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Differential effects in CGRPergic, nitrergic, and VIPergic myenteric innervation in diabetic rats supplemented with 2% L-glutamine

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ABSTRACT

The objective of this study was to investigate the effects of 2% L-glutamine supplementation on myenteric innervation in the ileum of diabetic rats, grouped as follows: normoglycemic (N); normoglycemic supplemented with L-glutamine (NG); diabetic (D); and diabetic supplemented with L-glutamine (DG). The ileums were subjected to immunohistochemical techniques to localize neurons immunoreactive to HuC/D protein (HuC/D-IR) and neuronal nitric oxide synthase enzyme (nNOS-IR) and to analyze varicosities immunoreactive to vasoactive intestinal polypeptide (VIP-IR) and calcitonin gene-related peptide (CGRP-IR). L-Glutamine in the DG group (i) prevented the increase in the cell body area of nNOS-IR neurons, (ii) prevented the increase in the area of VIP-IR varicosities, (iii) did not prevent the loss of HuC/D-IR and nNOS-IR neurons per ganglion, and (iv) reduced the size of CGRP-IR varicosities. L-Glutamine in the NG group reduced (i) the number of HuC/D-IR and nNOS-IR neurons per ganglion, (ii) the cell body area of nNOS-IR neurons, and (iii) the size of VIP-IR and CGRP-IR varicosities. 2% L-glutamine supplementation exerted differential neuroprotective effects in experimental diabetes neuropathy that depended on the type of neurotransmitter analyzed. However, the effects of this dose of L-glutamine on normoglycemic animals suggests there are additional actions of this beyond its antioxidant capacity.

Key words: Diabetic neuropathy, Glutamine, Ileum, Myenteric innervation.

INTRODUCTION

The myenteric and submucous plexus, the main components of the enteric nervous system (ENS), contain sensory neurons, interneurons, and motor neurons. Myenteric neurons are predominantly involved in the control of muscle contractions, and neurons from the submucous plexus mainly regulate mucosal secretomotor and vasomotor activities (Costa et al. 1996, Furness 2006).

Extensive experimentation allows correlation between the expression of identifiable neurochemicals and the function of the enteric neurons, a phenomenon known as the chemical code (Hansen 2003). The neurochemicals, often neurotransmitters, expressed varies with the functional class of enteric neuron, species, and gastrointestinal region. Additionally, the chemical code is plastic and changes can occur in response to pathophysiological conditions (Furness 2006, Hansen 2003, Olsson and Holmgren 2010).

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In addition to classic adrenergic and cholinergic neurotransmitters, more than 30 classes of transmitters have been identified in the ENS (Hansen 2003, Olsson and Holmgren 2010). Non-adrenergic non-cholinergic (NANC) neurotransmission is involved in neuromuscular transmission during the peristaltic reflex in the gastrointestinal tract. Principle transmitters are nitric oxide (NO) and vasoactive intestinal polypeptide (VIP), that act as inhibitory NANC neurotransmitters, and substance P (SP), an excitatory neurotransmitter (Fujimiya and Inui 2000, Nezami and Srinivasan 2010). The importance of neurotransmitters such as VIP in the neuroprotection of central and enteric neurons has been reported, in addition to the potent anti-inflammatory action of VIP (Ekblad and Bauer 2004). Calcitonin gene-related peptide (CGRP) is also a neuromodulator in the ENS. It is involved in both secretory and motor functions of the gastrointestinal tract (Chiocchetti et al. 2006, Holzer et al. 1989, Rasmussen et al. 2001).

Diabetes *mellitus* is a disease that alters motor function in the gastrointestinal tract by causing long-term modifications in neuronal function via changes in enteric neurotransmitters that contribute to abnormal motility (Adeghate et al. 2001, Chandrasekharan and Srinivasan 2007, Nezami and Srinivasan 2010). Different subpopulations of enteric neurons that are distinguishable based on their neurotransmitter content are differentially affected by diabetes (Shotton and Lincoln 2006). Diabetic complications are primarily attributable to oxidative stress. Hyperglycemia associated with diabetes is involved in the increased production of reactive oxygen species and concomitant reduction of antioxidant capacity (Vincent et al. 2004).

Substances that participate directly or indirectly in the reduction of oxidative stress in diabetes, such as vitamin C (De Freitas et al. 2008), vitamin E (Pereira et al. 2008, Roldi et al. 2009, Tronchini et al. 2010, 2012), *Ginkgo biloba* extract (da Silva et al. 2011, Schneider et al. 2007), aminoguanidine

(Shotton et al. 2007), α -lipoic acid (Shotton et al. 2004), γ -linolenic acid (Shotton et al. 2004), L-glutamine (Tashima et al. 2007, Alves et al. 2010, Pereira et al. 2011, Zanoni et al. 2011, Tronchini et al. 2013) and L-glutathione (Hermes-Uliana et al. 2014), have been studied to prevent chronic complications. L-glutamine is the precursor for glutamate, which is used in glutathione synthesis (Newsholme et al. 2003). Glutathione is a major endogenous antioxidant that plays an important role in cellular protection against oxidative damage (Newsholme et al. 2003, Vincent et al. 2004).

