Food restriction protects the myenteric nervous population of rats with type 2 diabetes mellitus

A restrição alimentar protege a população nervosa mioentérica de ratos com diabetes mellitus tipo 2

DOI:10.34117/bjdv8n4-111

Recebimento dos originais: 21/02/2022
Aceitação para publicação: 31/03/2022

Carlos Vinicius Dalto da Rosa
Doutor
Institution: Universidade Estadual de Londrina
Address: Rodovia Celso Garcia Cid, PR-445, Km 380 - Campus Universitário
Londrina - PR ,CEP: 86057-970
E-mail: carlosvinicius_xz@hotmail.com

Jessica Men de Campos
Doutora
Institution: Centro Universitário Metropolitano de Maringá (Unifamma)
Address: Av. Virgílio Manfélia - Jardim Ouro Cola, Maringá – PR, CEP: 87070-170
E-mail: jessicamen.salus@gmail.com

Gabriela Scomparin Goularte
Graduação
Institution: Universidade Estadual de Maringá, Centro de Ciências da Saúde
Departamento de Medicina.
Address: Av. Colombo, 5790, Jardim Universitário, Maringá, PR – Brasil
CEP: 87020-900
E-mail: gabi.scomparin.goularte@gmail.com

Vilma Aparecida Ferreira de Godoi
Doutora
Institution: Universidade Estadual de Maringá, Centro de Ciências Biológicas
Departamento de Ciências Fisiológicas
Address: Avenida Colombo, 5790, Zona 7, Maringá, PR – Brasil, CEP: 87020-900
E-mail: godoigazola@gmail.com

Maria Raquel Marçal Natali
Doutora
Institution: Universidade Estadual de Maringá, Centro de Ciências Biológicas
Departamento de Ciências Morfológicas
Address: Avenida Colombo, 5790, Zona 7, Maringá, PR – Brasil, CEP: 87020-900
E-mail: mrmnatali@gmail.com

ABSTRACT
Background: Type 2 diabetes affects the intestine. Food restriction (FR) promotes benefits, but the studies of its effects in rats are scarce. This study aims to analyze the influence of two type 2 diabetes models and FR over the intestine morphology. Methods:
Thirty Wistar rats formed three groups (n=10/group) treated for two months: C (control); DE (diabetic with streptozotocin+cafeteria-style diet); and DN (diabetic with streptozotocin+nicotinamide). These groups were subdivided into six during two months: CC (control), CCR (control+food restriction), DEC (diabetic+standard diet), DER (diabetic+food restriction), DNC (diabetic+standard diet) and DNR (diabetic+food restriction). FR was 50% of the average daily dietary intake of group C. Jejunum and ileum samples were collected for evaluation of wall morphometry, goblet cell number, number and profile of myenteric neurons and glia. Results: The DE model promoted wall reduction in both segments, while DN jejunum was increased. Goblet cells were reduced in the jejunum for both diabetic models and FR. Both intestinal segments presented reduction of neuronal and glial myenteric populations in diabetes. Conclusions: FR promoted protection of myenteric neurons and glial cells against diabetic damage. The jejunum and ileum respond differently to diabetes and FR. FR has positive effects over the small intestine, mainly over the enteric nervous system.

Keywords: dietary restriction, streptozotocin, enteric neurons, enteric glia, small intestine.

1 INTRODUCTION

Type 2 diabetes mellitus (T2DM) involves chronic metabolic disorders in the whole organism. In the last decades its incidence and prevalence increased considerably.
The major factors responsible for this increase are population growth, older population, urbanization, obesity and deficiency of physical activity [1].

T2DM can be induced in various ways [2], including by association of factors [3]. The induction also can occur by a carbohydrate and/or lipid-rich diet [4], or by the combination of hypercaloric diet and streptozotocin injection (STZ) [5].

The oxidative stress can affect individuals with T2DM. The increase in production of reactive oxygen species (ROS) and the reduction in the antioxidant capacity of the tissues are mainly results from hyperglycemia [6,7].

Although T2DM is the more frequent type of diabetes, it is the less studied with relation to the gastrointestinal tract (GIT) [8]. In the GIT, T2DM favors motor problems like constipation, diarrhea and fecal incontinence. These symptoms are mainly originated from diabetic neuropathy, derived from oxidative stress [9]. The neuropathy consists in neuronal death or alteration of its expression, which generates dysfunction and the common symptoms of T2DM [7].

