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ORIGINAL ARTICLE

# Diabetic neuropathy: An evaluation of the use of quercetin in the cecum of rats

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## Abstract

**AIM:** To investigate the effect of quercetin supplementation on the myenteric neurons and glia in the cecum of diabetic rats.

**METHODS:** Total preparations of the muscular tunic were prepared from the ceca of twenty-four rats divided into the following groups: control (C), control supplemented with quercetin (200 mg/kg quercetin body weight) (CQ), diabetic (D) and diabetic supplemented with quercetin (DQ). Immunohistochemical double staining technique was performed with HuC/D (general population)/nitric oxide synthase (nNOS), HuC-D/S-100 and VIP. Density analysis of the general neuronal population HuC/D-IR, the nNOS-IR (nitrergic subpopulation) and the enteric glial cells (S-100) was performed, and the morphometry and the reduction in varicosity population (VIP-IR) in these populations were analyzed.

**RESULTS:** Diabetes promoted a significant reduction (25%) in the neuronal density of the HuC/D-IR (general population) and the nNOS-IR (nitrergic subpopulation) compared with the C group. Diabetes also significantly increased the areas of neurons, glial cells and VIP-IR varicosities. Supplementation with quercetin in the DQ group prevented neuronal loss in the general population and increased its area ($P < 0.001$) and the area of nitrergic subpopulation ($P < 0.001$), when compared to C group. Quercetin induced a VIP-IR and glial cells areas ($P < 0.001$) in DQ group when compared to C, CQ and D groups.

**CONCLUSION:** In diabetes, quercetin exhibited a neuroprotective effect by maintaining the density of the general neuronal population but did not affect the density of the nNOS subpopulation.



**Key words:** Diabetes; Myenteric plexus; Neuroprotection; Neuronal nitric oxide synthase; Vasoactive intestinal polypeptide; Enteric glia

**Core tip:** The present study is the first to report a neuroprotective effect of the flavonoid quercetin in the general population of enteric neurons in the cecum of rats with experimental diabetes mellitus. Quercetin did not reduce the loss of nitrergic neurons in the diabetic rats. This observation suggests that selective changes in the neurochemical code of enteric neurons occur in the presence of quercetin. We propose a causal link between the area and number of glial cells and the size of VIP-IR (reduction in varicosity population) varicosities. Although this link is not fully understood, these observations provide a basis for further studies to clarify the link between glia and VIP-IR varicosities.

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## INTRODUCTION

Diabetes affects the gastrointestinal tract causing changes in its motility, such as diarrhea, constipation and gastroparesis[1]. These symptoms are related to damage to the Enteric Nervous System (ENS) caused by diabetic neuropathy. Diabetes affects subpopulations of enteric neurons differently[2,3], causing changes in neuronal size and density[2-6] and altering neurochemical code and neurotransmitters release[7]. The etiology of diabetic neuropathy is complex; hyperglycemia, which through various metabolic pathways including mitochondrial dysfunction and osmotic stress, can induce toxicity in neurons[8]; and oxidative stress, which results in a decrease in antioxidant capacity[9] through glucose oxidation, protein glycosylation and a decrease in the formation of reduced glutathione[10], are among the causative factors. Reductions in the levels of non-enzymatic antioxidants such as ascorbic acid[11] and vitamin E enhance oxidative stress[12].

Antioxidants are promising therapies for preventing and alleviating the debilitating clinical symptoms of diabetic neuropathy. Flavonoids, a family of polyphenolic compounds with a high antioxidant capacity[13], might effectively protect against the pathology of diabetes. Quercetin is a flavonoid that is naturally present in various foods, such as onions, apples, broccoli, tea and red wine[14,15]. Quercetin has shown several beneficial pharmacological properties, such as antiperoxidative, anticarcinogenic, anti-inflammatory and antioxidant activities[16]. Ganglion neurons and their bundles of nerve fibers in the ENS are surrounded by numerous glial cells[17]. These glial cells play an important role in gastrointestinal physiology and pathophysiology, contributing to intestinal homeostasis, serving as a link between the nervous and immune systems[18] and influencing neurochemical phenotype[19]. The number of glial cells and neurons in the ENS are reduced by diabetes[2].

The use of a potent antioxidant, such as quercetin, could mitigate this damage and alleviate the clinical symptoms of the disease. Because diabetes affects enteric neurons subpopulations differently[2,3], the aim of this study was to investigate the potential for quercetin to mitigate of neuropathy. This aim was achieved by comparing the areas of the varicosities of vasoactive intestinal polypeptide (VIP)-containing neurons and the distributions of neuronal nitric oxide synthase (nNOS) and HuC/D (general population) containing neurons and glial cells in the ceca of diabetic and control rats.

## MATERIALS AND METHODS

### Animals

All the procedures involving animals were conducted in accordance with the ethical principles adopted by the Brazilian College of Animal Experimentation (COBEA) and were reviewed and approved by the Ethics Committee on Animal Experiments (CEEA) at the Universidade Estadual de Maringá (State University of Maringá). For the present study, twenty-four adult male Wistar rats (Central Animal Facility at the State University of Maringá) were used. At 88 d of age, weighing 360 g on average, the animals were transferred to the vivarium Sector of the Morphological Sciences Department, where they were housed in individual cages maintained under controlled environmental temperature conditions of (22 ± 2 ℃) and light/dark cycle (12/12 h) with *ad libitum* access to a water fountain and food (Nuvilab®).