The present study investigated the effects of 2% L-glutamine supplementation on diabetes-induced changes in the neurons and varicosities of myenteric fibers in the ileum. Immunohistochemical techniques were used to localize HuC/D immunoreactivity in the total population of myenteric neurons (HuC/D-IR), neuronal nitric oxide synthase enzyme immunoreactivity in a subpopulation of nitrergic neurons (nNOS-IR), and the varicosities of nerve fibers immunoreactive to VIP (VIP-IR) and CGRP (CGRP-IR).

MATERIALS AND METHODS

ANIMAL PROCEDURES

All of the experimental procedures described in this work were supervised and approved by the Committee of Ethics in Animal Experimentation of the Universidade Estadual de Maringá. They were conducted in accordance with the ethical principles of the Brazilian Society of Science in Animal Lab (SBCAL).

Twenty male ninety-day-old Wistar rats (*Rattus norvegicus*) were grouped into groups: normoglycemic (N), normoglycemic supplemented with L-glutamine (NG), diabetic (D), and diabetic supplemented with L-glutamine (DG). The rats were kept in individual cages with controlled temperature ($24 \pm 2^\circ\text{C}$) and light (12 h/12 h light/dark cycle), food and water *ad libitum*. Non-

supplemented animals (N and D groups) received balanced standard Nuvital chow (Nuvilab, Colombo, PR, Brazil). L-glutamine (Ajinomoto, Tokyo, Japan) was incorporated into the standard chow at a concentration of 2% (20 g/kg of chow), which was offered to NG and DG groups.

Diabetes *mellitus* was induced at the age of 90 days by an intravenous injection of streptozotocin (35 mg/kg body weight; Sigma, St. Louis, MO, USA) dissolved in citrate buffer, pH 4.5 (10 mM) in the D and DG groups after a 14 h fast. After, glycemia was determined using the glucose oxidase method (Bergmeyer and Bernet 1974) to confirm the establishment of the experimental model. Only animals with glycemia greater than 250 mg/dl were used.

MATERIAL RESECTION AND PROCESSING

At the end of the experimental period (120 days), the animals were weighed and killed under thiopental anesthesia (40 mg/kg body weight, intraperitoneal; Abbott Laboratories, Chicago, IL, USA). Blood was sampled by cardiac puncture for the determination of glycated hemoglobin levels by using the ion-exchange resin method (Koenig et al. 1976). Two hours before sacrifice, the animals were injected with vincristine sulfate (0.5 mg/kg) to stabilize the microtubules of the cytoskeleton. The animals used in this study were the same as those used by Alves et al. (2010) and Pereira et al. (2011).

After laparotomy, the ileum of all of the animals were resected, washed in phosphate-buffered saline (PBS, 0.1 M, pH 7.3), and carefully inflated with Zamboni fixative (Stefanini et al. 1967) to fill the space previously occupied by feces, such that the tissues were not distended. The part of the ileum used was the terminal portion, approximately 6 cm from the cecum. Soon afterward, the ileums were maintained for 18 h in the same solution at 4°C. After fixation, the ileums were cut along the mesenteric

border and successively washed in 80% alcohol until the visible removal of fixative. Dehydration in a series of increasing alcohol concentrations (95 and 100%), clarification in xylol, and rehydration in a series of decreasing alcohol concentrations (100, 90, 80, and 50%) were performed. The tissue were then stored at 4°C in PBS with the addition of 0.04% sodium azide.

Tissues were dissected under stereomicroscopy to obtain whole-mount muscular layer preparations by the removal of the mucosal and submucosal layers. For the study of different subpopulations of varicosities in the myenteric plexus, the whole-mounts were immunohistochemically processed separately for VIP (Costa et al. 1980) and CGRP (Belai et al. 1996). HuC/D (Lin et al. 2002) and nNOS (Wrzos et al. 1997) immunohistochemistry was performed together by double-staining for the study of the total population of myenteric neurons and subpopulation of nitrergic neurons, respectively.

IMMUNOFLOUORESCENCE PROCEDURES

All whole-mounts were initially washed twice in PBS solution that contained 0.5% Triton X-100 (Sigma) for 10 min. They were then incubated for 1 h in blocking solution that contained PBS + 0.5% Triton X-100 + 2% bovine serum albumin (BSA; Sigma) + 10% goat serum.

DOUBLE-STAINING IMMUNOHISTOCHEMISTRY OF HuC/D PROTEIN AND nNOS ENZYME

For the double-immunolocalization of HuC/D protein and nNOS enzyme, the whole-mounts were incubated in a solution that contained both primary antisera: anti-HuC/D (produced in mouse; 1:500; Molecular Probes, Carlsbad, CA, USA) and anti-nNOS (produced in rabbit; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively. Negative control tissues were incubated in the same solutions without antisera. After 48 h of incubation, tissues were washed

twice in PBS solution that contained 0.5% Triton X-100 for 10 min and incubated for 2 h at room temperature with the following secondary antisera: Alexa Fluor 488 donkey anti-mouse IgG (1:500; Molecular Probes) and Alexa Fluor 546 goat anti-rabbit IgG (1:500; Peninsula Labs, Torrance, CA, USA). The whole-mounts were then washed twice in PBS solution, mounted on slides with buffered glycerol-gel (9:1), and stored in a refrigerator.