The enteric nervous system (ENS) is involved in GIT neuropathy. It is arranged in two main ganglionic plexus: the submucous and myenteric plexus. They modulate the absorption, secretion and motility of GIT [10] and are affected by T2DM [11].

Stenkamp-Strahm et al. [12] observed diabetic neuropathy in mice with a model of T2DM achieved by hyperlipidic diet. Besides the structural damage (neurofilament loss and axon swelling), there was reduction of myenteric neuron number and alteration in the expression of neuron subpopulations of duodenum [12]. Spangéus and El-Salhy [13] also found abnormalities in neuronal expression of vasoactive intestinal peptide and nitric oxide synthase (NOS) in the GIT of a spontaneous diabetic mice model.

In the small intestine (SI) of diabetic rats, it can also be observed numerical increase of serotoninergic enteroendocrine cells [14], besides morphologic (like hyperplasia) and enzymatic increase [8,15]. The mechanism of these alterations is not clear yet.

Food restriction (FR) consists of reducing food intake, maintaining minimum levels of nutrients. It benefits pancreatic β-cell function, the maintenance of blood glucose and other factors in patients with T2DM [16,17], and in animal models [2,18]. Therefore, FR is an easy alternative in the control of T2DM compared to other interventions such as bariatric surgeries, which have become more frequent due to the epidemic of obesity and T2DM [19].
In our previously study [20] we observed that both models of T2DM (association between STZ and cafeteria-style diet, and association of STZ and nicotinamide) produced consistent alterations such as weight deregulation, hyperglycemia, hyperphagia, polydipsia, biochemical parameters (glycation and hepatic damage), insulin production and response (insulin resistance), and oxidative stress. FR was able to partially reverse the observed damage of this study. From this, in the present study we aimed to evaluate the morphological alterations caused by these two models of T2DM in the jejunum and ileum, as well the effects of FR in these segments.

2 METHODS
2.1 DRUGS AND CHEMICALS

Streptozotocin and nicotinamide used in this study were obtained from Sigma-Aldrich, Missouri, USA. All reagents used had the best possible quality. The antibodies used were: primary anti-HuC/D (Molecular Probes, Oregon, USA), anti-nNOS (Santa Cruz Biotec., Texas, USA) and anti-S100 (Sigma-Aldrich); and secondary Alexa Fluor 546 and 488 (Invitrogen, California, USA).

2.2 ANIMALS AND TREATMENT

Thirty male Wistar rats (Rattus norvegicus), 90 days old and initial body mass of 328.2±21.8 g, from the Central animal house of the State University of Maringá, were kept individually in polypropylene boxes, with 12/12 hours light and dark cycles and temperature of 22±2ºC. All manipulation protocols followed the regulations of the Ethics Commission on the Use of Animals (protocol 7590050415).

The animals were treated during 4 months, split into 2 periods: pre-food restriction (months 1 and 2) and food restriction (months 3 and 4).

In the pre-food restriction period, the animals were distributed into 3 groups (n=10): C (control), DE (T2DM with diet) and DN (T2DM). Group C rats received intravenous saline, and were fed with standard diet ad libitum.

The rats from DE group rats were diabetized with an intravenous injection of 35 mg/kg of streptozotocin (STZ), and received a cafeteria-style diet (33% standard ration Nuvilab®, 33% Nestlé® condensed milk, 7% sugar and water), sugar water (32%) and normal water, ad libitum (adapted from Sahin et al. [21] and Trammel et al. [22]), as previously described [20].
The DN group rats were diabetized with intravenous injection of 60 mg/kg STZ, followed by injection of nicotinamide (NIC-80 mg/kg). One week later they received a new dose of 30 mg/kg of STZ and 40 mg/kg of NIC (adapted from Sharma et al. [23]). These animals received standard diet and water ad libitum, as previously described [20].

Insulin insufficiency [24] and fasting hyperglycemia greater than 200 mg/dL of blood [5] were observed for both diabetic models.

In the months 3 and 4 (food restriction period), group C formed the groups CC (control) and CCR (control + food restriction); the DE group formed DEC (diabetic + standard diet) and DER (diabetic + food restriction); and DN formed DNC (diabetic + standard diet) and DNR (diabetic + food restriction) (n=5) (Table 1). During this period, all animals received standard diet and water ad libitum.