After a 2-d period of adaptation to the new environment, rats were weighed and the 120-d trial period monitoring began. At this time, rats were divided randomly into four groups, each containing 6 animals, that received the following treatments: control (C), control supplemented with quercetin (200 mg/kg quercetin body weight) (CQ), diabetic (D) and diabetic supplemented with quercetin (DQ).

To induce diabetes, rats from the D and DQ groups were fasted for fourteen hours and then received an intravenous injection (penile vein) of streptozotocin (STZ) (Sigma, St. Louis, MO) at a dose of 35 mg/kg body weight, dissolved in citrate buffer 10 mmol/L (pH 4.5). Four days after the induction, blood glucose was measured (Accu-Chek Active, Roche Diagnostics GmbH, Mannheim, BW, Germany) to confirm the establishment of experimental diabetes. All the animals in the D and DQ groups had glucose levels above 210 mg/dL.

Starting on the fourth day of the experiment, animals in the CQ and DQ groups were weighed weekly and their water intake was measured. These measurements were used to calculate the dilutions required to ensure each animal in the CQ and DQ groups received 200 mg/kg per day of quercetin in their drinking water. Animals in C and D groups received water without supplementation. After 120 d (210-d-old), the rats were euthanized following anesthesia with Thiopental® (40 mg/kg *ip*; Abbott Laboratories, Chicago, IL). Blood was collected by cardiac puncture and blood glucose concentration measured using the glucose oxidase method[20].

### Cecum collection and processing

The ceca were removed, washed in phosphate buffered saline (PBS; 0.1 mol/L pH 7.4) and filled with and immersed in Zamboni fixative solution[21] for 18 h at 4 ℃. Following fixation, ceca were opened along their mesenteric borders and washed with 80% alcohol until the excess fixative was removed. Then, dehydration was performed in 95% and 100% EtOH, followed by diaphanization in xylene and sequential rehydration in 100%, 90%, 80% and 50% EtOH and finally PBS. Individual

**Table 1 Primary and secondary antibodies used for immunohistochemistry**

| Primary | Host | Dilution dose | Company | Secondary | Dilution dose | Company |
|---|---|---|---|---|---|---|
| HuC/D | Mouse | 1:500 | Molecular Probes, Invitrogen | Anti-mouse Alexa Fluor 488 | 1:500 | Molecular probes, Invitrogen |
| nNOS | Rabbit | 1:500 | Zymed | Anti-rabbit Alexa Fluor 546 | 1:500 | Molecular probes, Invitrogen |
| S-100 | Rabbit | 1:500 | Molecular Probes, Invitrogen | Anti-rabbit Alexa Fluor 546 | 1:500 | Molecular probes, Invitrogen |
| VIP | Rabbit | 1:500 | Península Laboratories, Inc. | Anti-rabbit Alexa Fluor 546 | 1:500 | Molecular probes, Invitrogen |

HuC/D: General population; nNOS: Neuronal nitric oxide synthase; VIP: Vasoactive intestinal polypeptide.

**Table 2 Circumference area of the cecum and the correction factor used to calculate the neuronal density (mean ± SEM)**

| Groups | *n* | Area ($cm^2$) | Correction factor |
|---|---|---|---|
| C | 6 | 8.1 ± 1.0 | Not applicable |
| CQ | 6 | 7.5 ± 0.5 | 0.9 |
| D | 6 | 13.1 ± 0.7[b] | 1.6 |
| DQ | 6 | 10. 9 ± 1.2 | 1.3 |

[b]$P < 0.01$ *vs* C group. C: Control; CQ: Control supplemented; D: Diabetic; DQ: Diabetic supplemented with quercetin.

ceca were cut into small segments (approximately 2 $cm^2$) that were subsequently microdissected under a stereomicroscope to remove the mucosa and submucosa and reveal the tunica muscularis.

### Immunohistochemistry

Three tissues sections per animal underwent immunohistochemical staining. One section was double-labeled to reveal immunoreactivity for HuC/D (general population) and nNOS. A second section was stained for HuC/D and S-100 (a glial protein), and a third section was stained to reveal immunoreactivity for vasoactive intestinal peptide (VIP). Tissues were washed twice in PBS containing 0.5% Triton X-100 for 10 min followed by one hour incubation in a blocking solution of PBS containing 2% BSA and 10% goat serum at room temperature under constant agitation. Tissues were then incubated for 48 h under agitation at room temperature in solutions of PBS containing primary antisera at the dilutions indicated in Table 1, 2% BSA, 0.5% Triton X-100 and 2% goat serum. Tissues were washed in PBS containing 0.5% Triton X-100 and incubated for 2 h at room temperature in solutions of PBS containing the appropriate secondary antisera at the dilutions indicated in Table 1, 2% BSA, 0.5% Triton X-100 and 2% goat serum. Tissues were then washed in PBS containing 0.5% Triton X-100 three times for 10 min and mounted on slides with 10% PBS in glycerol.