IMMUNOHISTOCHEMISTRY OF VIP AND CGRP

For the immunolocalization of VIP and CGRP, the whole-mounts were incubated separately with the following primary antisera: anti-VIP (produced in rabbit; 1:200; Bachem Americas, Torrance, CA, USA) and anti-CGRP (produced in rabbit; 1:200; Santa Cruz Biotechnology), respectively. Negative control tissues were incubated in the same solutions without antisera. After 48 h incubation with the respective primary antisera, tissues were washed twice in PBS solution that contained 0.5% Triton X-100 for 10 min and incubated for 2 h at room temperature with fluorescein-conjugated anti-rabbit secondary antiserum (IgG-FITC produced in goat; 1:200; Santa Cruz Biotechnology). The whole-mounts were then washed twice in PBS solution, mounted on slides with buffered glycerol-gel (9:1), and stored in a refrigerator.

QUANTITATIVE ANALYSIS

HuC/D-IR and nNOS-IR myenteric neurons were quantified using images randomly obtained from an intermediate region of the ileum (60°-120°, 240°-300°; intestinal circumference in each animal, with 0° as the mesenteric insertion) (Miranda Neto et al. 2001, Zanoni et al. 2005). The images were captured by an AxioCam high resolution camera (Zeiss, Jena, Germany) coupled to an Axioskop Plus light microscope (Zeiss), digitized on a computer using AxioVision version 4.1, and recorded onto a compact disc. The image-analysis software Image-

Pro Plus version 4.5.0.29 (Media Cybernetics, Silver Spring, MD, USA) was used for the quantification of myenteric neurons in the images. The number of HuC/D-IR and nNOS-IR neurons present per myenteric ganglion was determined. Fifty ganglia per animal were quantified using images captured with a 20X lens. The results enabled us to calculate the ganglionic density, which is expressed as the number of HuC/D-IR and nNOS-IR neurons per ganglion.

MORPHOMETRIC ANALYSIS

The areas of nNOS-IR myenteric neuronal cell bodies were measured using the same images as those used in the quantitative analysis, captured with a 20X lens. The area (in μm^2) of 100 neuronal cell bodies for each animal was measured using Image-Pro Plus, with a total of 500 areas per group.

VIP-IR and CGRP-IR myenteric varicosities were measured using images randomly obtained from an intermediate region of the ileum. Each varicosity, a symmetric expansion of a neurite that accumulates neurotransmitters along the myenteric nerve fiber, was measured using a 200X digital zoom and Image-Pro Plus software. The original calibration of the images was maintained. The area (in μm^2) of 400 varicosities for each animal and immunostain was measured, with a total of 2000 varicosities per group per immunostain. To facilitate the identification of single varicosities, only nerve fibers located within nerve tracks outside of ganglia were analyzed.

STATISTICAL ANALYSIS

The data were analyzed using Statistica 7.1 and GraphPad Prism 5.1 software and are expressed as the mean \pm standard error. Morphometric data and ganglionic densities were set in delineation blocks followed by Tukey's test. For the other data, we used one-way analysis of variance (ANOVA) followed by Tukey's test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Diabetic animals (D and DG groups) showed characteristic clinical signs of diabetes *mellitus* that were observed throughout the experiment. Non-treated diabetic animals were hyperglycemic (D group, 275.5 ± 3.8 mg/dl) compared with the N group (132.3 ± 6.6 mg/dl) ($P < 0.05$). L-glutamine supplementation did not change hyperglycemia in diabetic animals (DG group, 282.9 ± 4.5 mg/dl) ($P > 0.05$ when compared D and DG groups).

Additionally, glycated hemoglobin was significantly higher in diabetic animals (D and DG groups) compared with the normoglycemics (N and NG groups) ($P < 0.05$ respectively) (Fig. 1).

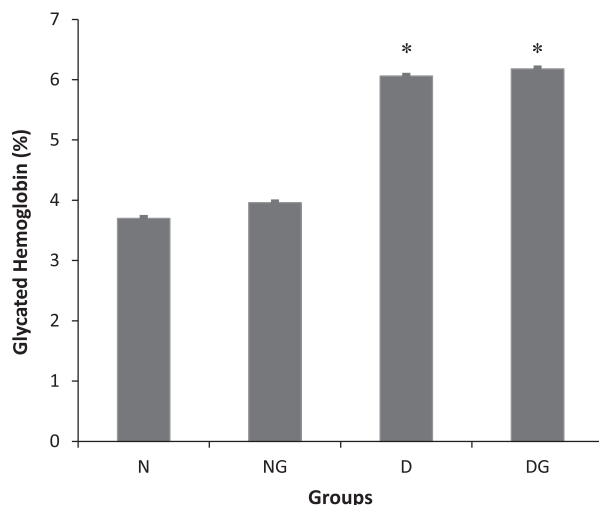


Figure 1 - Glycated hemoglobin (%) in normoglycemics (group N), normoglycemics supplemented with L-glutamine (group NG), diabetics (group D), and diabetics supplemented with L-glutamine (group DG). $n = 5$ rats per group. * $P < 0.05$, compared with groups N and NG.