The food restriction (FR) protocol consisted of receiving only 50% of the average food intake of the C group, as previously described [20], totalizing 16 g of standard chow daily, and water ad libitum. Throughout the treatment it was monitored: food and water consumption; body mass; and fasting/postprandial blood glucose [20].

2.3 SMALL INTESTINE SAMPLES COLLECTION, PROCESSING AND HISTOLOGICAL ANALYSIS

After anesthesia, vertical laparotomy was made and small intestine (SI) was collected and measured in its length. Subsequently, samples of jejunum (right after the duodenojejunal flexure) and ileum (in the final quarter of SI) were separated and washed in phosphate buffered saline (PBS, 0.1 M, pH 7.3), and fixed (4% paraformaldehyde).

After 6 hours of fixation, jejunum and ileum samples were histologically processed (dehydrated, diaphanized and paraffin-embedded) and 5 µm thick semiserial transversal sections were made with a microtome.

Table 1. Diets of experimental groups, controls and diabetics, during the treatment with food restriction

<table>
<thead>
<tr>
<th></th>
<th>Months 1 and 2 (pre food restriction period)</th>
<th>Months 3 and 4 (food restriction period)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group CC</strong></td>
<td>Standard diet&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Standard diet&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Group CCR</strong></td>
<td>Standard diet&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Standard diet (16 g)</td>
</tr>
<tr>
<td><strong>Group DEC</strong></td>
<td>Cafeteria-style diet + sugar water (32%)</td>
<td>Standard diet&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Group DER</strong></td>
<td>Standard diet&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Standard diet (16 g)</td>
</tr>
<tr>
<td><strong>Group DNC</strong></td>
<td>Standard diet&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Standard diet (16 g)</td>
</tr>
<tr>
<td><strong>Group DNR</strong></td>
<td>Standard diet&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Standard diet (16 g)</td>
</tr>
</tbody>
</table>

* ad libitum
2.4 MORPHOMETRIC ANALYSIS OF THE INTESTINAL WALL COMPONENTS.

To morphologic and morphometric evaluation of jejunal and ileal wall components, the histological sections were stained with hematoxylin and eosin (HE). The images were captured with a light microscope coupled to a high-resolution camera (under a 10× objective) to analyze the total wall thickness, mucosa, height of the villi, crypt depth, submucosa and muscular externa. One hundred measurements per parameter were performed of each intestinal segment per animal, using the program Image Pro Plus 4.5 (Media Cybernetics, Maryland, USA).

2.5 GOBLET CELLS COUNTING

To assess goblet cell number, another set of slides was submitted to Periodic Acid-Schiff (PAS) histochemical staining method to evidence neutral mucines of jejunum and ileum. The slides were analyzed under light microscope, under 20× objective. 2500 epithelial cells per animal were recorded to obtain the percentage of labeled to unlabeled cells. The goblet cell index was calculated by the number of labeled cells×100/total number of counted cells.

2.6 LABELING AND QUANTIFICATION OF IMMUNOREACTIVE MYENTERIC NEURONAL AND GLIAL CELLS

After fixation, whole-mounts from the muscular externa tunic were obtained by dissection of jejunum and ileum samples. The whole-mounts were washed and incubated in blocking solution. Then, the tissues were incubated with primary anti-HuC/D and anti-nNOS antibodies diluted (1:500) and with secondary antibody (Alexa Fluor 546 and 488 respectively - 1:500), as previously described [25], to mark general population and the nitrergic subpopulation, respectively. Another set of whole-mounts from each intestinal segment was incubated with an anti-S-100 antibody, followed by a secondary antibody (Alexa Fluor 488) to mark glial cells. The whole-mounts were mounted in slides with Prolong Gold Antifade Reagent.

Under a fluorescence microscope (Olympus FSX100), the neurons and glia found in 40 captured microscopic fields under 20× objective were counted, with a total analyzed area of 5.87 mm²/animal. Additionally, 100 cell bodies of each marking per animal were measured in their area, using Image Pro Plus 4.5.
2.7 STATISTICAL ANALYSIS

Using GraphPad Prism program (GraphPad Software, version 5.0, USA), the Kolmogorov-Smirnov normality test was applied over the data. Parametric data were subjected to analysis of variance (ANOVA) followed by Tukey's post-test. Kruskal-Wallis test followed by Dunns post-hoc test was adopted for the nonparametric data. The results were presented as mean ± standard error (SEM), and all analysis were considered significant at p<0.05.

