

Vitamin E supplementation in rats with experimental diabetes mellitus: analysis of myosin-V and nNOS immunoreactive myenteric neurons from terminal ileum

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Abstract The effect of vitamin E (1 g/kg body weight) supplementation on myosin-V and neuronal nitric oxide synthase (nNOS) immunoreactive myenteric neurons from the ileum of diabetic rats was investigated in the present study. Forty animals were divided into the following groups: normoglycemics (N), normoglycemics treated with vitamin E (NE), diabetics (D), and diabetics treated with vitamin E (DE). Quantitative and morphometric analyses were performed. The area of the tertiary plexus was also determined. Diabetes produced a 24% reduction in the number of myosin-V neurons in group D compared with group N, an effect that was accompanied by an increase in the tertiary plexus area ($P < 0.05$). Neuronal density was 27% higher in group NE than group N ($P < 0.05$). Nitroergic neuronal density was not altered as a consequence of either diabetes or vitamin E treatment. Myosin-V and nNOS immunoreactive neuronal cell body area increased significantly in group NE. The area of myosin-V and nNOS myenteric neurons also increased in group D. Vitamin E treatment (group DE) increased only the size of nitroergic neurons. The present results suggest that vitamin E elicited a neuroprotective and neurotrophic effect on the natural aging process, but with regard to diabetes, vitamin E supplementation exerted a neurotrophic effect only on nitroergic neurons.

Keywords Diabetes mellitus · Myenteric neurons · Myosin-V · Neuronal nitric oxide synthase · Vitamin E

Introduction

Neuropathy is one of the secondary complications of diabetes mellitus (DM), one of the most common human metabolic disorders. The etiology of diabetic neuropathy has been extensively studied. Romero (1996) hypothesized that three mechanisms are involved in the electrophysiological alterations of peripheral nerves associated with experimental DM: hyperglycemia, free radical production, and decreased Na^+/K^+ ATPase activity.

Chronic hyperglycemia, characteristic of all types of DM, is strongly related to the microvascular pathology specific to the disease, affecting not only peripheral nerves, but also the retina and renal glomeruli, and leading to debilitating neuropathies, blindness, and renal disease (Brownlee 2001). Free radical production and decreased endogenous antioxidant defenses during DM are also directly related to micro- and macrovascular alterations (e.g., atherosclerosis) considered to be the main causes of morbidity and mortality in diabetic patients (Son 2007). The enzymes hepatic superoxide dismutase, catalase, and glutathione peroxidase are examples of endogenous antioxidants that are decreased in experimental models of streptozotocin-induced DM (Yoshida et al. 2005). Several mechanisms appear to be involved in the genesis of oxidative stress in diabetes in animals and humans (type 1 and type 2 diabetic patients), including glucose autooxidation, protein glycation, advanced glycation endproduct formation, and the polyol pathway (Bonfont-Rousselot et al. 2000).

The development of neuropathy in the gastrointestinal tract affects different types of enteric neurons responsible for the control of motility, secretion, blood flow, mucosal growth, and aspects of the local immune system (Furness and Costa 1987). Consequently, the neurological

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alterations that reach the enteric nervous system are related to gastrointestinal manifestations such as constipation, diarrhea, dysphagia, heartburn, emesis, and nausea (Dyck et al. 1993; Zochodne 2007).

Previous studies have shown that DM reduces the number of myenteric neurons (Hernandes et al. 2000; Furlan et al. 2002; Zanoni et al. 2007; Tashima et al. 2007) and causes changes in cell body area (Zanoni et al. 2002, 2003; Fregonesi et al. 2005; Shotton and Lincoln 2006). Neuronal subpopulations are differentially affected by the disease. An incremental increase in vasoactive intestinal polypeptide and nitric oxide synthase (NOS) content in the myenteric plexus is observed after 8 weeks experimentally induced DM (Shotton et al. 2007). Neuronal subpopulations also react differentially to various diabetic neuropathy treatments (Shotton et al. 2003, 2004).

New treatment strategies have been shown to be effective against neurovascular dysfunction induced by diabetes. Substances with antioxidant action, such as ascorbic acid (Zanoni et al. 2002, 2003), aminoguanidine (Shotton et al. 2007), and L-glutamine (Tashima et al. 2007), have been tested to explore whether they can protect myenteric neurons against experimental DM. Vitamin E, a potent lipophilic antioxidant, has been shown to be effective against the development of neuropathy in experimental models and in the prevention of nerve conduction velocity deficits in animals with DM (Cameron and Cotter 1999; Sharma et al. 2001).