HuC/D-IR NEURONS

Diabetes reduced the number of HuC/D-IR neurons present per myenteric ganglion by 30.2% in the D group compared with the N group ($P < 0.0001$). In the DG group, L-glutamine supplementation did not prevent the reduction in ganglionic density compared with non-supplemented diabetic animals

(D group, $P > 0.05$). Normoglycemic animals supplemented with L-glutamine (NG group) had 26.86% fewer neurons per ganglion than the N group ($P < 0.0001$). The ganglionic density of HuC/D-IR neurons is shown in Fig. 2. Confocal photomicrographs of HuC/D-IR myenteric neurons are shown in Fig. 3.

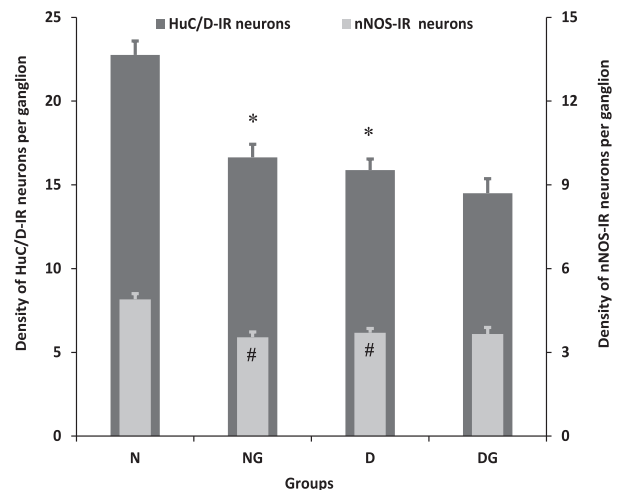


Figure 2 - Density per ganglion of HuC/D-IR and nNOS-IR myenteric neurons in normoglycemics (group N), normoglycemics supplemented with L-glutamine (group NG), diabetics (group D), and diabetics supplemented with L-glutamine (group DG). $n = 5$ rats per group. * $P < 0.0001$, compared with group N; # $P < 0.0001$, compared with group N.

nNOS-IR NEURONS

In myenteric ganglia, nNOS-IR neuron density decreased significantly (24.5%) with diabetes *mellitus* (D group) compared with the N group ($P < 0.0001$). No differences were found between the D and DG groups ($P > 0.05$). Ganglionic density was 27.8% lower in the NG group compared with the N group ($P < 0.0001$). The ganglionic density of nNOS-IR myenteric neurons is shown in Fig. 2.

The cell body area of nNOS-IR neurons increased significantly (54.3%) in the D group compared with the N group ($P < 0.0001$). L-glutamine supplementation in diabetic animals (DG group) prevented the increase in neuronal morphometry by 12.36% compared with the D