### Quantitative analysis of immunoreactive myenteric neurons

Analysis was performed by sampling the antimesenteric basal region[22]. High-resolution micrographs of stained tissue were captured using an AxioCam MRC camera (Carl Zeiss, Jena, Germany) coupled to an Axioshop Plus fluorescence microscope (Carl Zeiss, Jena, Germany) with Axio Vision software (v. 4.6). Images were subsequently analyzed using Image-Pro Plus (v. 4.5.029; Media Cybernetics, Silver Spring, MD) to quantify the neurons and glia. For each animal, all the neurons and glial cells present in 30 images captured at × 20 magnification were manually identified and counted. The area of each image was approximately 0.2041 $mm^2$ and the total quantified area was 6.123 $mm^2$. The results were expressed per square centimetre.

### Neuronal density correction

According to Cowen *et al*[23], pathological processes can change organ size, which can scatter the neurons. Therefore, the results of the neuronal and glial quantification were corrected for the changes in cecum size caused by diabetes (Table 2). For this correction, the cecum of each animal was outlined on cardboard and the images were transferred to the Image-Pro Plus software to measure the perimeter of each animal's cecum. The average area in $cm^2$ of the cecum in each group was used to calculate the correction factor and the factor was then applied to the quantitative results for each animal in the CQ, DQ and D groups (Tables 2 and 3).

### Morphometric analysis of immunoreactive myenteric neurons

Images of ganglia were captured using a 20 × objective for HuC/D-, nNOS- and S-100-immunoreactivity and a 40 × objective for VIP-immunoreactivity. Morphometric analyses were performed using the image analysis software Image Pro-Plus. The areas of 100 neuronal cell bodies (HuC/D-IR, nNOS-IR) and glial cells (S-100-IR) were measured per animal. For VIP, the areas of 400 varicosities per animal were measured. Varicosity measurements were performed using a digital zoom of × 800, maintaining the original calibration of the captured image.

### Statistical analysis

Statistical analysis of the quantitative data was performed

**Table 3 Neuronal and glial density in the myenteric plexus of the cecum (mean ± SEM)**

| Groups | HuC/D | nNOS | S-100 | Ratio HuC-D/ nNOS | Ratio HuC-D/ S-100 |
|---|---|---|---|---|---|
| C | 5492 ± 81 | 2028 ± 102 | 6343 ± 367 | 2.7 ± 0.09 | 1.2 ± 0.05 |
| CQ | 5104 ± 132 | 1826 ± 124 | 8615 ± 318[b] | 2.9 ± 0.20 | 1.7 ± 0.07[a,e] |
| D | 4121 ± 325[b] | 1511 ± 162[a] | 5708 ± 322 | 2.8 ± 0.18 | 1.4 ± 0.16 |
| DQ | 5060 ± 25[c] | 1500 ± 59[a] | 5998 ± 269 | 3.4 ± 0.16[a] | 1.2 ± 0.04[c] |

[a]$P < 0.05$, [b]$P < 0.01$ *vs* C group; [c]$P < 0.05$ *vs* D group; [e]$P < 0.05$ *vs* DQ group. C: Control; CQ: Control supplemented; D: Diabetic; DQ: Diabetic supplemented with quercetin.

using Statistica 7.1 and GraphPad Prism 3.1, with data expressed as the means ± SEM. Morphometric data were set in delineation blocks and analyzed by Tukey's test. For the other results, we performed one-way analysis of variance (ANOVA) followed by Tukey's test. The level of significance was 5%.

## RESULTS

Diabetes was induced by STZ administration in the D and DQ groups, as demonstrated by the assessment of blood glucose (Table 4). Hyperglycemia was accompanied by a significant reduction in the body weights in the D and DQ groups when compared with the control (C) group. Other symptoms typical of diabetes, such as polydipsia (increased water intake) and polyuria (increased urine output), were also observed (Table 4). In addition, the D group demonstrated a significant dilatation (61%) of the cecum compared with the C group ($P < 0.01$). Treatment with quercetin helped to reduce the diabetes-associated dilation of the cecum in DQ group to 34% of the controls (Table 2), which was not significantly different from the C group ($P > 0.05$). However, quercetin treatment did not affect the hyperglycemia, polydipsia or polyurea induced by STZ (Table 4).

### *Morphology of HuC/D, nNOS, S-100 and VIP immunoreactivity in the cecum myenteric plexus*

HuC/D-IR and nNOS-IR neuronal cell bodies were observed within the ganglia and along the interganglionary nerve tracks. The intensity of the immunofluorescence in neurons was heterogeneous within the four groups studied (Figure 1). We observed that the nitrergic population was usually located peripherally in the ganglion. Glial cells were present in all ganglia and along the nerve tracks. However, more glial cells were found in the nerve tracks of the CQ group than the other groups (Figure 2E and F). The VIP-IR varicosities were distributed throughout the tunica muscularis. However, in the D and DQ groups a reduction in the number of nerve fibers was observed (Figure 3C and D).

