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Vitamins C and E (ascorbate/α-tocopherol) provide synergistic neuroprotection in the jejunum in experimental diabetes

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ABSTRACT

The present study evaluated the synergistic effects of the association of ascorbic acid and α-tocopherol on myenteric in the jejunum of diabetic rats. The rats were randomly divided into four equal groups: untreated normoglycemic (UC), untreated diabetic (UD), ascorbic acid and α-tocopherol-treated normoglycemic (CAE) and ascorbic acid and α-tocopherol-treated diabetic (DAE). The rats from the CAE and DAE group received supplementation with ascorbic acid (1 g/L in water) and α-tocopherol (1% in chow). At 210-days-old, the animals were sacrified and their jejunum was collected and submitted to immunohistochemistry. Quantitative and/or morphometric analysis were performed. Supplementation with ascorbic acid and α-tocopherol prevented the cell loss of myenteric neurons expressing HuC/D and TrkA in an equivalent proportion. We also observed a reduction of the CGRP nerve fiber varicosities and the prevention of the increased cell body size of submucosal VIP neurons ($p < 0.05$). The association of ascorbic acid and α-tocopherol reduced the deleterious effects of diabetes promoting protection on the enteric neurons.

1. Introduction

The diabetic neuropathy is characterized by a heterogeneous group of disorders that affects the components of the autonomic peripheral nervous system and the enteric nervous system [1,2]. Several studies have reported changes in the myenteric plexus of several intestinal segments in diabetic rats in both the number of neurons and their size [3–7].

However, these cellular disorders differentially affect the various sub-populations of neurons, such as the VIP-ergic [8] and nitrergic [9], which undergo an increased synthesis of VIP (vasoactive intestinal peptide) and NO (nitric oxide) neurotransmitters, respectively, and also an increase in the cell body [6,10,11]. In contrast, other mediators show decreased expression levels due to neuronal loss and/or a downregulation; one such mediator is CGRP (calcitonin gene-related peptide), which depends on neurons that express NGF (nerve growth factor) to activate genes or RNAs, which encode precursor molecules for their integrity [12].

Therefore, disturbances, such as vomiting, nausea, abdominal distension diarrhea and constipation, are common in diabetic patients and are often correlated with neuronal lesions of the gastrointestinal tract [13–15].

Although hyperglycemia is the most important primary causal factor, several mechanisms, such as the vascular and metabolic ones, have been described in the development of the diabetic neuropathy [16,17].

Hyperglycemia in diabetes is due to the body's inability to properly maintain glucose levels, either due to a resistance to the action of insulin or a deficit of insulin caused by the failure of pancreatic beta cells [18,19].

The increase in blood glucose through the polyol pathway or AGEs (advanced glycosylation end-products) is also responsible for the formation of unstable compounds, such as the superoxide radi-