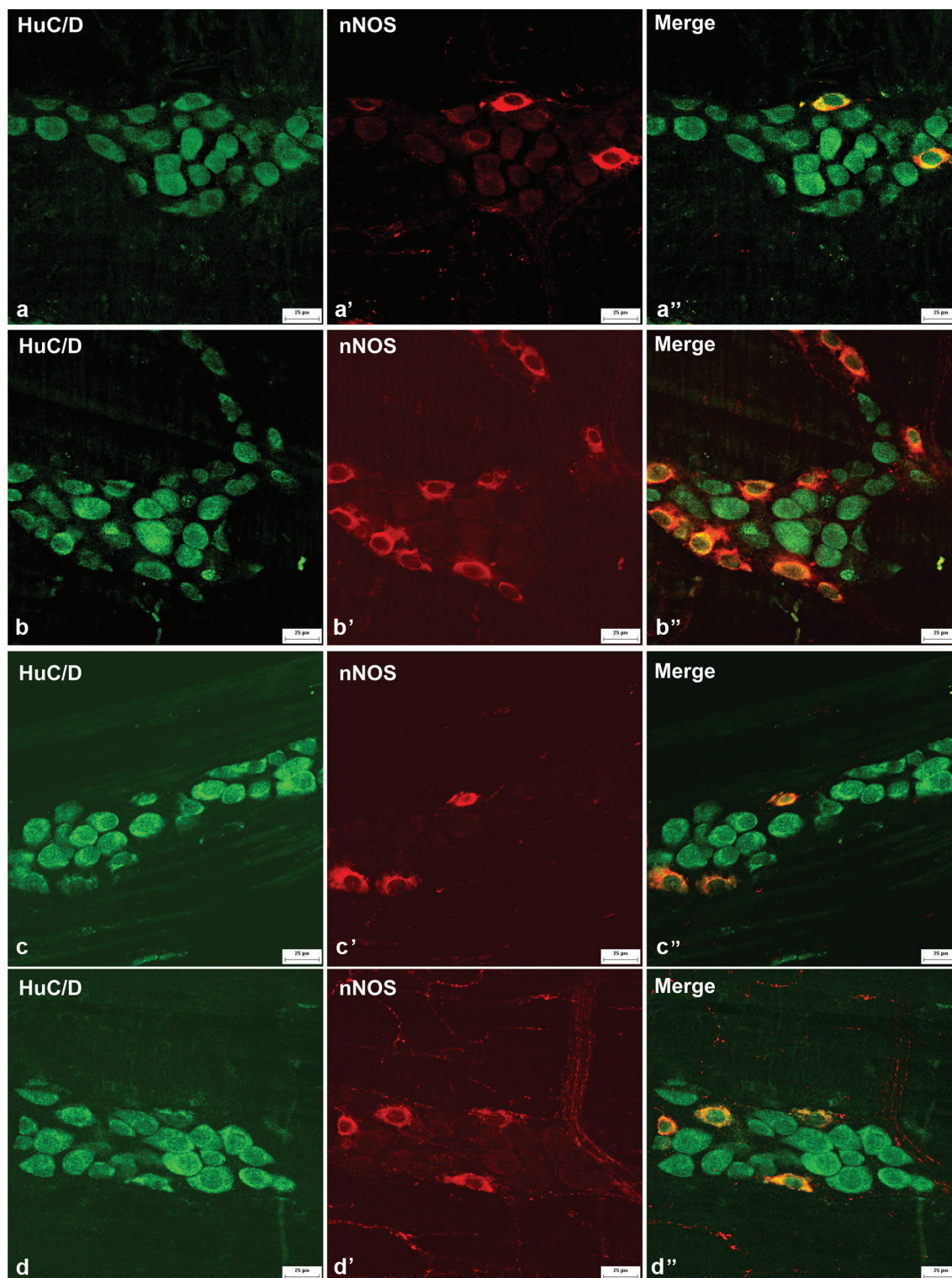


Figure 3 - Confocal photomicrographs of HuC/D-IR (**a**, **b**, **c**, **d**) and nNOS-IR (**a'**, **b'**, **c'**, **d'**) myenteric neurons in normoglycemics (group N) (**a-a''**), normoglycemics supplemented with L-glutamine (group NG) (**b-b''**), diabetics (group D) (**c-c''**), and diabetics supplemented with L-glutamine (group DG) (**d-d''**). Double-labeled HuC/D and nNOS neurons are shown in **a''**, **b''**, **c''**, **d''**. Calibration bar = 25 µm. (See the colors in the online version).

group ($P < 0.0001$). The neuron cell body area was 11.6% lower in the NG group than in the N group ($P < 0.003$). The morphometry of nNOS-IR neurons is presented in Table I. Most nNOS-IR neuron cell bodies showed sizes that ranged from 100 to 200 μm^2 in groups N, NG, and DG. In the D group, most nNOS-IR neuron cell bodies had areas that ranged from 200 to 300 μm^2 . Confocal photomicrographs of nNOS-IR myenteric neurons are shown in Fig. 3.

VIP-IR VARICOSITIES

The average area in μm^2 of VIP-IR myenteric varicosities is shown in Table I. Diabetes is associated with a significantly increased area of VIP-IR varicosities compared with group N ($P < 0.0001$). L-glutamine supplementation in diabetic animals (DG group) prevented this increase compared with the D group ($P < 0.0001$), and no significant differences were found between the DG and N groups ($P > 0.05$). In normoglycemics supplemented

with L-glutamine (NG group), the area of varicosities was lower than in the N group ($P < 0.0001$). Most VIP-IR varicosities in N and D groups had areas that ranged from 2 to 3 μm^2 . In the NG and DG groups, most VIP-IR varicosities showed sizes that ranged from 1 to 2 μm^2 . Confocal photomicrographs of VIP-IR varicosities are shown in Fig. 4.

CGRP-IR VARICOSITIES

The size of CGRP-IR varicosities were not different between group D and group N ($P = 0.07$). Supplementation with L-glutamine reduced the areas of CGRP-IR varicosities in both diabetic animals (group D compared to group DG; $P < 0.0001$) and normoglycemic animals (group N compared to group NG; $P < 0.0003$). The average area in μm^2 of CGRP-IR myenteric varicosities is shown in Table I. Most CGRP-IR varicosities had sizes that ranged from 1 to 2 μm^2 in all studied groups. Confocal photomicrographs of CGRP-IR varicosities are shown in Fig. 4.

TABLE I
Average area in μm^2 of myenteric neuronal cell bodies (nNOS-IR) and myenteric varicosities (VIP-IR and CGRP-IR) in normoglycemics (group N), normoglycemics supplemented with L-glutamine (group NG), diabetics (group D), and diabetics supplemented with L-glutamine (group DG).

Morphometric data	N	NG	D	DG
nNOS-IR neurons	163.1 \pm 2.57 ^a	144.2 \pm 2.25 ^b	251.7 \pm 5.69 ^c	220.6 \pm 4.67 ^d
VIP-IR varicosities	2.16 \pm 0.014 ^a	2.02 \pm 0.018 ^b	2.26 \pm 0.018 ^c	2.13 \pm 0.019 ^a
CGRP-IR varicosities	1.91 \pm 0.021 ^a	1.81 \pm 0.018 ^b	1.85 \pm 0.019 ^{a,b}	1.73 \pm 0.018 ^c

$n = 5$ rats per group.