### *Neuronal and glial density*

After correction for cecum dilatation (Table 2), we observed a reduction in the general population (HuC/D) of myenteric neurons in the ceca of diabetic rats compared with the C group ($P < 0.001$). The CQ and DQ groups were not significantly different from the C group ($P > 0.05$) (Table 3). There was a significant reduction in nNOS-IR neurons in D and DQ groups compared with the C group ($P < 0.05$). Quercetin supplementation did not alter the cell densities between the D and DQ groups in this subpopulation ($P > 0.05$). Double labeling showed that the ratio of HuC-D/nNOS was similar in the C, D and CQ groups but was significantly reduced in the DQ group (Table 3). Quantitative analysis of glia (S-100-IR) showed a significant increase in the CQ group compared with the C group ($P < 0.001$). The D and DQ groups were not different from the C group ($P > 0.05$). The ratio of glial cells to neurons (HuC-D/S-100) were similar in groups C, D and DQ, but were significantly increased in the CQ group when compared with the C and DQ groups ($P < 0.05$) (Table 3).

**Table 4 Animal parameters (mean ± SEM)**

| Groups | Starting weight (g) | Final weight (g) | Blood glucose (mg/dL) | Water intake (mL/d) | Feed intake (g/d) | Urine volume (mL/d) |
|---|---|---|---|---|---|---|
| C | 341 ± 9 | 517 ± 13 | 138 ± 5 | 42 ± 3 | 37 ± 3 | 11 ± 2 |
| CQ | 366 ± 14 | 510 ± 10 | 146 ± 8 | 45 ± 3 | 34 ± 5 | 12 ± 2 |
| D | 333 ± 4 | 301 ± 21[a] | 518 ± 19[a] | 113 ± 16[a] | 44 ± 6 | 66 ± 10[a] |
| DQ | 368 ± 6 | 292 ± 8[a] | 532 ± 35[a] | 103 ± 22[a] | 30 ± 7 | 53 ± 13[a] |

[a]$P < 0.05$ *vs* C group. C: Control; CQ: Control supplemented; D: Diabetic; DQ: Diabetic supplemented with quercetin.

### *Morphometric analysis*

**Neuronal population HuC/D-IR and nNOS-IR and glial cells:** The average HuC/D-IR and nNOS-IR neuronal areas in the C and CQ groups were not significantly different ($P > 0.05$). However, there was an increase in the neuronal area in the D group when compared with the C group ($P < 0.05$) and an even larger increase in the average area of the DQ group when compared with the D group ($P < 0.05$). The mean areas of the HuC/D-IR and nNOS-IR neuron cell bodies are shown in Table 5. We observed a significant increase in the average area of the glia in the D group compared with the C, CQ and DQ groups, and a significant decrease in the DQ group compared with the C, CQ and D groups (Table 5).

**VIP-IR varicosities:** Fluorescence intensity was lower in VIP-IR varicosities in the DQ group compared with the other groups (Figure 3). We found significant differences in the diameter of the varicosities among the four groups studied; the CQ group had an increase in the average area of varicosities compared with the C group ($P < 0.01$), a larger increase was observed in the D group compared with the C and CQ groups ($P < 0.01$). In contrast, a decrease in the area of varicosities was found in the DQ group compared with all the other groups ($P < 0.001$) (Table 5).

