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RAPID COMMUNICATION

Myenteric neurons and intestinal mucosa of diabetic rats after ascorbic acid supplementation

Priscila de Freitas, Maria Raquel Marçal Natali, Renata Virginia Fernandes Pereira, Marcilio Hubner Miranda Neto, Jacqueline Nelisis Zanoni

Priscila de Freitas, Maria Raquel Marçal Natali, Renata Virginia Fernandes Pereira, Marcilio Hubner Miranda Neto, Jacqueline Nelisis Zanoni, Department of Morphophysiological Sciences, Enteric Neurons Laboratory, State University of Maringá, Avenida Colombo, 5790, Maringá 87020-900, Paraná, Brazil

Author contributions: De Freitas P is the principal investigator; Zanoni JN and Miranda Neto MH designed the study; De Freitas P and Pereira RVF contributed data collection; Zanoni JN, De Freitas P and Natali MRM wrote the study and contributed analytic tools.

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Correspondence to: Priscila de Freitas, Department of Morphophysiological Sciences H-79, Enteric Neurons Laboratory, State University of Maringá, Avenida Colombo, 5790, Maringá 87020-900, Paraná, Brazil. priscilamga2@hotmail.com
Telephone: +55-44-30319319 Fax: +55-44-32614340

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Abstract

AIM: To investigate the effect of ascorbic acid (AA) dietary supplementation on myenteric neurons and epithelial cell proliferation of the jejunum of adult rats with chronic diabetes mellitus.

METHODS: Thirty rats at 90 d of age were divided into three groups: Non-diabetic, diabetic and diabetic treated with AA (DA) (1 g/L). After 120 d of treatment with AA the animals were killed. The myenteric neurons were stained for myosin-V and analyzed quantitatively in an area of 11.2 mm²/animal. We further measured the cellular area of 500 neurons per group. We also determined the metaphasic index (MI) of the jejunum mucosa layer of about 2500 cells in the intestinal crypts, as well as the dimensions of 30 villi and 30 crypts/animal. The data area was analyzed using the Olympus BX40 microscope.

RESULTS: There was an increase of 14% in the neuronal density (792.6 ± 46.52 *vs* 680.6 ± 30.27) and 4.4% in the cellular area (303.4 ± 5.19 *vs* 291.1 ± 6.0) respectively of the diabetic group treated with AA when compared to control diabetic animals. There were no significant differences in MI parameters, villi height or crypt depths among the groups.

CONCLUSION: Supplementation with AA in the diabetic animal promoted moderate neuroprotection. There was no observation of alteration of the cellular proliferation of the jejunum mucosa layer of rats with chronic diabetes mellitus with or without supplementation with AA.

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Key words: Ascorbic acid; Diabetes mellitus; Intestinal mucosa layer; Myenteric neurons; Myosin-V

Peer reviewer: Yasuji Arase, MD, Department of Gastroenterology, Toranomon Hospital, 2-2-2 Toranomonminato-ku, Tokyo 105-8470, Japan

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INTRODUCTION

The disease diabetes mellitus is characterized by abnormally high plasma glucose concentrations. Chronic hyperglycemia and the associated metabolic abnormalities are responsible for many disease complications, including damage to the blood vessels, eyes, kidneys and nervous system[1].

In diabetes mellitus, the mucosa of the small intestine undergoes morphological changes[2,3]. This impairs passage of food along the intestine, secretion of enteric juices and the absorption of the digestion products, all of which depend on the integrity of the epithelial lining. Intestinal epithelium is characterized by fast cellular renewal with continuous proliferation of stem cells inside Lieberkühn crypts, cellular migration along the crypt-villi axis, cellular differentiation, polarization, apical apoptosis and luminal loss[4].