In the same row, means followed by different superscript letters are significantly different ($P < 0.05$, Tukey's test).

DISCUSSION

The present experimental model of diabetes was confirmed by high levels of glycated hemoglobin and the establishment of diabetic autonomic neuropathy, reflected by a reduction of the total population of myenteric neurons in the ileum. The number of HuC/D-IR neurons in diabetic animals (D group) was lower per unit area (Pereira et al. 2011) and per myenteric ganglia compared with

normoglycemics (N group). A reduction in the number of neurons per unit area can be caused by hypertrophy or distention of the intestine, however, the quantification per ganglion is a parameter that is not influenced by changes in the gut wall area (Voukali et al. 2011). Because both approaches measured a reduction, the most likely explanation is that there is a loss of myenteric neurons diabetes-induced. Neuronal death is related to oxidative

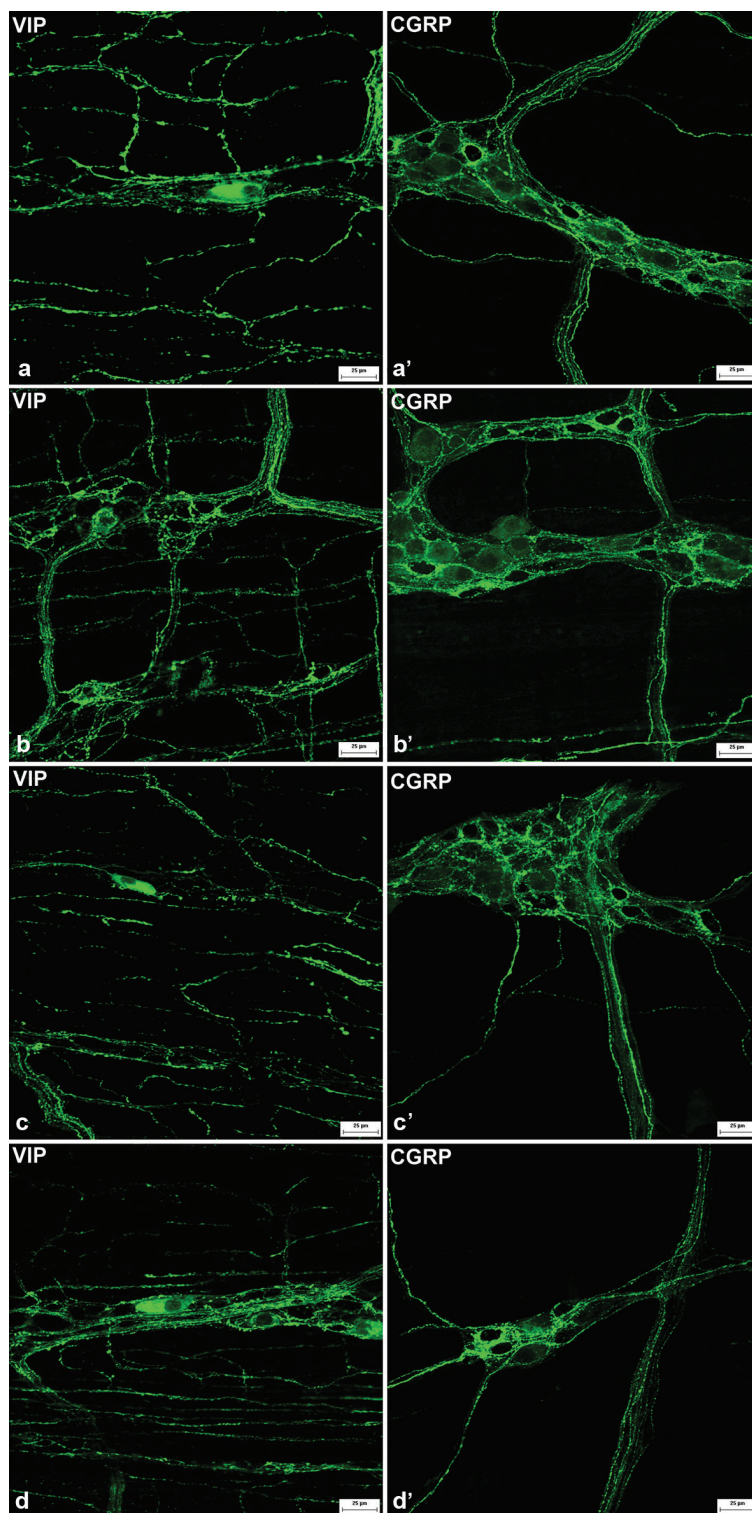


Figure 4 - Confocal photomicrographs of VIP-IR (**a**, **b**, **c**, **d**) and CGRP-IR (**a'**, **b'**, **c'**, **d'**) myenteric varicosities in normoglycemics (group N) (**a**, **a'**), normoglycemics supplemented with L-glutamine (group NG) (**b**, **b'**), diabetics (group D) (**c**, **c'**), and diabetics supplemented with L-glutamine (group DG) (**d**, **d'**). Calibration bar = 25 µm. (See the colors in the online version).

stress, considered the main factor responsible for diabetes-induced damage to the ENS (Kashyap and Farrugia 2011, Vincent et al. 2004). In diabetic patients, hyperglycemia raises oxidative stress, leading to neuron death by apoptosis and consequent changes in gastrointestinal tract motility, including cases of constipation and diarrhea (Chandrasekharan et al. 2011).