Vitamin E interferes with the process of lipid peroxidation, protecting polyunsaturated fatty acids against attack by free radicals (Yoshida et al. 2005). The presence of vitamin E in neuronal cells membranes, especially the inner mitochondrial membrane, suggests its possible use in the treatment of DM. The objective of this study was to investigate the effects of vitamin E supplementation on the general population of myenteric neurons (myosin-V immunoreactive) and on the subpopulation of nitrergic neurons (neuronal nitric oxide synthase [nNOS] immunoreactive) in the terminal ileum of experimental DM rats.

Materials and methods

Animals

All experimental procedures were conducted in accordance with the ethical principles of the Brazilian Academy of Animal Experimentation (COBEA) and approved by the Committee of Ethics in Animal Experimentation from the Universidade Estadual de Maringá.

Forty male Wistar strain rats (*Rattus norvegicus*) obtained from the Central Biotery of Universidade

Estadual de Maringá were used. The animals were divided into the following four groups: normoglycemics (N), normoglycemics treated with vitamin E (NE), diabetics (D), and diabetics treated with vitamin E (DE).

Ninety-day-old rats were kept in individual cages in the vivarium for a period of 120 days under a 12 h:12 h light cycle (lights on 6:00 am to 6:00 pm) and controlled temperature ($24 \pm 2^\circ\text{C}$). Food and water were available ad libitum. Non-treated animals (groups N and D) received balanced standard chow (Nuvital; Nuvilab, Colombo, PR, Brazil). For experimental supplementation of rats in groups NE and DE, vitamin E (Zhejiang NHU, China) was incorporated into the standard chow (1 g/kg body weight; Cotter et al. 1995). To determine the correct amount of vitamin E to be added to the food, the animals' weight and food intake were measured every 15 days.

Diabetes mellitus was induced in groups D and DE after a 14 h fast 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). After induction of DM, glycemia was determined by the glucose oxidase method (Bergmeyer and Bernet 1974) to confirm the establishment of the experimental model. All animals of groups D and DE exhibited glycemia greater than 250 mg/dl.

Material resection and processing

At the end of the 120-day experimental period, animals were weighed, anesthetized with a 40 mg/kg body weight intraperitoneal dose of thiopental (Abbott Labs, Chicago, IL, USA), and sacrificed. Blood was sampled for determination of glycemia and glycated hemoglobin by the ion-exchange resin method (Koenig et al. 1976). Twenty ileums, five for each experimental group, were resected and immunohistochemically processed for myosin-V; the other 20 ileums were immunohistochemically processed for nNOS.

Immunolocalization of neuronal myosin-V: a study of the general myenteric neuron population

The animals were perfused with cold 1.1% saline solution followed by fixative solution containing sodium periodate (10 mM), lysine (75 M), and paraformaldehyde (1%) in phosphate buffer, pH 7.4 (37 mM; McLean and Nakane 1974). Immediately after perfusion, ileums were resected, washed with saline solution until complete removal of feces, carefully inflated with the fixative solution such that the segments were not distended, and tied at the extremities with cotton thread. Afterward, the ileums were maintained in the fixative solution for 1 h, dehydrated in alcohol (50, 70, 80, 90, and 100%),

clarified in xylol, and rehydrated in a decreasing series of alcohol (100, 95, 90, 80, and 70%). Intestinal segments were dissected with the aid of a transillumination stereomicroscope to obtain whole-mount muscular layer preparations through the removal of mucous and submucous layers. Whole-mount preparations then were subjected to the following technique for detection of myosin-V immunoreactive myenteric neurons (Drengk et al. 2000). The preparations were washed four times in phosphate buffered saline (PBS), pH 7.4 (0.1 M), and blocked for 1.5 h in solution containing bovine serum albumin (BSA, 2%; Sigma), goat serum (1:50), Triton X-100 (0.5%; Sigma), and PBS. Preparations were sequentially incubated at room temperature in solution containing the primary antibody (1:750) specific for the medial tail of myosin-V. The polyclonal antibody generated against chicken recombinant myosin-V protein has been characterized previously (Espreafico et al. 1992). The anti-myosin-V antibody was produced at the University of São Paulo, Ribeirão Preto campus. After 48 h, the tissue was washed twice in PBS solution containing Triton X-100 (0.1%) and twice in PBS solution containing Tween-20 (0.05%). The tissues then were incubated for 24 h with secondary antibody (1:1000) conjugated with peroxidase (Pierce, Rockford, IL, USA) at room temperature. Finally, tissues were washed four times with PBS solution containing Tween-20 (0.05%). The immunoreaction was revealed by diaminobenzidine (DAB; Sigma), and the preparations were mounted in glycerol gel. Negative control was performed by omitting the primary antibody.