3 RESULTS

T2DM was obtained in both types of induction, STZ+cafeteria-style diet and STZ+NIC. In general, both models presented hyperglycemia, insulin resistance, reduced pancreatic insulin, altered serum biochemical parameters and oxidative stress [20]. GIT function was also affected, as seen by the frequent episodes of diarrhea, mainly in DEC group. Both models affected the small intestine.

3.1 SMALL INTESTINE LENGTH

The small intestine (SI) length was significantly increased (p<0.05) by T2DM only in DEC group (15.74%), regarding CC group. Food restriction (FR) significantly reduced SI length by 20.66% in DER group, when compared to DEC. The SI length of DNC group had no difference (p>0.05) when compared to CC group, but was 19.84% lower compared to DEC. FR did not affects this parameter on DNC group (Fig. 1).

Fig. 1 Small intestine length (cm) of control and diabetic rats submitted to food restriction after 4 months of treatment. Groups: control (CC), control with food restriction (CCR), diabetic+streptozotocin+cafeteria-style diet (DEC); diabetic+streptozotocin+cafeteria-style diet with food restriction (DER), diabetic+streptozotocin+nicotinamide (DNC) and diabetic+streptozotocin+nicotinamide with food restriction (DNR). Results expressed as mean±SEM (n=5/group). *p<0.05; ***p<0.001. One-way ANOVA and Tukey’s post-hoc test analysis.
3.2 JEJUNUM AND ILEUM WALL MORPHOMETRY

The histological organization of the intestine was preserved in all groups, for both ileum and jejunum. However, there were differences in the intestinal morphometric parameters between the groups.

The morphometry of jejunum and ileum data is presented in Table 2. It was observed that the jejunum of DEC rats was reduced in every parameter analyzed (total wall, mucosa, submucosa, muscularis externa, villus height and crypt depth) when compared to CC group. The DNC group presented increased jejunum wall parameters, except in submucosa and crypt depth, which were reduced, regarding CC group. Comparing the two diabetic groups, DEC and DNC, we noted higher (p<0.05) total wall, mucosa, villi height and muscularis externa, while the submucosa and crypt depth parameters were reduced.