It has been shown that the autonomic nervous system has a role in intestinal epithelium renewal, which can be assessed through the elimination of the parasympathetic[5], sympathetic[6] or myenteric innervation[7]. The enteric nervous system is a division

of the autonomic nervous system in the gastrointestinal tract; it can mediate independent reflexes (motility, absorption and secretion) and is made up of sensory neurons, interneurons and interconnected motor neurons[8]. Diabetes mellitus causes a decrease in enteric innervation with a consequent onset of the diabetic neuropathy[9-11].

Several theories have been proposed to explain the neuropathy in diabetes mellitus. They include: impaired metabolism of fatty acids, reduction in the blood supply to the nerves, advanced products of non enzymatic glycation, oxidative stress, inadequate trophic support and the activation of the polyol pathway[12,13]. Aldose reductase converts glucose excess into sorbitol[14,15] which is responsible for edema, neuronal lesions[14,16,17]. The sorbitol concentration can be reduced in the diabetes mellitus through the use of ascorbic acid (AA), an inhibitor of the enzyme aldose reductase[18,19].

Another factor that can be related to the paper neuroprotector of the AA is the antioxidant activity exercised by this vitamin, being of some importance in the diabetes mellitus seen that in this pathology the oxidative stress is intensified and the defense antioxidants are diminished. In this way, our aim was to verify the effect of supplementation with AA on the myosin-V immunoreactive myenteric neurons and on the intestinal mucosa of the jejunum of diabetic rats.

MATERIALS AND METHODS

Animal procedure

Albino male rats (*Rattus norvegicus*), Wistar strain weighing about 300 g and aged 90 d were used in this study. The rats were submitted to a 14-h-fast and then injected with streptozotocin (35 mg/kg of body weight) dissolved in citrate buffer (10 mmol/L, pH 4.5) in the penial vein to induce diabetes mellitus. The onset of diabetes mellitus was verified by the glycosuria and polyuria.

Thirty rats, divided into three groups of ten animals each, were used as follows: Non diabetic rats (ND), diabetic rats (D) and diabetic treated with AA (DA). The AA was given orally for 16 wk (from the 90 d of age) in water (1 g/L prepared daily)[20]. The rats were kept in individual cages with a photoperiod of 12 h (6:00 am-6:00 pm) and at room temperature (RT) (24 ± 2℃) with water and food (Nuvital® lab chow) *ad libitum*.

At 210 d of age, the rats were anesthetized intraperitoneally with sodium thiopental-Thionembutal® (40 mg/kg of body weight). The blood was collected by heart puncture for measuring the levels of glycated hemoglobin[21], glucose[22] and AA[23]. The animals used in this study were treated under the ethical principles adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Ethics Committee in Animal Experimentation of the State University of Maringá.

Myosin-V neuronal staining[24]

Fifteen animals were perfused with 1 mL/body weight of saline solution followed by perfusion with 1 mL/g per body weight of fixation solution containing sodium periodate (10 mmol/L), lysine (75 mmol/L), paraformaldehyde (1%) in phosphate buffer (PB) (37 mmol/L, pH 7.4)[25]. Immediately after perfusion, each treated fragment of jejunum was removed, rinsed with saline solution, flushed with fixative solution and tied in its extremities (balloons). Thirty minutes after immersion in fixative solution, the jejunum was opened and left in this solution for an additional 30 min. Subsequently, the segments were opened along the mesenteric border and dehydrated in alcohols (50%, 70%, 80%, 90%, 95% and 100%), remained in each solution 10 min, cleared in xylol (10 min), rehydrated back through the ethanol 100%, 95%, 90%, 80%, 70%, and stored in 70% ethanol. Afterwards, the segments were dissected under stereomicroscopy with trans-illumination, through the removal of the mucosa and submucosa layer, obtaining muscular layer whole mounts. These mixtures were washed four times in phosphate buffered saline (PBS) (0.1 mol/L, pH 7.4) and blocked for 2 h with PBS containing bovine serum albumin (2%), goat serum (2%) and Triton X-100 (0.5%) at room temperature. Later, the segments were incubated in eppendorfs in a solution containing 0.86 μg/mL of the myosin-V primary antibody (extracted from rabbits) (1:750) diluted in PBS, BSA (1%), Triton X-100 (0.1%) and goat serum (2%) at room temperature under agitation (48:00). After incubation, the tissues were rinsed twice in PBS (0.1 mol/L), Triton X-100 (0.1%) and twice in a solution of PBS (0.1 mol/L) + Tween-20 (0.05%). Then, the tissues were incubated in peroxidase-conjugated secondary antibodies (1 μg/mL) (1:1000) at room temperature under shaking (24 h). Finally, they were rinsed four times for 15 min in PBS (0.1 mol/L) + Tween-20 (0.05%). The staining with peroxidase-conjugated antibody was developed by incubation with 0.75 mg/mL diaminebenzidine and 0.03% of H_2O_2 in water (1 mL) and PBS 0.1 mol/L (1 mL) for 15 min at room temperature under shaking. Samples were mounted in glycerol gel, containing glycerol (50%), gelatin in water (0.07 g/mL) and phenol (2 μL/mL). The negative control was performed with the omission of the primary antibody.