Immunolocalization of nNOS: a study of nitrergic myenteric neurons

After the animals were sacrificed, the ileums were resected, washed in PBS, pH 7.4 (0.1 M), and inflated with Zamboni fixative (Stefanini et al. 1967). Soon afterward, the ileums were maintained for 18 h in the same solution. The segments then were cut along the mesenteric border and successively washed in 80% alcohol until the visible removal of fixative. The segments then were dehydrated in alcohol (95 and 100%), clarified in xylol, and rehydrated in a decreasing series of alcohol (100, 90, 80, 50%) and then PBS. The segments were dissected to obtain whole-mount muscular layer preparations. The preparations were subjected to the following immunohistochemical technique for detecting nitrergic myenteric neurons (Wrzos et al. 1997).

The preparations were initially washed three times in PBS solution containing Triton X-100 (0.5%). Soon afterward, the tissues were incubated in BSA (1% in PBS) for 1 h. Once blocked, the tissues were incubated for 48 h

at room temperature in nNOS-specific primary antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The tissues were washed three times in PBS and incubated for 2 h with secondary biotinylated antibody (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature. The tissues then were washed three successive times in PBS. Finally, the preparations were incubated with ABC avidin-biotin-peroxidase complex (1:1500) for 1 h, revealed by DAB containing H_2O_2 (0.02%), and mounted in glycerol gel. Negative control was performed by omitting the primary antibody.

Quantitative analysis of myosin-V and nNOS immunoreactive myenteric neurons

The quantification of myosin-V and nNOS immunoreactive myenteric neurons was performed using images obtained randomly from an intermediate region of the ileum (60° – 120° , 240° – 300° ; intestinal circumference in each animal, with 0° as the mesenteric insertion; Zanoni et al. 2005).

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 neuronal quantification of the images.

For each animal, all neurons present in 40 images, captured with a $20\times$ lens, were counted. The area of each image, measured with Image-Pro Plus, was approximately 0.14 mm^2 . Results are expressed as the number of neurons per cm^2 .

Neuronal density correction

The neuronal quantification results were corrected for alterations in the size of the intestine caused by intestinal growth as a consequence of natural aging and/or pathological processes that can “dilute” the number of neurons present in the analyzed area. For this correction of both immunohistochemistry techniques, a factor was calculated and applied to the quantitative results. The correction factor was calculated using the mean area in cm^2 of the small intestine of each group. The area, in turn, was obtained by measuring the intestinal length and width soon after each animal was sacrificed (Cowen et al. 2000) (Tables 1 and 2).

Morphometric analysis of myosin-V and nNOS immunoreactive myenteric neurons

The areas of myosin-V and nNOS immunoreactive myenteric neuronal cell bodies were measured using the same

Table 1 Areas of the small intestine (mean \pm SEM) and factors used to correct for the neuronal density of myosin-V immunoreactive myenteric neurons from the ileum in normoglycemics (group N), normoglycemics treated with vitamin E (group NE), diabetics (group D), and diabetics treated with vitamin E (group DE)

Group	Area (cm ²)	Correction factor
N	146 \pm 16.5 ^a	Standard
NE	190 \pm 12.8 ^a	1.30
D	246 \pm 32.9 ^b	1.68
DE	256 \pm 7.7 ^b	1.75

n = 5 rats per group

Means followed by different letters in the same column are statistically different ($P < 0.05$, Tukey's test)

Table 2 Small intestine areas (mean \pm SEM) and factors used to correct for neuronal density of nitrergic myenteric neurons from the ileum of normoglycemics (group N), normoglycemics treated with vitamin E (group NE), diabetics (group D), and diabetics treated with vitamin E (group DE)

Group	Area (cm ²)	Correction factor
N	99 \pm 15.1 ^a	Standard
NE	89 \pm 4.6 ^a	0.90
D	142 \pm 18.6 ^a	1.44
DE	136 \pm 11.5 ^a	1.37

n = 5 rats per group

Means followed by different letters in the same column are statistically different ($P < 0.05$, Tukey's test)

images, captured with a 20 \times lens, used in the quantitative analysis. The area (in μm^2) of 100 neuronal cell bodies was measured using Image-Pro Plus for each animal for each technique, with a total of 500 areas per group.