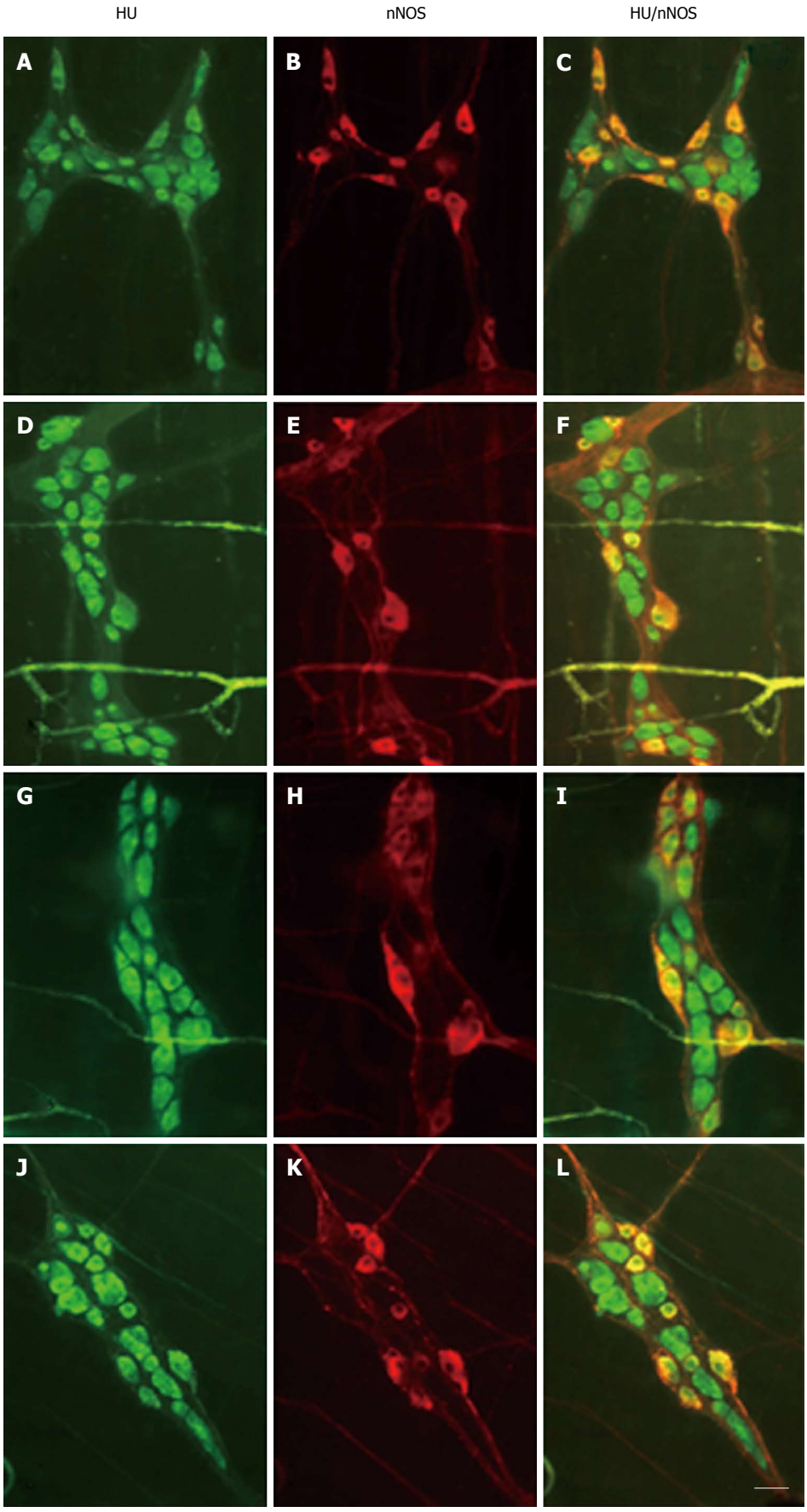


**Figure 1 Representative micrographs showing immunoreactivity to general (green) and nitrergic (red) in the myenteric plexus of the rat cecum:** A-C: Control group; D-F: Quercetin supplemented control group; G-I: Diabetic group; J-L: Quercetin supplemented diabetic group. Scale bar = 50 μm. nNOS: Neuronal nitric oxide synthase.