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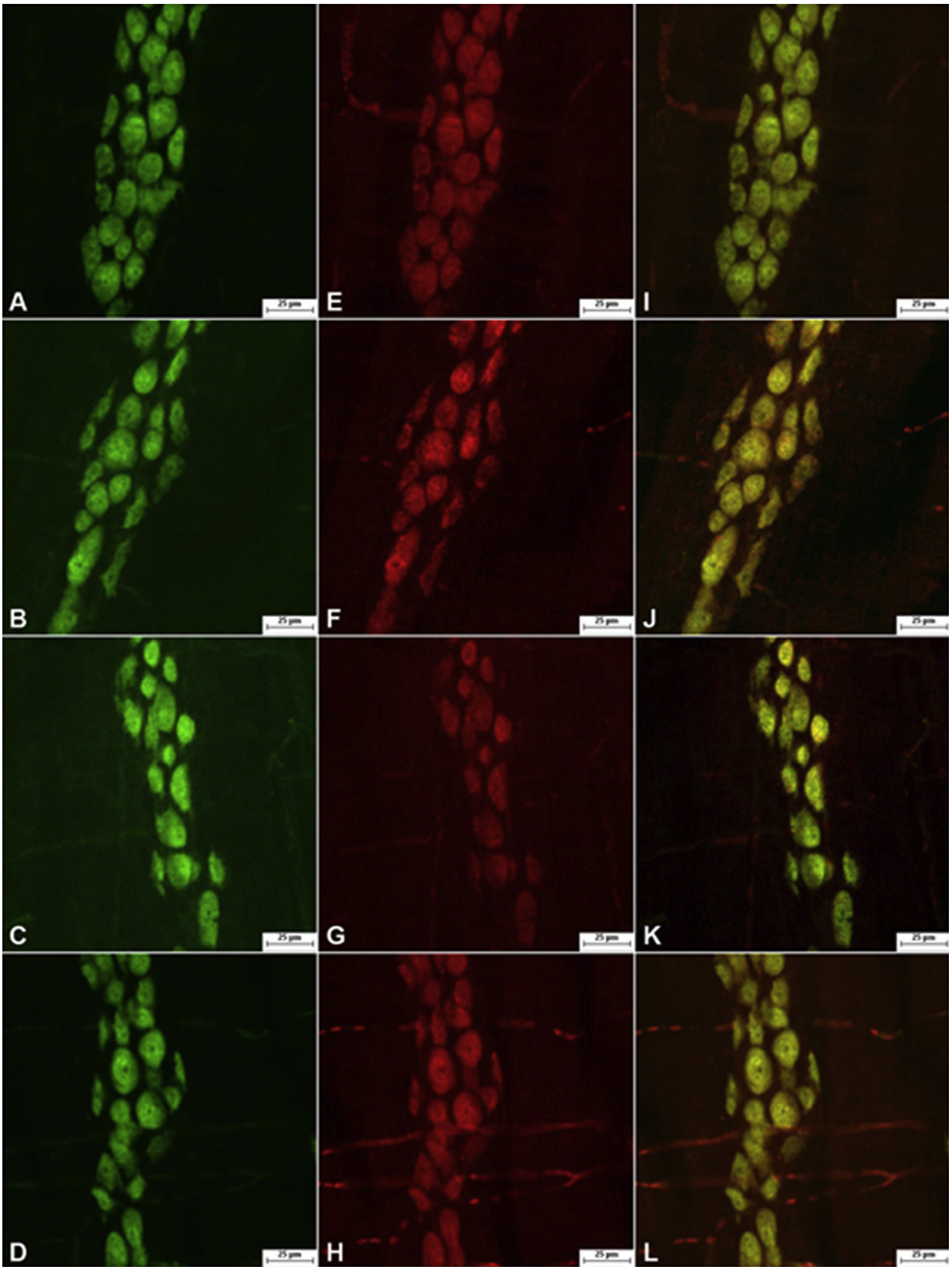

Fig. 1. Micrograph of the jejunum myenteric plexus immunostained for HuC/D (A–D) and TrkA (E–H) in the untreated normoglycemic (A, E and I), untreated diabetic (B, F and J), normoglycemic treated with ascorbic acid and α-tocopherol (C, G and K) and diabetic rats treated with ascorbic acid and α-tocopherol (D, H and L). $n = 5$ rats per group. Calibration bar: 25 μm.

cal, hydroxyl radical and hydrogen peroxide. These events, together with the decline of antioxidant defense systems, lead the patient to oxidative stress [20–22].

Moreover, the exacerbated production of superoxide anions with the increased release of NO causes a faster interaction, leading to the production of peroxynitrite, which can be decomposed into other reactive oxygen species, contributing to lipid peroxidation and, consequently, to tissue disorders, such as endothelial and neuronal injury. However, studies have shown that the administration of antioxidants may minimize such problems [23,24].

This study aimed at verifying the combined use of ascorbic acid with α-tocopherol, potent antioxidants, on the prevention of the diabetic neuropathy in HuC/D and TrkA immunoreactive myenteric neurons, myenteric nerve fibers that express CGRP and the VIP-ergic submucosal subpopulation in the jejunum of diabetic rats.

2. Material and methods

2.1. Animals

All the experiments described in this paper were reviewed and approved by the Ethics and Animal Experimentation Committee of the State University of Maringa. Twenty Wistar rats (*Rattus norvegicus*) at 90 days of age and weighing on average 313 ± 4.94 g were used. The rats were divided into four groups: untreated normoglycemic (UC), untreated diabetic (UD), normoglycemic treated with ascorbic acid and α-tocopherol (CAE), and diabetic treated with ascorbic acid and α-tocopherol (DAE). The animals were kept in individual cages, receiving water and food (lab Nuvital®) *ad libitum*, photoperiod (6:00 am–6:00 pm) and at controlled room temperature (24 °C ± 2 °C). Diabetes mellitus was induced in the UD and DAE groups by intravenous administration of 35 mg/kg body weight streptozotocin (Sigma, St. Louis, MO, USA), dissolved in citrate buffer 10 mmol/L (pH 4.5), after a 14 h fasting. The streptozotocin injection resulted in the diabetic syndrome characterized by polyuria, polyphagia and polydipsia; the blood glucose of each animal was assessed on the fourth day after the diabetes onset, resulting in the average value of 407.3 ± 10.01 mg/dL. The animals of the CAE and DAE group received ascorbic acid (Sigma, St. Louis, MO, USA) added to water (1 g/L) that was prepared daily, and the chow was supplemented (1%) with α-tocopherol (BASF—Lutavit E) and prepared weekly [3].