Area of the tertiary plexus

The area occupied by the tertiary plexus was determined using the same images, captured with a 20 \times lens, used for quantification of myosin-V immunoreactive myenteric neurons. The measurement was performed with 10 images per animal using Image-Pro Plus.

Table 3 Initial weight (90 days old), final weight (210 day old), glycemia, and glycosylated hemoglobin of normoglycemics (group N), normoglycemics treated with vitamin E (group NE), diabetics (group D), and diabetics treated with vitamin E (group DE)

Group	Initial weight (g)	Final weight (g)	Glycemia (mg/dl)	Glycosylated hemoglobin (%)
N	347 \pm 8.8 ^a	453 \pm 10.2 ^a	170 \pm 9.3 ^a	3.4 \pm 0.12 ^a
NE	312 \pm 11.2 ^a	413 \pm 7.9 ^a	146 \pm 5.2 ^a	4.3 \pm 0.45 ^a
D	323 \pm 6.7 ^a	271 \pm 8.2 ^b	415 \pm 18.4 ^b	5.9 \pm 0.24 ^b
DE	319 \pm 6.7 ^a	291 \pm 14.6 ^b	506 \pm 14.6 ^c	6.5 \pm 0.15 ^b

n = 10 rats per group

Means followed by different letters in the same column are statistically different ($P < 0.05$, Tukey's test)

To calculate the area occupied by the tertiary plexus (in μm^2), we measured the area localized between ganglia and fibers comprising the primary and secondary plexuses. Results are expressed as the percentage of the total area of each image (137.670 μm^2) occupied by the tertiary plexus.

Statistical analysis

Data were statistically analyzed using Statistica and GraphPad Prism and are expressed as mean \pm standard error. Morphometric data 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