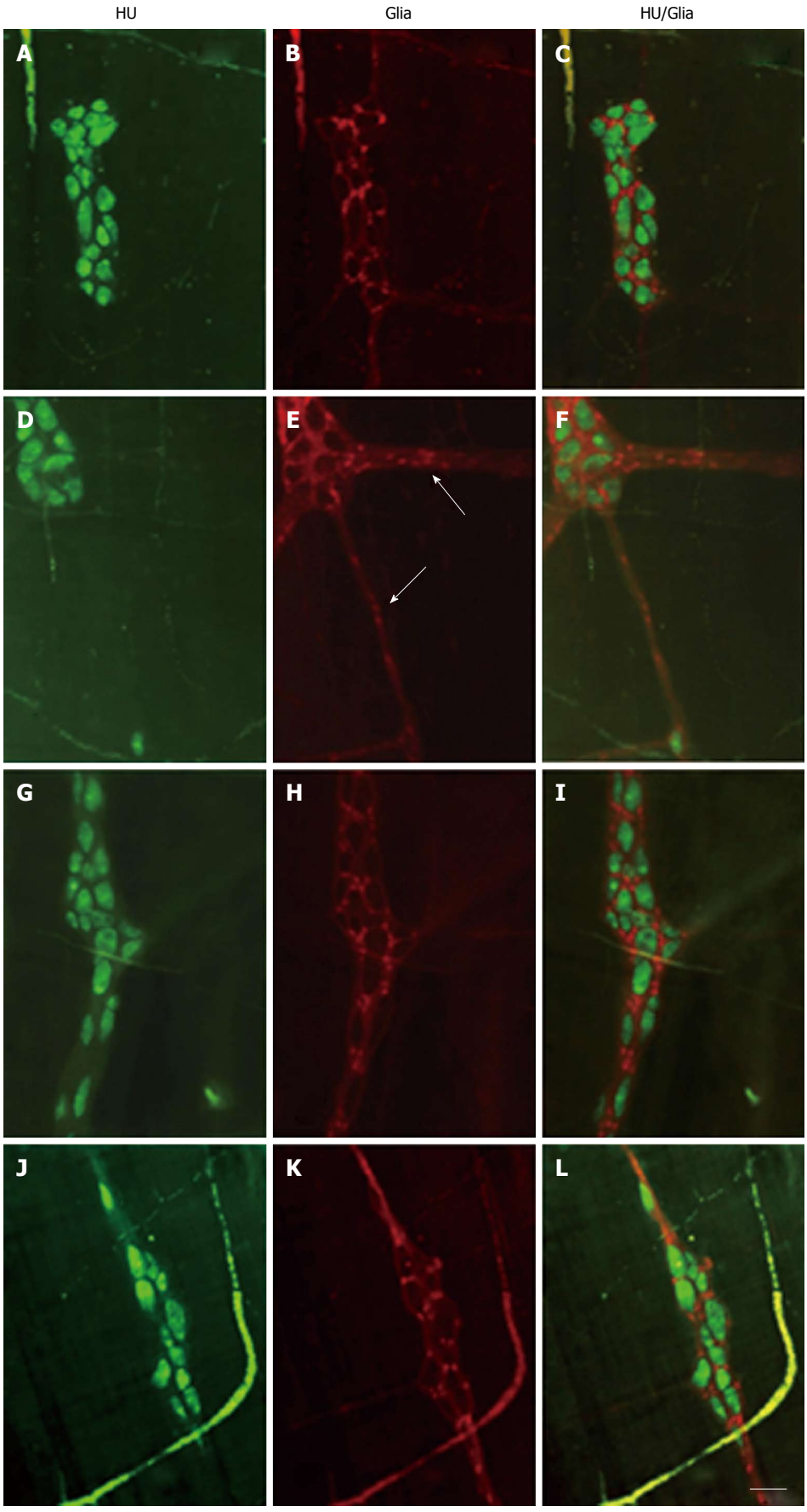


**Figure 2 Representative micrographs showing immunoreactivity to HuC/D (green) and S-100 (red) in the myenteric plexus of the rat cecum.** A-C: Control group; D-F: Quercetin supplemented control group; G-I, Diabetic group; J-L: Quercetin supplemented diabetic group. White arrows indicate glial cells present in nerve fibers. Scale bar = 50 μm.

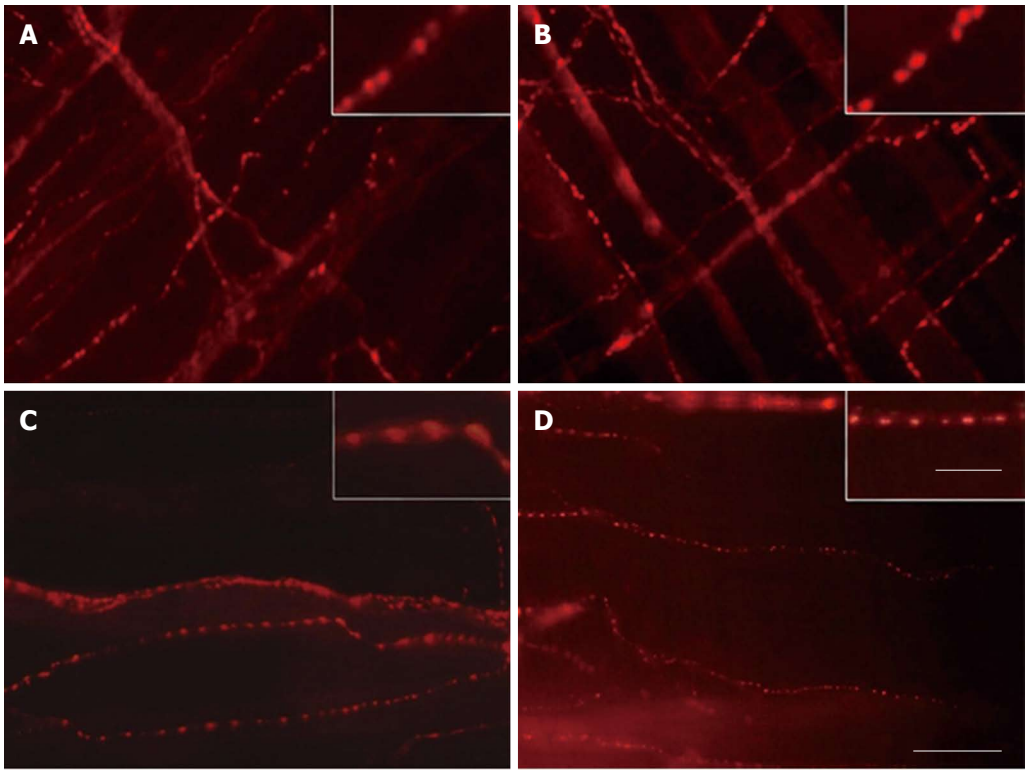


**Figure 3 Representative micrographs showing immunoreactivity to vasoactive intestinal peptide in the myenteric plexus of the rat cecum.** A: Control group; B: Quercetin supplemented control group; C: Diabetic group; D: Quercetin supplemented diabetic group. Magnified inserts show varicosities of the individual nerve fibers. Note the enlarged appearance of the varicosities in the diabetic group (C) and the reduced varicosities in the quercetin diabetic group (D) compared with the controls (A and B). Scale bars = 50 μm for main panels, 15 μm for inserts.

**Table 5 Mean cell body area of the neurons, glia and vasoactive intestinal polypeptide-IR varicosities (mean ± SEM)**

| Groups | HuC/D | nNOS | S-100 | VIP |
|---|---|---|---|---|
| C | 505 ± 11 | 455 ± 10 | 35.1 ± 0.5 | 4.63 ± 0.03 |
| CQ | 501 ± 12 | 430 ± 11 | 34.8 ± 0.4 | 4.88 ± 0.03[b] |
| D | 561 ± 13[a] | 539 ± 12[a] | 36.9 ± 0.5[a] | 5.12 ± 0.04[b] |
| DQ | 644 ± 16[b,c] | 606 ± 15[b,c] | 29.6 ± 0.4[b] | 3.97 ± 0.03[b] |

[a]$P < 0.05$, [b]$P < 0.01$ *vs* C group; [c]$P < 0.05$ *vs* D group. C: Control; CQ: Control supplemented; D: Diabetic; DQ: Diabetic supplemented with quercetin; HuC/D: General population; nNOS: Neuronal nitric oxide synthase; VIP: Vasoactive intestinal polypeptide.