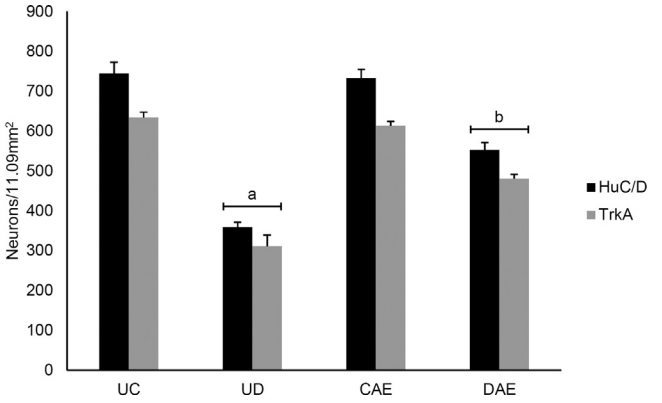

Fig. 2. Number of myenteric neurons immunoreactive for the Hu protein (HuC/D) and the NGF receptor (TrkA) in an area of 11.09 mm² per technique observed in the jejunum of rats. Groups: untreated normoglycemic (UC), normoglycemic treated with ascorbic acid and α-tocopherol (CAE), untreated diabetic (UD), diabetics treated with ascorbic acid and α-tocopherol (DAE). All results were expressed as mean ± standard error. $n = 5$ rats per group for each technique.
[a] $p < 0.05$ for the detection of Hu protein and TrkA receptors when comparing the UC, CAE and DAE groups.
[b] $p < 0.05$ for the detection of Hu protein and TrkA receptors when comparing the UC, UD and CAE groups.

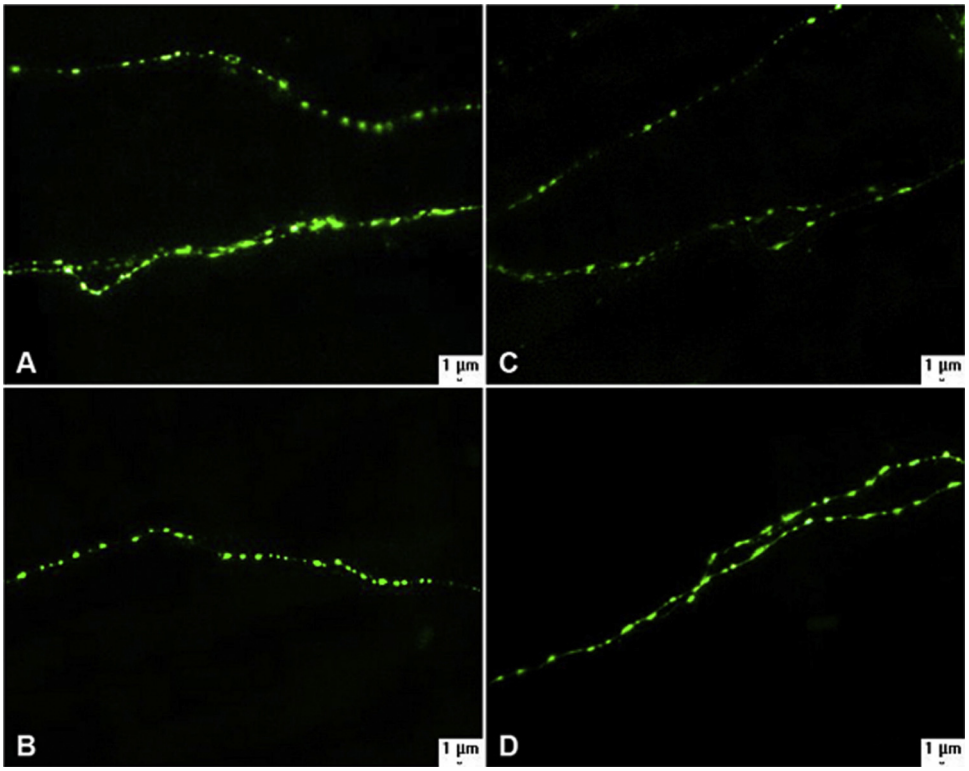

Fig. 3. Micrograph of the jejunum myenteric plexus immunostained for CGRP in groups: untreated normoglycemic (A), untreated diabetic (B), normoglycemic treated with ascorbic acid and α-tocopherol (C) and diabetic rats treated with ascorbic acid and α-tocopherol (D). $n = 5$ rats per group. Calibration bar: 1 µm.

2.2. Collection

At 210 days of age, the animals were anesthetized with thiopental (40 mg/kg body weight). Blood was collected by cardiac puncture for plasma glucose [25]. Later, the jejunum was collected and fixed for 18 h in Zamboni fixative solution [26] to perform the immunohistochemistry.