Quantitative and morphometric analyses of myosin-V stained myenteric neurons

Quantitative analysis: Measurements were made in the intermediate area of the jejunum intestinal circumference (60º-120º; 240º-300º), considering the mesenteric intersection as 0º[26]. Myenteric neurons were counted in 50 random fields, under a light microscope (Olympus BX40) with a 40× lens. The area of each microscope field was 0.224 mm^2.

Morphometric analysis: The images were taken with a high-resolution camera, transmitted to a microcomputer and recorded to a compact disk. The area (μm^2) of 100 cell bodies of the jejunum, in a total of 500 neurons per group, was measured through an image analysis system,

the Image-Pro-Plus 4.0. The neurons were distributed in intervals of 100 μm^2.

Morphometric analysis of the intestinal mucosa: An additional 15 animals were injected with vincristine, a blocking agent of the mitotic fuse, at a dose of 1 mg/kg of body weight, in the penial vein 2 h before segment collection.

The jejunum was collected, open along the mesenteric border and washed with saline solution, fixed in buffered paraformaldehyde (10%) for 6 h. After fixation, they were dehydrated and immersed in resin 2-hydroxyethyl-methacrylate. Later, 2 μm transverse semi-serial sections were made (a section for each five discarded), using a Leica RM 2145 microtome, with a glass razor. The resin sections were stained by HE.

Metaphasic index (MI): MI shows the number of metaphasic cells/total number of cells (%) that was obtained from the interphasic and metaphasic epithelial cells in the crypts of the jejunum. Twenty five thousand cells per animal were quantified under a light microscope.

Villi and crypts: We measured the height of 30 villi and the depth of 30 crypts per animal, in a total of 900 measurements (300 measurements/treatment), under a light microscope with an ocular micrometer. The heights of the villi were measured from the crypt-villus junction to its apex. The depth of the crypts was measured considering the extension between the crypt-villus junctions to its base.

Chemical products

AA, serum albumin bovine (BSA), diaminebenzidine (DAB), streptozotocin (STZ), Tween-20, paraformaldehyde and Triton X-100 were obtained at Sigma Chemical Company, USA); Vincristine sulfate (Eli Lilly of Brazil, Brazil); Historesin kit (Leica); secondary antibody conjugated with peroxidase serum anti-rabbit IgG (Pierce, Rockford, USES). The polyclonal antibody anti myosin-V was characterized by Espreafico *et al*[27].

Statistical analysis

The data were submitted to analysis of variance (ANOVA) and the test of Tukey as a post-test to compare the means. Since the areas of the cell bodies of the neurons did not have an even distribution, we used the Kruskal-Wallis test to compare the means. The analyses were accomplished with the prism 3.0 software. The data are shown as mean ± SE as an indicator of the observation number (*n*). The level of significance was $P < 0.05$.