<p>| Table 2. Morphometry of jejunum wall of control and diabetic rats submitted to food restriction. |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>CC</th>
<th>CCR</th>
<th>DEC</th>
<th>DER</th>
<th>DNC</th>
<th>DNR</th>
</tr>
</thead>
<tbody>
<tr>
<td>TW</td>
<td>653±3.09</td>
<td>637±2.99</td>
<td>548.1±3.28*</td>
<td>582±3.89</td>
<td>757.7±3.68**</td>
<td>583±2.81**</td>
</tr>
<tr>
<td>M</td>
<td>535.0±2.46</td>
<td>526.0±2.79</td>
<td>437.9±2.52*</td>
<td>485.3±3.02**</td>
<td>644.2±3.15**</td>
<td>500.4±3.07**</td>
</tr>
<tr>
<td>VH</td>
<td>382.2±50.19</td>
<td>375.6±61.05</td>
<td>349.4±46.97*</td>
<td>375.3±49.92*</td>
<td>509.3±56.85*</td>
<td>401.9±58.93**</td>
</tr>
<tr>
<td>CD</td>
<td>109.6±0.79</td>
<td>95.09±0.76*</td>
<td>99.39±0.59*</td>
<td>92.22±0.39**</td>
<td>96.93±0.76**</td>
<td>83.18±0.56**</td>
</tr>
<tr>
<td>SM</td>
<td>40.14±0.34</td>
<td>41.18±0.34</td>
<td>35.61±0.40*</td>
<td>41.29±0.40**</td>
<td>26.38±0.21**</td>
<td>32.53±0.37**</td>
</tr>
<tr>
<td>ME</td>
<td>74.27±0.79</td>
<td>75.67±0.67</td>
<td>81.51±0.81*</td>
<td>64.59±0.73</td>
<td>78.30±0.75**</td>
<td>51.04±0.72**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC</td>
<td>CCR</td>
<td>DEC</td>
<td>DER</td>
<td>DNC</td>
<td>DNR</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>------------------------</td>
<td>-------------------------</td>
<td>-------------------------</td>
<td>-------------------------</td>
<td>-------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>TW</td>
<td>666.6±2.75</td>
<td>588.5±2.61*</td>
<td>604.2±2.62*</td>
<td>544.5±3.19**</td>
<td>659.3±3.35**</td>
<td>486.6±2.92**</td>
</tr>
<tr>
<td>M</td>
<td>551.6±2.11</td>
<td>472.2±2.11*</td>
<td>499.8±2.07*</td>
<td>429.9±2.70**</td>
<td>542.7±2.32**</td>
<td>410.9±2.53**</td>
</tr>
<tr>
<td>VH</td>
<td>340.5±1.3</td>
<td>272.1±1.72*</td>
<td>291.1±1.35*</td>
<td>242.2±1.80**</td>
<td>351.2±2.26**</td>
<td>264.5±1.59**</td>
</tr>
<tr>
<td>CD</td>
<td>189.6±1.04</td>
<td>156.5±0.88*</td>
<td>173.4±1.00*</td>
<td>156.9±1.26**</td>
<td>162.6±0.99**</td>
<td>125.0±0.83**</td>
</tr>
<tr>
<td>SM</td>
<td>30.73±0.33</td>
<td>34.80±0.24*</td>
<td>28.24±0.25*</td>
<td>32.47±0.30**</td>
<td>20.40±0.21**</td>
<td>20.37±0.21**</td>
</tr>
<tr>
<td>ME</td>
<td>76.26±0.51</td>
<td>76.99±0.64</td>
<td>76.53±0.55*</td>
<td>72.56±0.67*</td>
<td>73.79±0.83*</td>
<td>49.79±0.55**</td>
</tr>
</tbody>
</table>

* Measurements of total wall thickness (TW), mucosa (M), submucosa (SM), muscularis externa (ME) tunics, villus height (VH) and crypt depth (CD) after 4 months of treatment. Groups: control (CC), control with food restriction (CCR), diabetic+streptozotocin+cafeteria-style diet (DEC); diabetic+streptozotocin+cafeteria-style diet with food restriction (DER), diabetic+streptozotocin+nicotinamide (DNC) and diabetic+streptozotocin+nicotinamide with food restriction (DNR). Results expressed as mean±SEM (n=5/group). * p<0.05 vs CC; # p<0.05 vs DEC; † p<0.05 vs DNC. Kruskal-Wallis and Dunn’s post-hoc test analysis.

FR did not produce significant morphometric changes in the jejunum of CCR group, except a reduction in crypt depth. With regard to diabetic groups, FR presented an
increase of most wall parameters of DER group when compared to DEC. However, most of these values still presented significative difference in relation to CC group. The DNR group showed that FR promotes reduction (p<0.05) of jejunum layers in this specific type of T2DM, except for submucosa, regarding the DNC group. Except in the villus height, all jejunum parameters of DNR group remained lower than the values of CC group.

DEC group showed that T2DM induced by streptozotocin and cafeteria-style diet promotes general reduction (p<0.05) of ileum tunics (Table 2). In the other model, DNC rats showed no alteration (p>0.05) of total wall and mucosa measurements, while we observed a reduction of submucosa, muscularis externa and crypt depth, compared to CC group. The villus height was increased in the DNC group, regarding control.

FR promoted changes in the ileum wall. CCR presented general reduction (p<0.05) in the ileum, when compared with CC, except for the submucosa tunic. FR also promoted general reduction in DER group regarding CC. When compared to DEC group, DER rats showed that FR exacerbates the ileum mucosa reduction (p<0.05). In DNR group, FR promoted strong reductions in all wall parameters analyzed, except for the submucosa tunic, compared to CC and DNC groups.

3.3 GOBLET CELL NUMBER ANALYSIS

The number and index of PAS\(^+-\) epithelial cells of jejunum (Fig. 2 a and b) was reduced due to both T2DM models and FR, regarding CC group. We also observed fewer goblet cells in DNC and DNR groups when compared to DE. The goblet cell number and index of ileum (Fig. 2 c and d) did not change between groups.