2.3. Processing the material for immunohistochemical techniques

After fixation, the segments were opened along the mesenteric border and washed successively in 80% alcohol until complete removal of the fixative. Next, the segments were dehydrated in ethanol (95 and 100%) for 10 min, cleared in xylol (10 min), rehydrated with ethanol 100, 90, 80, and 70%, and stored in phosphate buffered saline (PBS 0.1 M). Next, the jejunum was cut into slices of 1 cm to be microdissected, and the muscular layer was isolated to obtain whole mount preparations that were used in the HuC/D [27], TrkA [28] and CGRP [29] immunohistochemistry. Submucosal plexus whole mounts were used to detect VIP [30].

The whole mounts – separated by technique (double staining HuC/D—TrkA, CGRP and VIP) – were washed three times in 0.1 M PBS, pH 7.4 and blocked for 30 min with phosphate-buffered saline (PBS), containing 2% bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA), 2% goat serum and 0.5% Triton-X-100 (Sigma, St. Louis, MO, USA) at room temperature (RT).

2.4. Assay of HuC/D and TrkA-immunoreactive myenteric neurons

The segments were incubated in polypropylene tubes at RT (48 h) in a solution containing mouse anti-HuC/D primary antibody (1:500) (Molecular Probes, Carlsbad, CA, USA) and rabbit anti-TrkA receptor (1:500) (Santa Cruz Biotechnology, CA, USA), diluted in PBS. Next, the whole mounts were washed three times in 0.1 M PBS and incubated with secondary anti-mouse Alexa Fluor 488 (1:500) (Molecular Probes, Carlsbad, CA, USA) and anti-rabbit 546 antibodies (1:500) (Peninsula Labs, Torrance, CA, USA) diluted in PBS and incubated for 1 h at RT. They were then washed three times and mounted with buffered glycerol (9:1).

Table 1
Means and standard errors of the cell body areas of myenteric neurons immunoreactive for HuC/D and TrkA, VIP submucosal and varicosities of myenteric CGRP neurons for the groups: untreated normoglycemic (UC), normoglycemic treated with ascorbic acid and α-tocopherol (CAE), untreated diabetic (UD), diabetics treated with ascorbic acid and α-tocopherol (DAE). $n = 5$ rats per group for each technique.

	UC	UD	CAE	DAE
HuC/D	340.5 ± 5.53	406.0 ± 17.64*	322.2 ± 6.07	358.5 ± 4.29
TrkA	334.9 ± 8.01	385.4 ± 18.21**	314.9 ± 4.71	350.8 ± 6.89
CGRP	2.08 ± 0.12	1.19 ± 0.03*	2.40 ± 0.11	1.75 ± 0.13
VIP	315.8 ± 9.78	394.3 ± 10.10**	309.7 ± 8.39	336.5 ± 10.24

* $p < 0.05$ when compared to the UC, CAE and DAE groups.
** $p < 0.05$ when compared to the UC and CAE groups.

2.5. Assay of CGRP-immunoreactive myenteric neurons and VIP-immunoreactive submucosal

The segments were incubated at RT for 24 h in a solution containing primary rabbit anti-CGRP (1:200) (Santa Cruz Biotechnology, CA, USA), diluted in PBS for the CGRP staining technique. For the VIP expression, the samples were incubated for 16 h in a solution containing rabbit primary anti-VIP (1:200) (Bachem Americas, Torrance, CA, USA), diluted in PBS. The whole mounts were then washed three times in 0.1 M PBS at pH 7.4 and incubated with secondary antibody Alexa Fluor 488 diluted in PBS and incubated for 2 h at RT. They were then washed three times and mounted on slides with buffered glycerol (9:1).