Intestinal tissues become even more susceptible to oxidative stress in view of the deficiencies of antioxidants associated with diabetes. In the colon of diabetic patients, the quantity of non-enzymatic antioxidants, such as reduced glutathione, is decreased (Chandrasekharan et al. 2011). L-glutamine is an amino acid precursor of glutathione, one of the most important endogenous antioxidants responsible for the neutralization of reactive oxygen species (Curi et al. 2005, Newsholme et al. 2003, Vincent et al. 2004). Previous studies conducted in our laboratory found promising results of L-glutamine supplementation at a concentration of 1% in diabetic rats, but the effect was not significant (Tashima et al. 2007, Zanoni et al. 2011). Supplementation with 2% in DG group promoted neuroprotection of 18.24% in the total population of myenteric neurons per unit area (Pereira et al. 2011). This same neuroprotection was not observed in the ganglia in the present study, which may indicate the reduced availability of the amino acid to ganglionic neurons.

A reduction in the density of total neuronal population was observed per myenteric ganglion in the NG group compared to the N group. This reduction may be related to increased L-glutamine availability in animals with normal glycemic levels. The opposite occurs in diabetic animals (DG group), in which there appears to be a lower availability of L-glutamine because of its use by other metabolic pathways, including the liver and kidneys (Ardawi 1988). Thus, L-glutamine supplementation in the diet may not have reached the expected concentrations in ganglionic myenteric

neurons in the DG group. On the other hand, in normoglycemic animals supplemented with L-glutamine (NG group), the increased availability of L-glutamine can generate high amounts of glutamate in neurons. Glutamate is essential for the normal function of neurons and is the main neurotransmitter involved in central nervous system excitatory transmission (Dobrek and Thor 2011). Glutamatergic neurons are present in ENS and glutamate is also involved in enteric neurotransmission (Liu et al. 1997, Srinivasan and Wiley 2000). Because it is a precursor of glutathione, it may play a role in the antioxidant defense of cells (Newsholme et al. 2003). However, at high concentrations, glutamate is associated with neurotoxicity and can cause neuronal injury and cell death (Dobrek and Thor 2011, Kirchgessner et al. 1997). This toxicity glutamate-mediated has been described both in the central nervous system and in the ENS (Kirchgessner et al. 1997, Srinivasan and Wiley 2000). In addition, it is possible that the conversion of glutamate to glutathione can be reduced because glutathione concentrations may be normal in normoglycemic animals. Comparing supplementation with 1% and 2% L-glutamine in normoglycemic animals, we infer that the 1% concentration is more effective under aging conditions. The preservation of myenteric neurons has been demonstrated using this concentration in the duodenum (Zanoni et al. 2011) and jejunum (Tashima et al. 2007).

The present results regarding the ganglionic density of HuC/D-IR myenteric neurons in the NG group differed from results previously found for the ileum (Pereira et al. 2011). This demonstrates a differential effect of 2% L-glutamine supplementation within the same segment. Differences between different intestinal regions have been previously demonstrated by Belai et al. (1991), who found that diabetes differentially affected the ileum and distal colon in rats. However, different results within the same segment had not been reported.

Previous studies from our laboratory showed that the number of nNOS-IR neurons present per unit area does not change with diabetes in the stomach (Fregonesi et al. 2005), duodenum (de Mello et al. 2009), jejunum (Tronchini et al. 2012) and ileum (Pereira et al. 2008, Zanoni et al. 2003). In the present study, when nNOS neurons were quantified per ganglion, which was not completed in the previous studies, a significantly 24.5% reduction induced by diabetes was found. nNOS-IR neurons are particularly involved in the oxidative damage characteristic of enteric neuropathies through the excessive production of NO. An increase in NO production is triggered by the excessive increase in intracellular Ca^{2+} levels because the nNOS enzyme is Ca^{2+} -dependent (Rivera et al. 2011). In the present study, we observed an increase in the cell body area of nNOS-IR neurons in the D group, an effect associated with neuronal loss in myenteric ganglia, likely indicating an increase in nNOS activity and NO production. The increased size of nNOS-IR neurons diabetes-induced has been demonstrated in the stomach (Fregonesi et al. 2005), jejunum (Tronchini et al. 2012) and ileum (Pereira et al. 2008, Shotton et al. 2007, Shotton and Lincoln 2006, Zanoni et al. 2003). L-glutamine supplementation in diabetic animals (DG group) did not alter ganglionic density compared with the D group but prevented the increase in the cell body area of nNOS-IR neurons. These results indicate the possible control of NO production, demonstrating a neuroprotective effect of 2% L-glutamine in diabetes. According to Albrecht et al. (2010), L-glutamine modulates the synthesis of NO in the central nervous system. Supplementation in normoglycemic animals (NG group) caused a reduction in the ganglionic density and size of nNOS-IR neuron cell bodies, as in the total neuronal population.