## DISCUSSION

STZ administration to animals in the D and DQ groups promoted typical characteristics of diabetes mellitus, including hyperglycemia, polyuria, polydipsia and weight loss. Quercetin treatment did not affect these measurements in control or diabetic rats suggesting that supplementation with this antioxidant did not influence metabolic pathways linked to weight gain or the mobilization of energy substrates in these animals.

Rats in the D group exhibited 61% cecum dilation compared with animals in the C group, so we used a correction factor to quantify neuronal density. The correction factor was required to ensure the results presented reflected real changes in the neuronal/glial population density rather than the dispersion of these populations as a consequence of the dilation. Previous studies have shown that diabetes promotes dilation of the small intestine[6,24] and the large intestine[4,25,26].

A reduction (25%) in the density of myenteric neurons that were immunoreactive to HuC/D in the D group compared with the C group was observed. Earlier studies in our laboratory showed neuronal loss in different gastrointestinal segments, including the stomach[27], duodenum[28], ileum[6], proximal colon[29] and cecum[4,26]. These alterations may be attributed to the reduction in antioxidant defenses and the concomitant intensification of oxidative stress in the cells[10]. Free radicals can react with DNA, proteins and lipids and these reactions could cause nerve damage[30], which results in the gastrointestinal motility disorders that are typical of diabetes[31].

In the DQ group, quercetin promoted a preservation of neuronal density (HuC/D-IR) of 18% compared with the D group. The density in the DQ group was similar that observed in the C group. This preservation may be attributed to the antioxidant potential of quercetin[16], which would minimize oxidative stress, preventing cell death by necrosis or apoptosis[32]. The ability of quercetin to reduce superoxide anions ($O_2^{\bullet}$), singlet oxygen and hydroxyl radicals ($HO^{\bullet}$), could also prevent lipid peroxidation caused by these molecules[33]. Finally, quercetin may induce the gene expression of antioxidant enzymes, increasing glutathione levels (GSH) and conferring neuroprotection[34]. In the present study, we

found a significant reduction in the density of nitrergic neurons in the cecum of the rats from the D group (25%) and the DQ group (26%) compared with the C group. These decreases in nitrergic density may be attributed to the duration of the diabetes (17 wk). Previous studies have shown an accumulation of advanced glycation end-products, which result in oxidative stress and in neuronal apoptosis, begins in the twelfth week of diabetes[35,36]. Reductions in the number of neurons and/or nNOS activity were also observed in the stomach of diabetic rats[37,38]. However, depending on the model, the duration of diabetes and the techniques used to assess these changes, we can find an increase[39], a decrease[35,37], or no change[6] in the number of nitrergic neurons and/or nNOS levels and activity in different regions of the gastrointestinal tract. According to Shotton and colleagues[3], these inconsistencies may be explained by regional or neuronal subpopulation differences and/or by the existence of multiple stages in the development of neuropathy. In the current study, quercetin treatment did not prevent the reduction of nitrergic neurons compared with D group as it did for the general neuronal population (HuC/D-IR). These findings are of great interest, as quercetin seems not only to prevent neuronal loss but also to direct the chemical coding of the neurons it protects.

In the CQ group, we observed an increase in the VIP-IR varicosity areas compared with the C group ($P < 0.01$), similar data were obtained by Alves *et al*[40] who studied the effect of supplementation with L-glutamine in the jejunum of normoglycemic and diabetic rats. VIP is an inhibitory neuropeptide that has an important role in regulating glial cell proliferation, modulating cell plasticity, stimulating the release of neuroprotective factors and secreting gliotransmitters/gliopeptides that are involved in intercellular communication[41]. In the present study, there was a significant increase (36%) in the number of glial cells in the CQ group compared with the C group ($P < 0.001$). In the CNS, VIP promotes astrocytic proliferation[41,42]; a quercetin-induced increase in VIP may cause an increase in enteric glia in the same way. Furthermore, VIP is capable of stimulating the production of neurotrophic factors by glia in the CNS[43]. An interesting finding in the present study was that there was a greater increase in the number of glia within the fiber tracts rather than within the ganglia in the CQ group. Fiber tracts also contain numerous VIP-IR varicosities.

However, the observation that diabetic rats (group D) demonstrated an increase in the area of VIP-IR varicosities compared with the C group ($P < 0.01$), without a concomitant increase in the density of glial cells argues against the hypothesis of a direct causal relationship between VIP and glia. These seemingly conflicting observations may be explained by other factors that might be present in the diabetic state. There are published reports of increased expression and release of interleukin (IL)-1 beta in STZ-induced diabetic rats[44] and in human monocytes treated *in vitro* with different concentrations of glucose[45]. Studies by Rühl and colleagues[46] demonstrated a combined response of IL-1 beta and IL-10 that lead to a reduction in glial cell proliferation. Thus, despite an increase in IL-10 resulting from increased VIP expression the diabetic state could also be promoting an increase of IL-1 beta and together these two cytokines would inhibit glial cell proliferation in the D group. Regardless of the relationship between VIP and glia, which requires further study, the increase in the size of the VIP-IR varicosities in the diabetic rats may be explained by an increased expression of VIP as a compensatory effect due to neuronal loss[47], or it may be a reflection of neuronal plasticity to maintain the survival of the neurons in response to the pathophysiological conditions of diabetic neuropathy[48]. This conclusion could be supported by studies demonstrating an important role for VIP in neuroprotection[49,50], perhaps by scavenging reactive oxygen species as demonstrated *in vitro*[49] and *in vivo*[51].