3.4 IMMUNOHISTOCHEMISTRY FOR MYENTERIC NEURONS AND GLIAL CELLS

Samples of the immunohistochemistry staining are presented in Figure 3. The immunohistochemistry data are presented in the Figures 4 and 5. In the jejunum (Fig. 4), reduction the number of HuC/D\(^+\) neurons and in the nNOS\(^+\) subpopulation was observed in DEC and DNC, accompanied by a reduction of glial cell (S-100\(^+\)) number. The morphometry of cellular area increased in HuC/D\(^+\) neurons and glial cells only in the DEC group, and not in the DNC. The neuronal profile of nNOS\(^+\) neurons was increased in both diabetic groups, DEC and DNC, regarding CC.
Fig. 2 Goblet cell number of small intestine of control and diabetic rats submitted to food restriction after 4 months of treatment. (a) Total goblet cell number counted in 2500 epithelial cells of jejunum. (b) Goblet cell index of jejunum. (c) Total goblet cell number counted in 2500 epithelial cells of ileum. (d) Goblet cell index of ileum. (e) Representative photomicrograph of PAS-stained goblet cells (arrows). Periodic Acid-Schiff histochemical staining; 100X magnification. Groups: control (CC), control with food restriction (CCR), diabetic+streptozotocin+cafeteria-style diet (DEC); diabetic+streptozotocin+cafeteria-style diet with food restriction (DER), diabetic+streptozotocin+nicotinamide (DNC) and diabetic+streptozotocin+nicotinamide with food restriction (DNR). Results expressed as mean±SEM (n=5/group). **p<0.01; ***p<0.001. One-way ANOVA and Tukey’s post-hoc test analysis.

FR did not influence (p>0.05) the jejunum of the control group (CCR). In T2DM, FR prevented the decrease in number and the increase of neuronal profile (p<0.05) of HuC/D+ neurons only in DER group, while both T2DM models (groups DER and DNR) had the number and neuronal profile of nNOS+ preserved by FR. In the S-100+ glial population FR preserved the cell number in both diabetic groups (DER and DNR) compared to the diabetic controls. The glial profile was unaltered (p>0.05) in DER and reduced (p<0.05) in DNR when compared to DNC and CC groups.
Fig. 3 Representative photomicrographs of myenteric ganglia. Myenteric ganglia (200x magnification) from jejunum of diabetic rats submitted to food restriction (after 4 months of treatment). Myenteric ganglia (a) double immunostained myenteric ganglia for HuC/D+ (red) and nNOS+ neurons; (b) HuC/D+ single immunostain; (c) nNOS+ single immunostain; and (d) S-100+ glial cells

Fig. 4 Myenteric neurons and glia from jejunum of control and diabetic rats submitted to food restriction after 4 months of treatment. (a) Number of HuC/D+ neurons. (b) Cell body area of HuC/D+ neurons. (c) Number of nNOS+ neurons. (d) Cell body area of nNOS+ neurons. (e) Number of S-100+ glia. (f) Cell body area of S-100+ glia. Groups: control (CC), control with food restriction (CCR), diabetic+streptozotocin+cafeteria-style diet (DEC); diabetic+streptozotocin+cafeteria-style diet with food restriction (DER), diabetic+streptozotocin+nicotinamide (DNC) and diabetic+streptozotocin+nicotinamide with food restriction (DNR). Results expressed as mean±SEM (n=5/group). *p<0.05; **p<0.01; ***p<0.001. One-way ANOVA and Tukey's post-hoc test analysis.
The ileum (Fig. 5) presented reduction in the number and increase of neuronal profile of HuC/D⁺ and nNOS⁺ neurons, in both diabetic groups (DEC and DNC), regarding CC group. The S-100⁺ glia cell number was reduced, while cell profile increased only in DEC group, regarding the control.

The only alteration observed in the ileum of CCR group, by the action of the FR, was an increase (p<0.05) of glial profile. FR preserved only HuC/D⁺ neurons in the ileum of DER and DNR groups, when compared to their respective diabetic control groups. The glial S-100⁺ cell number was not affected by FR in the DER group. On the other hand, the DNR group presented an increase of glial cell number, and reduction of glial profile when compared to the normal (CC) and diabetic (DNC) controls.