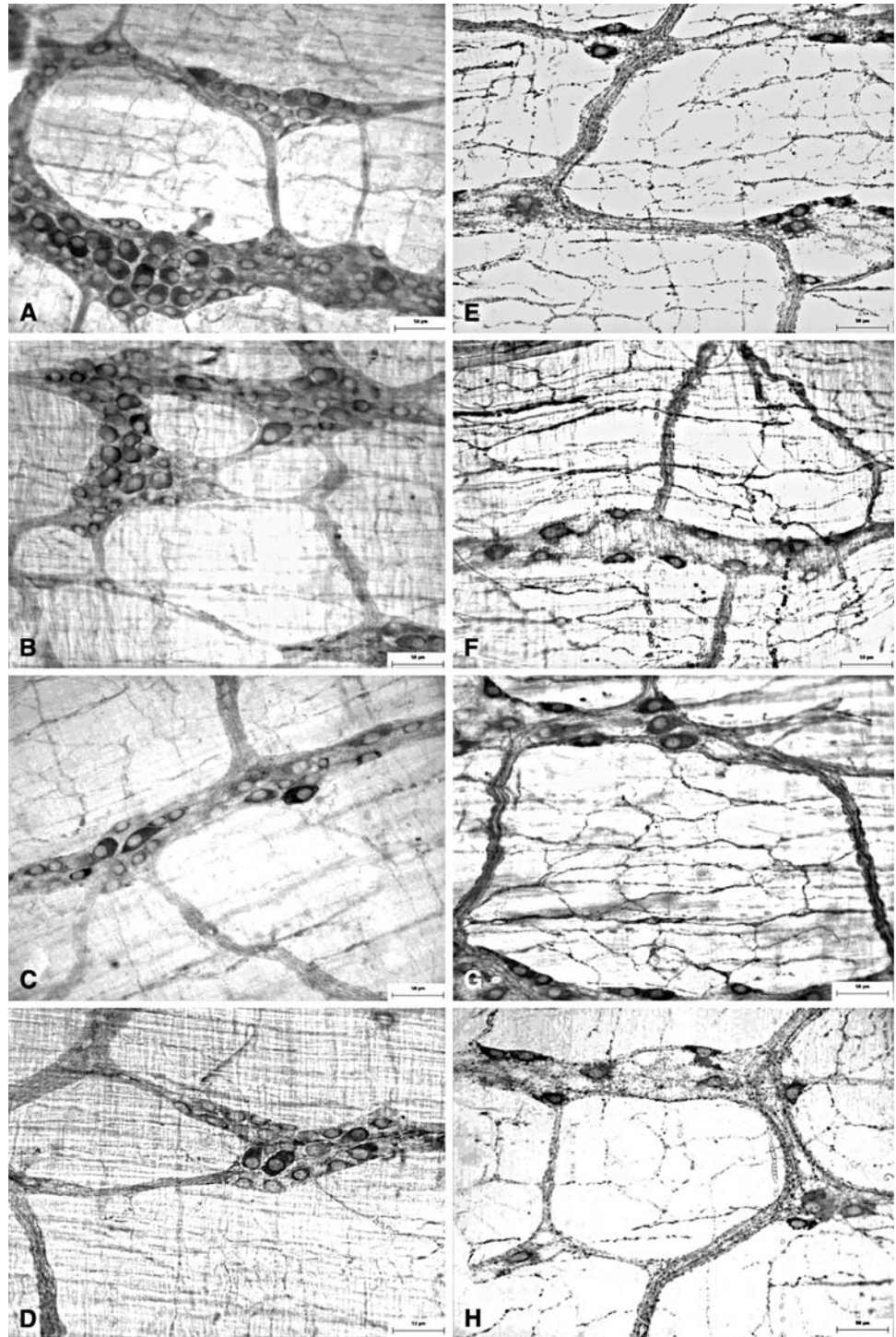
Streptozotocin induced a diabetic syndrome in groups D and DE characterized by polyuria, polydipsia, and polyphagia throughout the experiment. After 120 days, a significant increase in glycemia and glycosylated hemoglobin was observed in groups D and DE, in addition to a reduction of body weight, compared with normoglycemic animals (Table 3). Vitamin E supplementation did not significantly alter glycemia levels or glycosylated hemoglobin. In contrast, group DE exhibited minimal weight loss compared with untreated diabetic animals ($P > 0.05$) (Table 3).

The mean small intestine areas of group D and group DE diabetic animals, assessed with both the myosin-V and nNOS techniques, increased 33% ($P < 0.05$) and 32% ($P > 0.05$) respectively, compared with the mean values obtained for groups N and NE (Tables 1 and 2).

Morphology of enteric nervous system components of the myenteric plexus from ileums subjected to myosin-V and nNOS immunohistochemistry

Myosin-V immunohistochemistry allowed us to detect the primary and secondary components and small fibers

Fig. 1 Myosin-V (a–d) and nNOS (e–h) immunoreactive myenteric neurons from the intermediate region of the ileum of normoglycemics (a, e), normoglycemics treated with vitamin E (b, f), diabetics (c, g), and diabetics treated with vitamin E (d, h). Calibration bar: 50 μ m



representing the tertiary component of the ileum myenteric plexus. Neuronal cell bodies were observed in nervous ganglia and/or along interganglionic fibers. Heterogeneous intensity in immunohistochemistry staining of neuronal cell bodies expressing myosin-V protein was also visualized. The neurons of diabetic animals (groups D and

DE) were found to be less intensively stained than normal animals (groups N and NE) (Fig. 1).

Intensively stained neuronal cell bodies were observed in whole-mount preparations of all groups subjected to nNOS immunohistochemistry. Neurons were found to be isolated or occupying the peripheral region of the myenteric ganglia.

Primary and secondary components were readily visualized. Small varicosities were observed along tertiary plexus fibers (Fig. 1).

Neuronal density of myosin-V and nNOS immunoreactive myenteric neurons

Diabetes mellitus reduced the number of myosin-V immunoreactive neurons by 24% in group D compared with normoglycemic animals (group N; $P < 0.001$). Group DE exhibited a 12% greater neuronal density compared with group D ($P > 0.05$). Neuronal density in normoglycemic supplemented animals (group NE) was 21.2% greater than group N ($P < 0.001$) (Fig. 2). No differences in nitrergic neuronal density were observed between groups ($P > 0.05$) (Fig. 2).