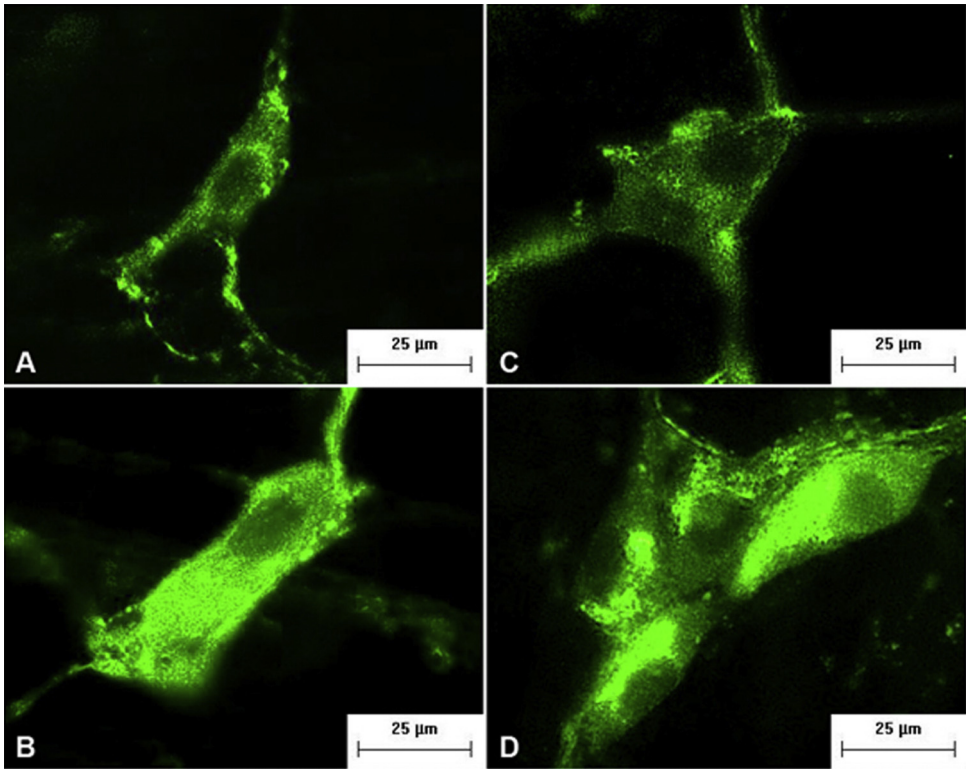

Fig. 4. Micrograph of the jejunum submucosal plexus immunostained for VIP in groups: untreated normoglycemic (A), untreated diabetic (B), normoglycemic treated with ascorbic acid and α-tocopherol (C) and diabetic rats treated with ascorbic acid and α-tocopherol (D). $n = 5$ rats per group. Calibration bar: 25 μm.

2.6. Quantitative analysis of HuC/D and TrkA myenteric neurons

The quantitative analysis was performed on 30 images/animal taken with 40× objective lenses at the intermediate region of the intestinal circumference (60–120°, 240–300°), considering the mesentery insertion at 0° [31] through a Plus Axioskop fluorescence microscope (Zeiss, Jena, Germany) with immunofluorescence filters (488 and/or 546) and a high resolution camera AxioCam (Zeiss, Jena, Germany) coupled to a microcomputer. Quantification and determination of the image area was performed using the analytical program Image Pro Plus 4.5.0.29 (Media Cybernetics, USA).

2.7. Morphometric analysis of HuC/D and TrkA myenteric neurons, CGRP neuronal varicosities and submucosal VIP neurons

The images taken for HuC/D and TrkA were analyzed in Image Pro Plus 4.5.0.29; the cell area (μm^2) of 100 cell bodies per animal was measured for a total of 500 neurons per group analyzed for each technique.

The area of 400 varicosities (μm^2) per animal of CGRP-immunoreactive nerve fibers was measured, totaling 2000 varicosities per group. The measurements were performed using 800× digital zoom, keeping the original calibration of the taken image.

The area (μm^2) of 100 cell bodies of immunoreactive VIP-ergic neurons was also measured for the studied groups.

2.8. Statistical analysis

The results were analyzed statistically using the Statistica 7.1 and GraphPad Prism 5.1 software and shown as the mean (M) ± standard error (SE). A randomized block design was performed to assess the morphometric data, which was followed by the Tukey's test. The one-way ANOVA was performed for the other data and followed by the Tukey's test. Values of $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Quantification of HuC/D and TrkA immunoreactive myenteric neurons

The HuC/D and TrkA immunohistochemistry showed the presence of cell bodies inside the ganglia of the jejunum myenteric plexus (Fig. 1). The neuronal staining intensity for the technique HuC/D was similar for all groups. For TrkA, there was a greater staining intensity in the diabetic rats (UD and DAE) when compared to the controls (UC and CAE). The UD and DAE groups also had neurons with more defined cell contours when compared to normoglycemic groups, UC and CAE, for HuC/D and TrkA techniques.

The ascorbic acid and α-tocopherol supplementation prevented HuC/D neuronal death in the DAE group (35.09%) compared to the diabetic rats (UD) ($p < 0.001$). No significant differences were observed when the control groups (UC and CAE) were compared ($p > 0.05$) (Fig. 2).

Ascorbic acid and α-tocopherol supplementation prevented the loss of 35.29% ($p < 0.001$) of the neurons expressing TrkA when comparing the DAE to the UD group. The ascorbic acid and α-tocopherol supplementation did not influence the neuronal density of the CAE group compared to control (Fig. 2).