RESULTS

Streptozotocin promoted the onset of the diabetic syndrome with accentuated hyperglycemia. The diabetic rats and the diabetic-treated with AA had similar levels of glycated hemoglobin ($P > 0.05$). There was a reduction of 48.7% in the plasmatic level of AA in the diabetic animals when compared to the non-diabetics ($P < 0.05$). The supplementation in group DA increased their level of AA in 25.7% when compared to the non-diabetics ($P > 0.05$), and 61.9% when compared to the diabetic group ($P > 0.05$) (Table 1).

Table 1 GLI, GHb and AA for animals with 210 d old in groups, mean ± SE (*n* = 10)

Treated group	GLI (mg/dL)	GHb (%)	AA (μg/mL)
ND	129 ± 3.9	4.1 ± 0.3	24.58 ± 5.5
D	466.4 ± 24.6	8.1 ± 0.2	12.6 ± 1.9
DA	493.0 ± 10.1	7.9 ± 0.5	33.1 ± 2.5

Table 2 Distribution of myosin-V stained myenteric neurons, classified according to their cellular profile in intervals of 100 μm^2 in animals from groups (*n* = 5 rats per group)

Size (μm^2)	Groups		
	ND	D	DA
< 100	7	2	0
101-200	113	117	96
201-300	175	172	190
301-400	125	136	114
401-500	53	53	62
501-600	19	17	30
601-700	6	2	7
> 700	2	1	1
Total of neurons	500	500	500

Quantitative analysis of the myosin-V stained myenteric neurons

Experimental diabetes mellitus caused a reduction in the myenteric neuronal density in an area of 11.2 mm^2 in the jejunum, as shown in Figure 1A ($P < 0.001$). The AA supplementation in the diabetic animals caused a 14% increase in the density of the myosin-V stained neurons when compared to the non-treated diabetic animals ($P > 0.05$).

Cellular area of myenteric neurons stained with myosin-V

The results regarding the neuronal area means (μm^2) were 289.41 ± 5.19 for the non-diabetic group, 290.1 ± 6.02 for the diabetic group and 303.4 ± 5.19 for group DA (Figure 1B). The means of the cellular areas were similar for the three studied groups ($P > 0.05$). The 201-300 μm^2 interval was the predominant cellular area for all groups (Table 2).

Morphometric analysis of the intestinal mucosa

The intestinal mucosa, assessed through the determination of the MI and measurements of the height of the villus and depth of crypts, did not show statistically significant differences among the groups.

MI of the diabetic group was 1.5% higher when compared with the non-diabetic group ($P > 0.05$). The MI obtained for group DA was 1.77% smaller than the one observed in the diabetic group ($P > 0.05$). The quantification data are presented in Figure 1C.

The villus height (μm) of the diabetic animals was 7% inferior in relation to the non-diabetic group. The