Morphometry of myosin-V and nNOS immunoreactive myenteric neurons

The mean areas of myosin-V immunoreactive neurons of groups N, NE, D, and DE were 270.8 ± 5.0 , 313.6 ± 5.5 , 309.2 ± 5.3 , and $299.9 \pm 5.1 \mu\text{m}^2$, respectively. A significant difference was observed between groups N and NE ($P < 0.05$). Significant differences in nitrergic neuronal cell bodies were observed between groups ($P < 0.05$): group N ($253.8 \pm 3.9 \mu\text{m}^2$), group NE ($287.9 \pm 4.5 \mu\text{m}^2$), group D ($311.1 \pm 4.8 \mu\text{m}^2$), group DE ($346.1 \pm 5.0 \mu\text{m}^2$).

Relative frequencies allowed us to verify that myosin-V and nNOS immunoreactive myenteric neurons were

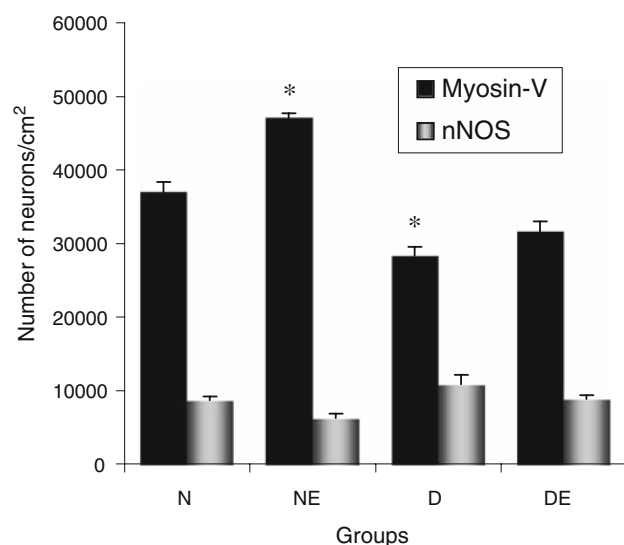


Fig. 2 Density of myosin-V and nNOS immunoreactive myenteric neurons observed with a $20\times$ lens in the intermediate region of the ileum of normoglycemics (group N), normoglycemics treated with vitamin E (group NE), diabetics (group D), and diabetics treated with vitamin E (group DE). $n = 5$ rats per group in each immunohistochemistry technique * $P < 0.05$ when compared to group N

distributed in an area ranging from 201 to $300 \mu\text{m}^2$ (Figs. 3, 4).

Area of the tertiary plexus

The percentages of total image area ($137.670 \mu\text{m}^2$) occupied by the tertiary plexus were $81 \pm 0.6\%$ and $81 \pm 2.1\%$ for groups N and NE, respectively ($P > 0.05$). Diabetes mellitus significantly changed, by 7%, the area occupied by the tertiary plexus in group D ($88 \pm 0.5\%$) compared with normoglycemics (group N) ($P < 0.01$). No significant

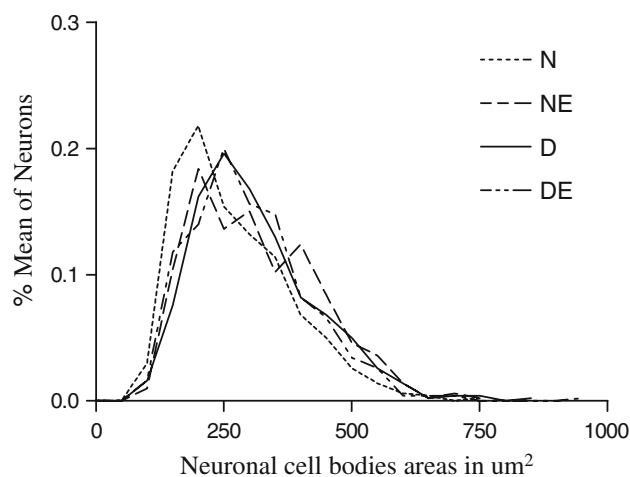


Fig. 3 Relative frequencies distribution of cell bodies areas of myosin-V immunoreactive myenteric neurons from the ileum of normoglycemics (group N), normoglycemics treated with vitamin E (group NE), diabetics (group D), and diabetics treated with vitamin E (group DE). $n = 5$ rats per group