Morphometric analysis of the general neuronal population (HuC/D-IR) and the nitrergic (nNOS-IR) subpopulation showed a significant increase in the neuronal area in the D group compared with the C group. An increase in neuronal area is a frequent finding in diabetic animals[4-6]. Hypertrophy was also observed in subpopulations of enteric neurons in the diabetic rats, including the VIP-IR neurons[40,47], nNOS-IR[3] and NADH diaphorase-positive neurons[52]. In the present study, we observed a significant increase in the neuronal area of the general (HuC/D) and nitrergic (nNOS-IR) populations in the DQ group when compared with the other studied groups (Table 5). This event could be explained a the reduction in glial function and metabolism, which was suggested by the decrease in glial cell area and which could reduce the production of neurotrophic factors leading to a loss of control over the processes of synthesis, potentially changing the neurochemical phenotype of the neurons. Evidence that enteric glial cells can produce neurotrophic factors, such as nerve growth factor, brain derived neurotrophic factor and neurotrophin 3, that modulate neuronal gene expression and possibly the enteric neurophenotype has been observed[53]. Additionally, neurotrophic factors play a critical role in regulating the synthesis of neurotransmitters and neuropeptides and in influencing neuronal morphology[54].

In summary, we concluded that in diabetic rats, quercetin exhibited a neuroprotective effect due to its antioxidant action. This action is independent of diabetes-induced changes in hyperglycemia, polydipsia, polyurea and weight loss. Interestingly, while quercetin was able to reduce the loss of myenteric neurons, it did not reduce the loss of nitrergic neurons suggesting a selective change in the neurochemical coding of the enteric neurons during quercetin treatment. Quercetin treatment increased the area of VIP-IR varicosities and concurrently increased the density of enteric glia in control animals. In diabetic rats, there is a disconnection between these observations: quercetin does not increase glial density, but does decrease VIP-IR varicosity area. While there are

data in the literature to support a causal link between VIP and glia and to suggest a connection to neuronal loss and changes in chemical coding, the molecular mechanisms and the relationships between these observations remain to be elucidated.

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## COMMENTS

### Background

Hyperglycemia from diabetes mellitus (DM) may cause long-term neuropathic abnormalities that affect the autonomic nervous system. In the gastrointestinal tract, neurodegeneration and morphological changes in the neurons and glia cells of the enteric nervous system (ENS) are observed. These changes are related to the oxidative stress of diabetes. Hyperglycemia is responsible for an increase in oxidative stress and a decrease in antioxidant capacity. In this context, the use of flavonoids, a family of polyphenolic compounds with a high antioxidant capacity, might be effective in protecting against the pathology of diabetes. Quercetin is a flavonoid that is present in various foods, such as grapes, and their derivatives. Several studies have revealed the beneficial pharmacological effects of quercetin in biological systems, including its potent antioxidant effect. Thus, the use of quercetin is a promising therapy for the prevention of neurological disorders and can reduce the pathological conditions of diabetes.

### Research frontiers

Currently, diabetes is considered an epidemic that affects more than 300 million people worldwide. Its chronic nature combined with the severity of its complications and the necessary means to control them, makes diabetes a very costly disease for patients and for the healthcare system. Autonomic neuropathies, which are among the complications of diabetes, can trigger a wide range of gastrointestinal problems, such as nausea, vomiting, diarrhea, constipation and fecal incontinence, that cause discomfort and deeply affect the quality of life of patients with DM. In this context, studies evaluating therapeutic strategies that have the potential to improve or mitigate the degenerative damage to the enteric nervous system, such as the use of the flavonoid quercetin, may eventually contribute to an improved quality of life for these patients.

### Innovations and breakthroughs

Previous studies in their research group, using simple neuronal marking techniques, reported the absence of an effect of antioxidants on the cecum of rats with experimental DM. In this study, using immunohistochemical techniques, the authors could observe an effect of antioxidant treatment on the cecum in a rat DM model. In addition, using these techniques we observed this effect in both the general neuronal population and in specific neuronal subpopulations of the cecum ENS. Although, studies suggest a neuroprotective effect of quercetin in the central nervous system, there are only a few studies examining the effect of quercetin in the ENS of rats with experimental DM.

### Applications

The present study shows that quercetin could improve antioxidant capacity and thus protect the enteric nervous system in the cecum of streptozotocin-induced diabetic rats *in vivo*. When these effects are confirmed by further research, future application of quercetin as a therapeutic in the treatment of diabetic neuropathy may be merited. Another aspect of the present study is the identification of an apparent causal relationship between VIP and glia. This observation could provide a basis for the clarification of other research.

### Terminology

ENS is made of sensory neurons, interneurons and motorneurons and is divided into two major plexuses in the gastrointestinal tract: the myenteric and submucosal. VIP and the enzyme Nitric Oxide Synthase are neuronal subpopulations of the ENS that express inhibitory neurotransmitters and are non-adrenergic and non-cholinergic. Enteric glia: a set of cells, which are similar to the astrocytes of the CNS, that are adhered to the ENS ganglion neurons and their nerve fiber bundles.

### Peer review

This paper concerns an interesting issue, however introduction is repetitive, and discussion is too long and should be reduced.

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