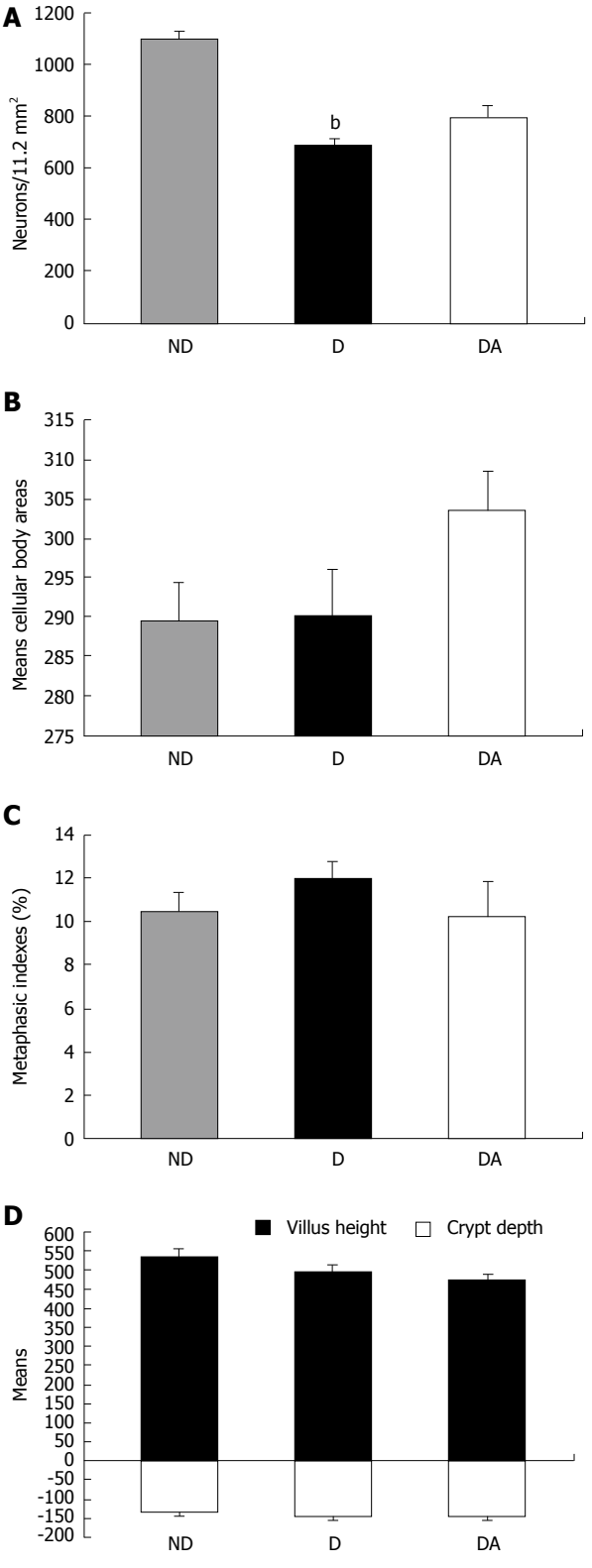

Figure 1 Quantitative and morphometric neurons meyenteric stained of myosin-V analyses. A: Number of myenteric neurons myosin-V stained quantified in 11.2 mm^2 in the jejunum of rats from groups: ND, D and DA, mean ± SE (*n* = 5). $^bP < 0.001$, *vs* the corresponding values in group ND; B: Means (μm^2) of neuronal area of myosin-V-stained myenteric neurons in animals from groups: ND, D and DA, mean ± SE (*n* = 5). There were no significant differences when comparing the three groups by Kruskal-Wallis test; C: IMs (%) of animals from groups: ND, D and DA, mean ± SE (*n* = 5). There were no significative differences when comparing the three groups by test of Tukey; D: Villus height (μm) and crypt depth (μm) in the jejunal mucosa of animals from groups: ND, D and DA, mean ± SE (*n* = 5). There were no significant differences when comparing the three groups by test of Tukey.

animals from the DA group had villi with lower heights than the non-diabetic and diabetic groups (11% and 4% lower, respectively) ($P > 0.05$).

The crypts of the diabetic group were 10% deeper than the crypts observed in the non-diabetic group. The animals from group DA showed a 17% reduction in the depth of their crypts when compared to the diabetic group, and an 8% reduction when compared to the non-diabetic group ($P > 0.05$). The data are shown in Figure 1D.

DISCUSSION

We observed the onset of diabetes mellitus in the diabetic animals and the DA group, characterized by polyuria, polydipsia and polyphagia, thus, validating this experimental model of streptozotocin-induced diabetes mellitus. The onset occurred in the first days of the induction and is associated with the elevation of glucose levels observed by the glucose oxidase and glycated hemoglobin test.