VIP-IR varicosities increased in size with diabetic neuropathy (D group). Similar results were observed in the ileum (Shotton et al. 2007)

and jejunum (Alves et al. 2010) in rats after 8 and 16 weeks of diabetes, respectively. The increase in the area of VIP-IR varicosities may reflect enteric neural plasticity. An increase in VIP expression likely occurred in surviving neurons, in view of the important neuroprotective role that the peptide can play (Alves et al. 2010). In colitis induced by trinitrobenzene sulfonic acid, the increase in the number and proportion of VIP-IR neurons per myenteric ganglia also indicates a protective role of the peptide in the inflamed bowel (Linden et al. 2005). The treatment with VIP of streptozotocin-induced diabetes in mice showed that VIP regulates the expression of cytokines, reduces oxidative stress, and improves total antioxidant capacity in this condition (Yu et al. 2011). However, the increased production of VIP may also be associated with greater intestinal relaxation, in view of its role as an inhibitory neurotransmitter. Supplementation with 2% L-glutamine in diabetic animals (DG group) in the present study effectively prevented the increase in the size of VIP-IR varicosities. In normoglycemic animals supplemented with L-glutamine (NG group), different results were found from the other studies conducted in our laboratory (Alves et al. 2010) because a reduction in the area of varicosities was found compared with the N group. We believe that in view of this decrease in the ileum, contrary to what was observed in the jejunum, 2% L-glutamine supplementation in normoglycemic animals must be carefully analyzed. The increase in the size of varicosities and/or cell bodies of VIP-IR neurons with antioxidant supplementation in aging was considered positive in previous studies (Veit and Zanoni 2012, Zanoni and Freitas 2005), in view of the anti-inflammatory, neuroprotective, and homeostatic restorative effects of increased VIP expression.

The area of CGRP-IR varicosities decreased in diabetic animals (D group). This result, however, was not significant, showing a tendency toward a decrease with diabetes ($P = 0.07$). The reduction

in CGRP-IR myenteric fibers in the ileum was observed 8 weeks (Belai and Burnstock 1990), 12 weeks (Belai et al. 1996, Shotton et al. 2004), and 16 weeks (Belai and Burnstock 1990) after the diabetes induction. CGRP-IR neurons show regulatory activity in different functions of the gastrointestinal tract. When located in the myenteric plexus, CGRP-IR neurons are intrinsic primary afferent neurons and project to intestinal mucosa, mainly controlling intestinal motility (Mitsui 2009). We cannot infer, however, that the reduction in the size of CGRP-IR varicosities in the NG, D, and DG groups is involved in the changes in bowel function because the present methodology cannot determine whether a reduction occurs in the synthesis of this neuropeptide or whether an increase occurs in its release during neurotransmission.

We conclude that 2% L-glutamine supplementation exerted a differential neuroprotective effect in experimental diabetes neuropathy that depend on the type of neurotransmitter analyzed. Newly observed in this study is the effect of L-glutamine to reduce neuronal density, cell areas and areas of VIP and CGRP varicosities of normoglycemic animals. Collectively, these results suggest there are additional actions of this amino acid beyond its antioxidant capacity and support the conclusion that there may exist a narrow therapeutic window for L-glutamine in the treatment of diabetic neuropathy.

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RESUMO

O objetivo deste estudo foi investigar os efeitos da suplementação com L-glutamina 2% na inervação

mioentérica do íleo de ratos diabéticos, agrupados da seguinte forma: normoglicêmico (N); normoglicêmico suplementado com L-glutamina (NG); diabético (D); e diabético suplementado com L-glutamina (DG). Os íleos foram submetidos a técnicas imunohistoquímicas para localizar neurônios imunorreativos à proteína HuC/D (HuC/D-IR) e enzima óxido nítrico sintase neuronal (nNOS-IR) e para analisar varicosidades imunorreativas ao polipeptídeo intestinal vasoativo (VIP-IR) e peptídeo relacionado ao gene da calcitonina (CGRP-IR). A L-Glutamina no grupo DG (i) preveniu o aumento na área do corpo celular de neurônios nNOS-IR, (ii) preveniu o aumento na área das varicosidades VIP-IR, (iii) não preveniu a perda de neurônios HuC/D-IR e nNOS-IR por gânglio, e (iv) reduziu o tamanho das varicosidades CGRP-IR. A L-Glutamina no grupo NG reduziu (i) o número de neurônios HuC/D-IR e nNOS-IR por gânglio, (ii) a área do corpo celular de neurônios nNOS-IR, e (iii) o tamanho das varicosidades VIP-IR e CGRP-IR. Suplementação com L-glutamina 2% exerceu efeitos neuroprotetores diferenciais na neuropatia diabética experimental que dependem do tipo de neurotransmissor analisado. Entretanto, os efeitos desta dose de L-glutamina em animais normoglicêmicos sugerem que existem ações adicionais desta além da sua capacidade antioxidante.

Palavras-chave: Neuropatia diabética, Glutamina, Íleo, Inervação mioentérica.

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