3.2. Measurement of HuC/D and TrkA immunoreactive myenteric neurons, varicosities of CGRP neurons nerve fibers and the bodies of VIP submucosal neurons

The ascorbic acid and α-tocopherol supplementation prevented an increase of 13.25% ($p < 0.05$) in neurons HuC/D immunoreactive of the diabetic group (DAE) compared to untreated rats (UD). No differences were found when the UC and CAE groups were compared ($p > 0.05$) (Table 1).

There was an increase of 15.08% in the area of the cell body of TrkA neurons in the UD group compared to the UC group ($p < 0.05$). No significant differences were found when the UD and DAE groups were analyzed ($p > 0.05$) (Table 1).

The CGRP immunoreactive fibers showed a reduction in the brightness intensity in animals supplemented with ascorbic acid and α-tocopherol (CAE and DAE) compared to untreated groups (UC and UD). More dispersed nerve fibers and with varicosities were also seen in smaller numbers for UD and DAE groups compared to the UC and CAE groups (Fig. 3).

The analysis of the varicosity area (μm^2) revealed a 42.94% ($p < 0.001$) and 32.19% ($p < 0.01$) reduction for the UD group when compared to the UC and DAE groups, respectively. There was no difference when the UC and CAE groups were compared ($p > 0.05$) (Table 1).

The VIP-ergic neurons in the submucosal plexus had an increased immunofluorescence intensity in the cell bodies in the UD and DAE groups. These groups also showed cell neurons with less defined contours compared to the UC and CAE groups (Fig. 4).

The ascorbic acid and α-tocopherol supplementation prevented an increase of 17.18% ($p < 0.05$) in VIPergic neurons from the diabetic group (DAE) when compared to untreated rats (UD). No significant differences were found when the UC and CAE groups were analyzed ($p > 0.05$) (Table 1).

When analyzing the relative frequencies of myenteric neurons, the HuC/D technique showed a distribution ratio of 301–400 μm^2 in the cell body area for the UC, CAE and DAE groups and of 401–500 μm^2 for the UD group. For TrkA neurons, an area ranging from 201 to 300 μm^2 was found for the UC and CAE groups and from 301 to 400 μm^2 for the UD and DAE groups. The varicosities (CGRP) were between 1 and 2 μm^2 for the UC and UD groups and 2–3 μm^2 for the CAE and DAE groups. The VIP-ergic neurons showed an area ranging from 201 to 300 μm^2 for the UC, CAE and DAE groups and 301–400 μm^2 for the UD group (Fig. 5).

4. Discussion

The hyperglycemia of diabetic rats led to diabetic neuropathy that causes changes in the number and size of the jejunum enteric neurons.

HuC/D immunohistochemistry was used to identify the total population of myenteric neurons. The HuC/D protein is a pan-neuronal antigen and is found in both central and peripheral neurons [27]. Thus, the total neuronal population was reduced in the group UD. Several authors have also shown these decreases in various segments of the gastrointestinal tract in chronic streptozotocin-induced diabetic rats [5,6,32,33]. Chronic hyperglycemia produces metabolic changes that culminate in changes in enteric innervation. There are several mechanisms proposed to explain the relationship between hyperglycemia and neuropathy. However, the main factor is pointed oxidative stress, which may result from high production of precursors of reactive oxygen species and/or low efficiency of enzyme systems [34,35]. In an attempt to keep the intestinal physiological functions, the remaining neurons of animals UD group suffered hypertrophy in the cell body area. Apparel previously reported in experimental models