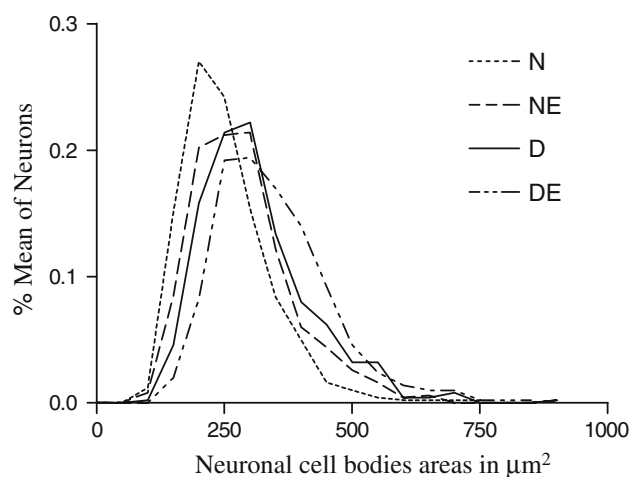


Fig. 4 Relative frequencies distribution of cell bodies areas of nitrergic myenteric neurons from the ileum of normoglycemics (group N), normoglycemics treated with vitamin E (group NE), diabetics (group D), and diabetics treated with vitamin E (group DE). $n = 5$ rats per group

difference in the area occupied by the tertiary plexus was observed between groups D and DE ($86 \pm 0.7\%$).

Discussion

Streptozotocin administration in the animals of groups D and DE promoted the typical characteristics of diabetes mellitus, including polyuria, polydipsia, polyphagia, weight loss, and high levels of blood glucose and glycated hemoglobin. Vitamin E treatment did not affect these parameters in either group NE or group DE.

All neuronal densities obtained in this study were corrected by a factor calculated from the area of the small intestine, ensuring that our quantitative results were a consequence of DM rather than simply neuronal dispersion. Group D animals used for myosin-V and nNOS immunohistochemistry exhibited a mean increase in intestinal area of approximately 41% and 30%, respectively, compared with group N, consistent with previous studies (Lincoln et al. 1984; Schmidt 2002). This effect may be explained by the increased expression of inhibitory neurotransmitters as DM evolves, such as vasoactive intestinal polypeptide and nitric oxide (NO) (Shotton et al. 2007), and the subsequent relaxation of gastrointestinal smooth muscles.

The general population of myenteric neurons in the present study was stained by an immunoreaction involving a primary antibody specific to myosin-V, a motor protein found in neuronal cell bodies and processes (Cheney et al. 1993). Different staining intensities were observed using this technique in the four groups of animals. This heterogeneity may be related to different neuronal activities (Drengk et al. 2000). According to our previous results, the reduction in staining intensity might be caused by a decrease in myosin-V protein content in neural tissue (Tashima et al. 2007).

A neuronal density of 37005 ± 1379 myosin-V immunoreactive neurons/cm² was observed in normoglycemic animals (group N). In group NE, the neuronal number was 21.2% higher. Our neuronal density results are discussed in proportions (%) rather than absolute numbers because we used a 20 \times panoramic objective for the quantitative analysis. In our earlier study of the ileum (Zanoni et al. 2003), we found a 24.3% reduction in the number of myenteric neurons, possibly reflecting the natural aging process. In the present study, vitamin E supplementation was effective in the aging process by preserving myenteric neurons in group NE, suggesting a neuroprotective action of vitamin E. Vitamin E has antioxidant properties and decreases the amount of free radicals (Bramley et al. 2000; Aksoy et al. 2005) that damage cell structures, including lipids, membranes, proteins, and DNA, and lead to neuronal death (Valiko et al. 2007).

Diabetes mellitus reduced the density of myosin-V immunoreactive myenteric neurons by 24% in group D compared with group N ($P < 0.001$). Several previous studies described similar results in different gastrointestinal segments, including the stomach (Fregonesi et al. 2005), ileum (Hernandes et al. 2000; Zanoni et al. 2003), and proximal colon (Furlan et al. 2002; Zanoni et al. 2007; Tashima et al. 2007). Diabetes mellitus is a pathological condition that affects the enteric nervous system, especially the myenteric plexus. An important factor related to diabetic neuropathy is oxidative stress (Vinson et al. 1989), which is pronounced in DM (Cameron and Cotter 1999). Additionally, the simultaneous reduction in antioxidant defenses beyond glutathione and glutathione peroxidase, including vitamins A, C, and E, contributes to this process (Rahimi et al. 2005).