We also verified a sharp reduction of the plasma level of AA due to the diabetes mellitus, in agreement with Young *et al*[28], Garg *et al*[29] and Lindsay *et al*[30]. This reduction might be associated with the increased consumption of the AA due to the increase of the oxidative stress caused by the diabetes mellitus[20,31]. The supplementation with AA allowed the rats from the DA group an increase of 61.9% in their plasmatic level when compared to the diabetic rats, maintaining the glycemia and typical state of diabetes mellitus for these animals.

In order to examine the possible neuroprotector action of the AA in the population of myenteric neurons, we used the immunohistochemical technique for staining the neurons that had myosin-V, since these neurons have a high concentration of this protein, similar to the central nervous system[24].

The loss of neurons suffered by the diabetic animals (680.6 ± 30.27 neurons/11.2 mm^2, group D), resulting in the pathology, was 37.9% in relation to the non diabetic rats (1097 ± 29.79 neurons/11.2 mm^2) ($P < 0.001$) while in the DA group (792.6 ± 46.52 neurons/11.2 mm^2) the loss of neurons was 27.7% in relation to the same group. Treatment of the DA group with AA, resulted in an upper number of myosin-V neurons at 14% in relation to group D ($P > 0.05$). The AA acts by reducing the activity of the aldose reductase. It is the first rate-limiting enzyme of the polyol pathway[32]. Under euglycemic conditions, aldose reductase plays a minor role in glucose metabolism; however, during diabetes, its contribution is significantly enhanced[33,34] leading to a conversion of excess glucose to sorbitol in insulin independent tissues like myenteric neurons. The hyperglycemia, increases sorbitol accumulation and osmotic stress because of the slower oxidation of sorbitol to fructose by sorbitol dehydrogenase. The importance of the AA is also related to its antioxidant activity; therefore, in diabetes mellitus there is an increase of the oxidative stress and a reduction of the levels of antioxidants, endogenous

and exogenous[35]. Cotter *et al*[36], using vitamin C (150 mg/kg per day), demonstrated a 36% prevention in the reduction of the speed of motor nervous conduction provoked by the DM ($P < 0.001$). According to the author, the levels of glycemia observed in experimental diabetes and the consequent increase of free radicals, are excessively higher than the relatively controlled observed one in patients with diabetes. This would explain the statistically significant neuroprotection exerted for the vitamin, which was not observed in our animals of group DA ($P > 0.05$). Due to the fact of a differential effect of streptozotocin-diabetes on different regions of the rat intestine[9], our research group carried out experiments in different intestinal segments. Our work developed in the Department of Morphophysiological Sciences of the State University of Maringá, Paraná, Brasil in whole mounts of diabetic rats supplemented with AA (1 g/L). Zanoni *et al*[37] and Zanoni *et al*[10] verified a reduction of the area of the cellular body from enteric neurons VIP-immunoreactives and NADPH-d positives of the ileum, respectively. Also it was verified 74.3% and 33.4% of neuroprotection in NADH-d myenteric neurons of the duodenum[38] and of the proximal colon[39], respectively. This percentage supports the neuroprotection in the number of myenteric neurons in animals of group DA, when compared to group D.

When analyzing the results obtained through the mean cellular area, we verified there were no significant differences when the three groups were compared. When considering specifically the diabetic condition with the non-diabetic group, we noticed some divergences in our results. Hernandes *et al*[7] and Fregonesi *et al*[40] observed an increase in the cellular area. They attributed as a possible cause the increase on the sorbitol concentration that would lead to changes of the intracellular osmolality, increasing it and resulting in edema and neuronal lesion. Similar to our results, Zanoni *et al*[37] also did not observe differences when they compared the cellular area of the myosin-v-stained myenteric neurons of the ileum of diabetic rats with the acid ascorbic diabetic-treated rats. On the other hand, VIP-ergic neurons of the ileum showed an increase in their cellular area in the diabetic rats and the supplementation with AA prevented this increase[37]. We believe that AA supplementation has an effect on the neuronal population. However, this happens in a specific way, depending on the subpopulation studied: for example, it is evident that it affords neuroprotection Vip-ergic neurons.