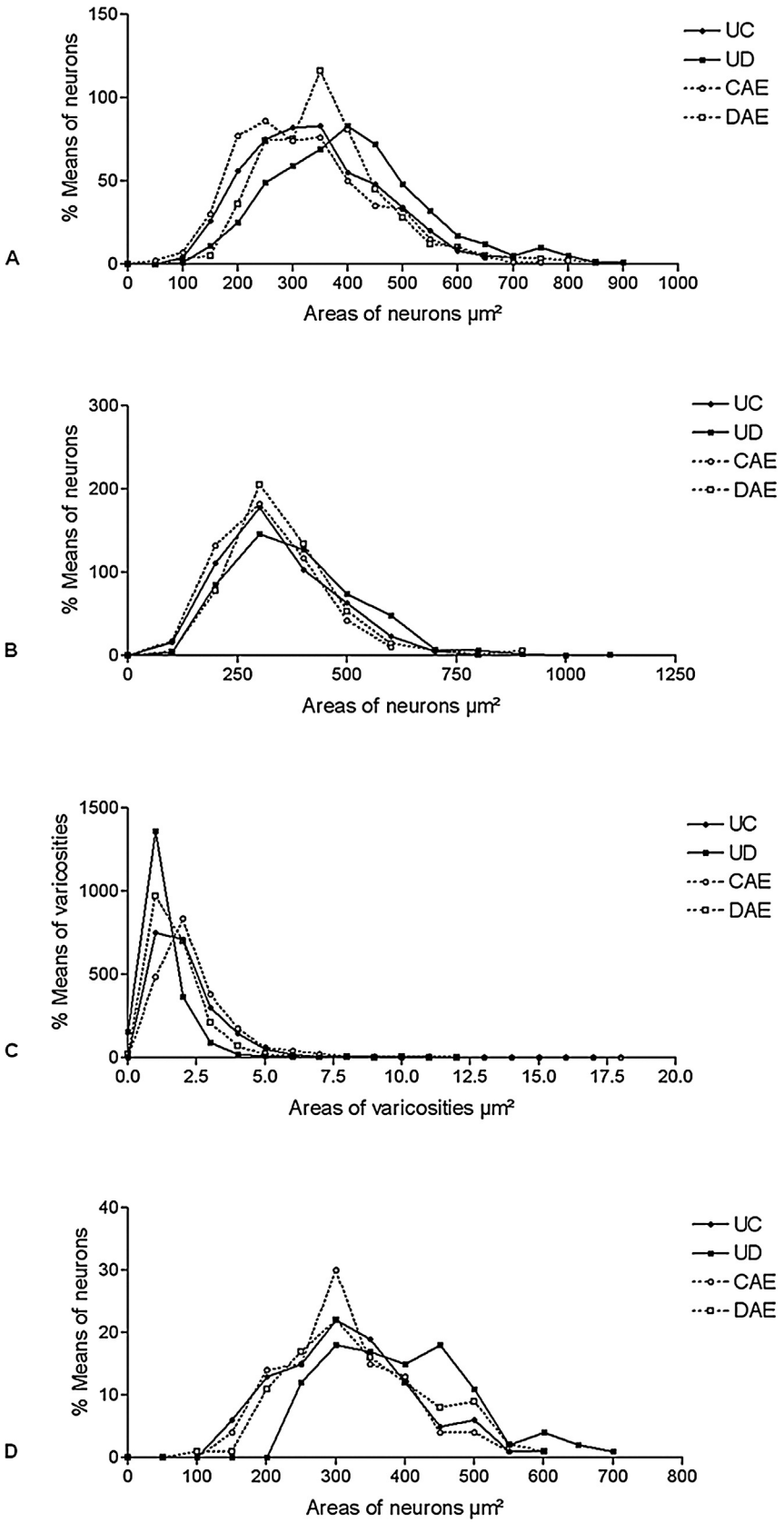

Fig. 5. Relative frequency distribution of the cell body area of myenteric neurons for HuC/D (A) and TrkA (B), varicosities of CGRP neurons (C) and cell bodies of submucosal VIP-ergic neurons (D) in untreated normoglycemic (UC), untreated diabetic (UD), ascorbic acid and α-tocopherol treated normoglycemic (CAE) and ascorbic acid and α-tocopherol diabetic treated rats (DAE). $n = 5$ rats per group.

of diabetes, which is associated with increased protein synthesis machinery that lead to compensatory adaptation mechanisms of lost neurons, thus ensuring its function and maintain the target tissue [4,5].

The ascorbic acid and α-tocopherol supplementation used in this study acted as a prophylactic on neuronal injury induced by diabetes when comparing the DAE group to the UD group. The α-tocopherol possibly prevented the adverse effects generated by free radicals on cell membranes, forming the tocopheroxyl radical, a less reactive agent that can regenerate into α-tocopherol in the presence of reducing agents, such as ascorbic acid [36,37]. In addition, α-tocopherol acts on cellular membranes due to its lipophilicity; in contrast, ascorbic acid has hydrophilic properties and inhibits the radicals in aqueous solutions, such as the cytoplasm of neuronal cells. In addition to its role as an antioxidant, ascorbic acid also inhibits aldose reductase to enhance neuronal health by decreasing the level of sorbitol formed [6]. The present findings suggest a synergistic effect that may prevent the changes caused by diabetes, corroborating previous study that observed neuroprotection enteric in the small intestine with the combined supplementation with ascorbic acid and α-tocopherol [3].

This study analyzed the NGF receptor, TrkA. It was found that the reduction in the total neuronal density (HuC/D neurons) was accompanied by a reduction of neurons immunostained for TrkA in diabetic animals. The relative ratio of neurons expressing the TrkA receptor was 85.16% of the total population of myenteric neurons (HuC/D).

In contrast, an increase in the area of TrkA immunoreactive neurons in the UD group was also observed. NGF plays an important role in the survival, differentiation and regeneration of neurons [38,39]. The NGF belongs to a family of growth factors called neurotrophins (NT), which also includes BDNF (brain-derived neurotrophic factor) [40], NT-3 [41] and NT-4/5 [42]. The receptors for NTs belong to the tyrosine kinase family (TrkA, TrkB and TrkC), with NGF having a higher specificity for TrkA [43].