Data verified for the area occupied by the tertiary plexus are consistent with results obtained for the number of myosin-V immunoreactive myenteric neurons. A decrease in neuronal density is paralleled by an increase in the area occupied by the tertiary plexus composed of axons that innervate the longitudinal muscular tunica located between the primary and secondary components of the myenteric plexus (Lepard 2005). Vitamin E treatment in group DE resulted in a 12% higher number of myosin-V immunoreactive myenteric neurons compared with group D. The neuronal density in group DE was preserved at 50% compared with the neuronal loss exhibited by group D. Cotter et al. (1995), using a vitamin E dosage similar to the present study, reported that vitamin E treatment prevented the decrease of motor nerve conduction velocity caused by DM. The authors suggested that glycemic levels observed in experimental diabetes and the consequent increase of free radicals are excessively higher than those observed in patients with controlled diabetes. These results may explain why statistically significant neuroprotection promoted by vitamin E was not found in the animals of the present study.

Nitric neurons expressing NO produced from reactions catalyzed by nNOS have an important inhibitory effect on smooth musculature of the gastrointestinal tract (Bult et al. 1990). In the present study, the number of nitric neurons in group N was 8693/cm², representing 23.5% of the general neuronal density of the myenteric plexus determined by myosin-V immunohistochemistry (37005 neurons/cm²). This result was similar to Cracco and Filogamo (1994); Miranda Neto et al. (2001), and Zanoni et al. (2005), who observed proportions of 24, 21, and 25.5%, respectively.

Nitric neuronal density was similar in all groups in the present study ($P > 0.05$), indicating the high resistance of this neuronal subpopulation to the action of free radicals found at elevated concentrations during DM

hyperglycemia. These results are consistent with the literature with regard to the stomach (Fregonesi et al. 2005), ileum (Wrzos et al. 1997; Zanoni et al. 2003), and distal colon (Yoneda et al. 2001). A situation similar to that of pathologies like DM is described for the natural aging process once nNOS immunoreactive neurons survive through age (Santer 1994; Belai et al. 1995; Zanoni et al. 2005). The proportion of nitrergic myenteric neurons in diabetic rats (group D) relative to myosin-V immunoreactive neurons (general population) was 38.2%, indicating that other neuronal subpopulations may be affected by DM, while nitrergic neurons are resistant to DM. In contrast, the relative proportion of nitrergic neurons in group DE was 27.9%, similar to that observed in group N.

The area of myosin-V immunoreactive myenteric neuronal cell bodies increased 14% in group D compared with normoglycemic animals (group N), suggesting altered neuronal activity induced by DM. Vitamin E did not significantly alter the size of myosin-V immunoreactive neurons in group DE compared with group D.

Additionally, our results showed that the area (μm^2) of nitrergic neuronal cell bodies in diabetic animals (group D) was 18.4% higher than normoglycemic animals (group N) ($P < 0.05$). These results are consistent with Fregonesi et al. (2005) in the stomach and Zanoni et al. (2003) and Shotton and Lincoln (2006) in the ileum. According to Fregonesi et al. (2005), increased synthesis of neuronal NO compensating for diabetes-induced low availability of NADPH for the enzyme nNOS may explain the increase in the area of nitrergic neuronal cell bodies. This hypothesis was supported by the increased expression of nNOS in DM determined by Western blotting (Adeghate et al. 2003). Shotton et al. (2003) and Shotton et al. (2007) also verified an increase in nNOS activity through radiochemical detection, further supported by measurements of cell body area in the present study. Vitamin E supplementation induced a significant increase in the area of nitrergic neurons in group DE compared with group D, an effect that may be a compensatory response resulting from the relatively reduced proportion of neuronal density in this group.

In conclusion, vitamin E exhibited neuroprotective and neurotrophic effects in the natural aging process. Vitamin E supplementation induced a neurotrophic effect on nNOS immunoreactive neurons in group DM. We cannot completely dismiss the possibility that vitamin E may exert a neuroprotective effect on diabetic neuropathy because higher dosages than the one used in this experiment must be tested.

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