Fig. 5 Myenteric neurons and glia from ileum of control and diabetic rats submitted to food restriction after 4 months of treatment. (a) Number of HuC/D⁺ neurons. (b) Cell body area of HuC/D⁺ neurons. (c) Number of nNOS⁺ neurons. (d) Cell body area of nNOS⁺ neurons. (e) Number of S-100⁺ glia. (f) Cell body area of S-100⁺ glia. Groups: control (CC), control with food restriction (CCR), diabetic+streptozotocin+cafeteria-style diet (DEC); diabetic+streptozotocin+cafeteria-style diet with food restriction (DER), diabetic+streptozotocin+nicotinamide (DNC) and diabetic+streptozotocin+nicotinamide with food restriction (DNR). Results expressed as mean±SEM (n=5/group). *p<0.05; **p<0.01; ***p<0.001. One-way ANOVA and Tukey’s post-hoc test analysis.
4 DISCUSSION

Both diabetic models (STZ+cafeteria-style diet and STZ+NIC) presented hyperglycemia, insulin resistance and oxidative stress, characterizing the T2DM state on our previous results [20].

T2DM affects the GIT, like the intestine wall morphology [26,27], enteroendocrine cell number [28], carbohydrate-related enzymes [8] and the myenteric neuron population [13,29]. However, the literature is still scarce about T2DM impacts over the small intestine. The present study focused morphological characteristics of the wall and the ENS generated by two types of T2DM models, in the jejunum and ileum.

The SI length were increased only in the DEC group (STZ + cafeteria-style diet). It is known that the increase of the intestine length is linear accordingly to the glycemia [30]. The higher levels of advanced glycation ending products (AGE) [20], when compared to the other T2DM model, probably are the responsible for the morphological remodeling in diabetes [31]. FR was able to prevent this AGE-related increase, probably by the positive impact of FR in glycation previously evaluated [20].

The biomechanical proprieties of the wall, also related to the integrity of the tunics, are important to the intestine functions [32], though studies involving T2DM and intestine morphometry are scarce. In our study, jejunum and ileum morphometries presented reduction of the intestinal wall in DEC group. This goes against the increase in the SI length observed in the same group. These results could mean a total deregulation of proliferation of SI, which is controlled by intrinsic innervation (ENS), and is affected by T2DM [33]. Another possible factor is the failure in the mechanical control of the wall, mainly the muscularis externa, which could affect these morphological characteristics. Hadzijahic et al. [34] confirmed that both mucosa and muscularis externa thickness are influenced by the reduction of myenteric neuron number. On the other hand, STZ+NIC-induced diabetes (DNC) promoted increase of jejunum total wall, even with crypt depth reduction.

With relation to the ileum, this model of T2DM caused a disruption of mucosa balance, with an increase of villus height and reduction of crypt depth, possibly by changes in proliferation and apoptosis. Verdam et al. [26] showed that human patients with obesity and T2DM presented increased enterocyte mass and simultaneous enterocyte loss, corroborating our hypothesis about the deregulation of the wall. Others studies already showed that obesogenic diet promotes hypertrophy in the jejunum of rats [35], increased small intestine weight, length [8] and proliferation [36] in rodents, as well as
T2DM causes villus edema [27] and impaired mucosal barrier integrity [37] in the SI of human subjects.

These results means that different durations and intensities in models of T2DM promote disparate characteristics, and even affects the distinct intestinal segments in several ways. Our results could be justified by differences in T2DM severity and disturbance of the diet, since each model affected the blood parameters, and the whole body differently [20].

Some studies relate FR with pancreatic beta-cell parameters [2,17,38] and oxidative stress [39,40], but few considerate its effects over the intestine wall. Goodlad et al. [41] observed that just three days of starvation promoted a great reduction of crypt cell production rate and proliferation of small intestine epithelial cells. Another study showed that a type of restriction promoted reduction of parameters of mucosa and an increase of apoptosis of small intestine epithelial cells of mice [42]. These data corroborates with our study, with a general reduction observed in the small intestine, due to FR. Also, in our study, the ileum seems more vulnerable to FR effects than jejunum, as seen in CCR group, possibly due the smaller role of ileum concerning absorptive functions, compared to the jejunum, saving nutrients and energy for other segments [43]. Also, the wall reduction alone cannot be considered a type of damage.