Previous studies have shown a lower expression of NGF in diabetic animals, which supports another hypothesis for the induction of the enteric neuropathy [44]. Rush et al. [45] and Mendell et al. [46] showed that the activation of Trk receptors by NGF in the CNS was involved in neuroplasticity processes and may regulate the size of the cell body.

Our study also showed an increase in the intensity of TrkA staining in neurons from the UD and DAE groups. This may be correlated to the increased expression of TrkA because Terenghi et al. [47] found a decrease in the NGF synthesis in diabetes, which increases the expression of tyrosine kinase receptors. We also found that the ascorbic acid and α-tocopherol supplementation did not significantly reduce the cell body area of TrkA neurons, when comparing the DAE to the UD group. This might have occurred not only because of the oxidative stress, but also because of the neuronal communication. Thus, the increased expression of TrkA could increase the availability of NGF binding, which could be reduced as a result of diabetes mellitus.

The reduction in the size of the varicosities in the nerve fibers of diabetic animals may be related to the reduction of NGF. According to Donnerer et al. [48], the NGF reductions inhibited the maintenance of CGRP levels in neuronal cells. Reinshagen et al. [49] found that the presence of CGRP in the colon was significantly reduced after the immunoneutralization of NGF. Several studies have reported the presence of CGRP in various tissues, such as the heart, blood vessels, thyroid, lung and gastrointestinal tract. Its main functions are neuromodulation, vasodilatation, skeletal development, and cardiac and intestinal contractility [50,51].

Reduced levels of CGRP leads to the reduction in the release of somatostatin [52]. Somatostatin, found at high levels in the brain and gut, has several functions, such as its inhibitory effect on the gut and on the release of VIP, with the latter being an important inhibitory mediator of the gastrointestinal tract [53].

The reduction in the area of the varicosities in CGRP nerve fibers could be reversed with the ascorbic acid and α-tocopherol supplementation in animals from the DAE group. The increased size of the varicosity is related to an improved neuronal communication, i.e., the interaction between NGF and its receptor (TrkA), which has a role in controlling the production of CGRP [12].

According to Dockray [8], the VIP-ergic neurons represent about 50% of neurons in the submucosal plexus. This sub-population plays an important role in controlling the secretion of water and electrolytes by the intestinal mucosa. Neuronal changes could result in gastrointestinal disorders [54].

Based on a study by Zanoni et al. [55], the increase in the cell body was correlated with an increase in the synthesis of the VIP neuropeptide by neurons because an increase in the immunoreactivity of cell bodies was observed. Those authors have also reported that the loss of VIP-ergic neurons in diabetes could involve an increase in the synthesis process and, consequently, in the gain of cell volume. In studies using denervation of the myenteric plexus, See et al. [56] observed an increase in the size of the cell body of VIP-ergic neurons in the submucosal plexus. These authors speculated that, in normal conditions, the myenteric neurons would have an indirect inhibitory control in the submucosal plexus to which they are connected. According to Furness [57], despite the plexuses being spatially separated, they are connected to form an integrated unit. Our studies report changes in the NGF through the analysis of its receptor (TrkA) and the CGRP myenteric innervation acting indirectly on the VIP-ergic neurons of the submucosal plexus.

The increase in the size of the cell body of VIP-ergic neurons may be related to the reduction of varicosities (CGRP) found in diabetic animals (group UD) because this reduction leads to a decrease in the release of somatostatin, which, in turn, is an important inhibitor agent of VIP secretion [52] to promote an increase in the cell body of VIP-ergic submucosal neurons in the UD group. This increase was attenuated by the administration of ascorbic acid and α-tocopherol in animals from the DAE group. Our laboratory has studied the behavior of VIP-ergic neurons in diabetic rats and also after supplementation with antioxidants. Positive results have been found when ascorbic acid [55] and L-glutamine [58] were used as antioxidants. We believe that the most significant results found in this study are due to the combined action of the antioxidant agents. Within our hypothesis, we suggest that the improvement of the CGRP neurotransmitter synthesis may be reflected in the greater control of the neurotransmitter VIP synthesis because CGRP has an indirect inhibitory control on VIP.

Therefore, it can be concluded that the combination of ascorbic acid and α-tocopherol reduced the deleterious effects of diabetes on the myenteric neurons when comparing the DAE to the UD group. The supplementation also prevented the increase in the cell body area of the total myenteric population and VIP-ergic submucosal neurons, besides the decrease of CGRP varicosities area, which was due to the neuroprotective effects of these agents to improve neuronal communication.

Conflicts of interest

None.

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