the lack of differences in glial cells, which are responsible for the production of neurotrophic factors that are important for neuronal development and maintenance as age advances [9]. The loss of glial cells precedes neuronal loss [36], justifying the unchanged number of HuC/D and nNOS neurons in the C12 animals. Conversely, in the 12-mo-old rats subjected to dietary restriction, a significant decrease was found in the HuC/D neuron population and glial cells, whereas the nNOS subpopulation did not change compared with the other groups.

No changes were found in the number of nNOS neurons with age or in response to the dietary restriction, indicating that these neurons are indeed more resistant to the aging process [7] and the imposed nutritional condition.

The literature has proposed several reasons for neuron loss, including the increased production and accumulation of free radicals [3], a decrease in neurotrophic factors derived from glial cells [37], apoptosis activation, changes in gene expression or mutation in certain genes [38], alterations in Ca$^{2+}$ signaling [38], and mitochondrial dysfunction [37,39].

Conversely, caloric restriction has been shown to decrease oxidative damage to macromolecules, improving repair [35], leading to lower rates of mitochondrial free radicals, decreasing cell metabolism and apoptosis [12], and improving neurotrophic signaling [40]. Neuronal loss in animals subjected to dietary restriction did not corroborate the aforementioned benefits. Some hypotheses could be formulated to explain this neuronal loss. One hypothesis is that the number of glial cells was decreased, compromising their ability to produce neurotrophic factors that are important for neuron maintenance [9] and the decrease of which has been linked to neuronal death [37]. Another hypothesis is that the imposed nutritional status could have hindered neuron–glia homeostasis. Glia act by regulating extracellular pH by buffering the levels of extracellular ions, such as K$^+$ and Na$^+$, participating in the immune response, capturing neurotransmitters such as glutamate and γ-amino-butyric acid, and providing substrates for neurotransmitter synthesis [41,42].

Variations in the cell area of enteric neurons demonstrate the significant plasticity of these cells in response to different experimental manipulations and changes in the luminal environment of the gastrointestinal tract, with a decrease [43] or maintenance [8] in malnourished rats and a decrease [27] or maintenance [28] in obese rats.

Nevertheless, with the exception of nitrergic neurons, the population of which remained as expected [4,36], the animals subjected to dietary restriction exhibited a decrease of the cell area of the HuC/D population and larger glial cell bodies. An increase in the activity of the remaining glial cells resulted in hypertrophy of their cell bodies, whereas neurons exhibited a decrease of activity and cell atrophy attributable to the influence of dietary restriction.

Hypertrophied glial cells in the brain (i.e., astrocytes) with greater proliferation of their extensions have been associated with an increase in the synthesis of glial fibrillary acidic protein and vimentin after brain injury [44]. These central nervous system effects support our hypothesis that enteric glia hypertrophy may also occur because of greater intracellular protein synthesis in the cytoskeleton, increasing functional capacity.

In neural lesion models, prolonged dietary restriction has apparently neuroprotective effects and promotes functional recovery. One hypothesis presented by Sharma and Kaur [45] suggests that dietary restriction combined with mild stress improves cell defense systems and protects neurons against subsequent severe stress, such as brain lesions.

Thus, unlike studies that used caloric restriction as a neuroprotective agent, the present results indicate that the experimental model used herein, in which rats were supplied with 50% of the normal daily ration for 5 mo, may be considered a severe, rather than mild, form of stress, with significant morphologic repercussions on the population of HuC/D neurons and glial cells, sparing nitrergic neurons given their proved greater resistance.

**Histologic analysis**

The absence of an effect of age on the intestinal area, total wall, mucosa tunic, villus height, and crypt depth indicates the preservation of functions that are aimed primarily at absorption [46]. A decrease of the muscular coat was observed in the C12 group compared with the C7 group. This result differs from that of Marese et al. [9], in which no muscular coat changes were observed at different ages. Muscle hypertrophy has been observed in denervation models [47], and responses similar to the present results have been found in malnutrition models [48].

In animals subjected to dietary restriction (RA12 group), significant decreases in the thickness of the intestinal wall, mucosa tunic, crypt depth, and villus height were detected. However, no differences were found in intestinal area and muscular coat thickness between the C12 and RA12 groups. The results obtained for the muscular coat and its elements are common in research that involves dietary manipulation, particularly studies that decrease the food supply, because nutrient availability in the intestinal lumen acts as a trophic factor [14].

The presence of nutrients in the lumen stimulates the growth of villi and crypts [49]. Given the decreased intestinal parameters and lower villus/crypt ratio, a decrease in digestion and intestinal absorption was observed in those animals.
A decrease in the metaphase index was observed in the C12 and RA12 groups compared with the C7 group. The duration of the progenitor cell cycle in the intestinal epithelium has been shown to increase with age [50], with changes in the number of intestinal epithelia [51], demonstrating that age influences cell proliferation. Despite the absence of differences between the RA12 and C12 groups, the cellular kinetics of the mucosa may also have undergone disequilibrium. The renewal of intestinal epithelium cells is a dynamic process that results from two cytologic events associated with cell renewal and renewal. Whenever the intestine responds to a given condition by increasing the extrusion rate, with an unchanged or decreased proliferation rate, villus height decreases, with consequential functional compromise [14].

The rate of goblet cells did not vary among the groups. Although villus height and crypt depth did not change in aged animals and were lower in animals subjected to dietary restrictions, the population of caliciform cells did not change, and the production of mucins was preserved, indicating that other factors may be related to the kinetics of this cell type.

Conclusion

The aging process alters oxidative status, resulting in increased lipid peroxidation that in turn is reversed by dietary restriction that also lowers the levels of cholesterol and triacylglycerol. The present dietary restriction model elicited morpho-quantitative changes in the myenteric plexus and histology of the ileum, with likely effects on intestinal function. The intestine responds to a given condition by increasing the extrusion rate, which is reversed by dietary restriction. The renewal of intestinal epithelium cells is a dynamic process that results from two cytologic events associated with cell renewal and renewal. Whenever the intestine responds to a given condition by increasing the extrusion rate, with an unchanged or decreased proliferation rate, villus height decreases, with consequential functional compromise [14].

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