The relation between intestine, diabetes and FR is scarce in the literature. Mao et al. [36] found that caloric restriction can reverse diabetes-induced morphometric alterations of intestine. In the present study, FR promoted contrary effects in the jejunum of the two models of T2DM. FR partially avoided the diabetes-induced reduction of wall in DER, while promoted a strong reduction in DNR. Regarding the ileum, all groups submitted to FR showed reduction of the wall. This could mean the ileum would have a limited adaptive capacity compared to the jejunum.

The goblet cells are important in maintaining a good environment to the intestinal activities, being responsible for protecting the epithelium from damage [44]. However, data about goblet cells related to T2DM and FR are scarce. Our results show that both T2DM and FR caused reduction of goblet cell number in jejunum, while in the ileum they were unaltered. The reduction of goblet cells in the jejunum indicates that probably there are less neutral mucines available in the luminal surface, which affects the normal functions of SI. The goblet cell and mucines reduction were observed in mice subjected to high-fat diet, being related to the alteration of microbiota and inflammation [45,46]. The FR could reduce the goblet cell and mucus of the epithelial surface by the lower food
intake and consequent lower demand. Schoffen et al. [47] also observed the reduction of neutral mucines by FR, but in the proximal colon. Based on this, FR has an overwhelming effect over T2DM changes. However, this cannot be considered a positive effect, but an adaptive response. The difference between the intestinal segments should be further studied in the future.

High-fat diets [48,49] and the main diabetes types, types 1 [25] and 2 [12,22,29], promote losses in the enteric neuron population, generating neuropathy [50], which disrupts the GIT regulation, generating alterations in function, like diarrhea [7], and its morphology [34]. Our results corroborate the literature, showing reduction of general and nitricergic neuron number, as well as the glial density, in both segments and T2DM models, except in the glia from ileum of DNC group. Except in the HuC/D+ neurons and glial cells from jejunum of DNC, all other populations analyzed, in both intestinal segments, presented an increase in their cellular profile. Even after two months of standard diet, the diabetic animals from DEC group maintained the adaptations caused by the T2DM. This indicates that only normalizing dietary conditions has no efficacy over this type of damage.

The neuron and glia loss are attributed to the oxidative stress, since these cells are more vulnerable this type of damage [6]. Glial cells are important in the maintenance of neuronal environment, including in diabetes [51] and therefore their alteration leads to neuronal disturbance [52,53]. Stenkamp-Strahm et al. [54] showed that high-fat diet affected only mucosal glial cell populations, while myenteric glia was unaffected. This difference could be related to the type of damage, as well the duration of the study. The increase of neuronal profile, can be related to the neuronal loss as a compensatory adaptation to the lack of cells in order to maintain the minimal control over the tissue.

FR had little or no effect over the small intestine of control animals regarding the neuronal and glial population. In the other hand, FR promoted great benefits over the two diabetic models, preventing alterations in the number and profile of neurons and glia in most of the situations. The jejunum was more susceptible to FR protection, mainly in DER group. The nNOS+ subpopulation of ileum had no benefits from FR. The protective effect of FR over neurons is considered by other studies [55], but to our knowledge this is the first study correlating the effects of T2DM and FR over intestine and enteric neurons.

The alterations observed in the nervous tissue of the present study explains, in part, the other results. The reduced innervation decreases the control over the absorption
and musculature, leading to the observed characteristics like diarrhea and altered morphology of the intestine wall.

5 CONCLUSIONS

Both T2DM models change the morphology of intestine wall and in the ENS, leading to alteration of neuron and glia number and morphometry in the myenteric plexus. FR protects the intestine from diabetic damage, although FR itself generates some adaptations. Different segments of the gut present distinct responses to T2DM and FR. More studies are necessary to verify the full potential and safety of FR over the intestine, since it is a complex and vital organ.

ACKNOWLEDGMENTS

The authors are grateful for the technical support provided by the Departments of Morphological and Physiological Sciences of the State University of Maringá.

CONFLICT OF INTERESTS

The authors have declared that no competing interest exists

FINANCIAL SUPPORT

This work was supported by CAPES (Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior – grant number 40004015001M9) and from PRONEX (Programa de Apoio a Núcleos de Excelência), during the conduct of the study.
REFERENCES