The onset of the experimental diabetes mellitus in the jejunum of rats did not cause alterations in the MI or in the morphometry of the intestinal mucosa (villi height and depth of crypts) of our animals. The literature data reveal that the small intestine of the rat responds to the experimental with hyperplasia and hypertrophy of the mucosa[41,42] and an increase of the thickness of the submucosa layer and total wall[42]. These results cannot be considered contradictory to ours, since these authors maintained acute diabetes. They also agree partially with the data of Zoubi *et al*[3], who did not detect alterations in the morphometry of the intestinal crypts in the small intestine of rats after 84 d of inducing the diabetes mellitus. However, they observed an increase in the mitotic index.

As the intrinsic enteric nervous system mediates signals of the autonomic nervous system that regulate the dynamics of the intestinal epithelium carrying out an inhibitory control about the same one. Zucoloto *et al*[43] and Hernandes *et al*[44] carried out denervation of the myenteric plexus with benzalkonium chloride in rats. Zucoloto *et al*[43] did not observe differences in the cellular growth of crypts after 5 mo and Hernandes *et al*[44] observed an increase in cellular proliferation of the mucosa 15 d after denervation, but not after 23 d. We believe that the results found for the cellular proliferation of the intestinal mucosa may be related to an adaptation of the mucosa layer to the chronic pathogenesis of diabetes mellitus (if we consider the long period, 120 d, of maintaining diabetes mellitus).

Despite maintaining the morphometry of the mucosa layer, we presume that the diabetes mellitus did not lead to functional damage of the jejunum revealing an important mechanism of intestinal adaptation to chronic diabetes mellitus. On the other hand, the absence of morphometric variations on the intestinal mucosa of the diabetic rats supplemented with AA is explained by the effect of this antioxidant on the myenteric neurons being too little to promote significant changes.

The conclusion was that supplementation with AA in the diabetic animal promoted a moderate neuroprotection. Alteration of the cellular proliferation of the jejunum mucosa layer of rats was not observed, with chronic diabetes mellitus with or without supplementation with AA.

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COMMENTS

Background

Diabetes mellitus (DM) is caused by inadequacy of insulin production in the pancreas, when this organ either totally stops producing insulin or does not produce enough of the hormone. When badly controlled, the glucose level increases abnormally and causes harm, such as dysfunction and failure of several organs, especially nerves, kidneys, eyes, heart and blood vessels. In DM, the concentration of free radicals and/or the oxidative stress increases in an uncontrolled way, causing lesions on the neurons. The oxidative stress in DM is intensified due to the reduction of antioxidant levels, an increase of non-enzymatic glycation, and also frequent inflammations. Drugs that reduce the oxidative stress may have a relevant role in the treatment of the neurological complications of DM. Ascorbic acid (AA) is one of these substances, and it seems to be a promising one, not in the sense of curing diabetes, but of contributing to the maintenance of better conditions of neural conduction.

Research frontiers

The repercussions of the DM on the longevity and quality of life of individuals

in this condition, and their economic implications affect the productive potential and overburden the health system, has acted as motivation for many searches that seek to assess substances which might collaborate in the reduction of degenerative processes arising from DM. Results with AA have been promising, not to cure diabetes, but at least contribute to maintenance of better neural conditions, and reduction of oxidative stress common to this pathology.

Innovations and breakthroughs

Due to the fact of a differential effect of streptozotocin-diabetes on different regions of the rat intestine, our group carried out experiments in different intestinal segment in whole mounts of jejunum of diabetic rats supplemented with AA (1 g/L) and found that supplementation with AA in the diabetic animal promoted moderate neuroprotection.

Applications

The authors understand that the confirmation of the real benefits of AA will be consolidated or discarded based on new research, but will also be of some importance to the disclosure of reports of cases where this product was administered to treat patients, thus contributing to publication of a history containing comments on its use.

Peer review

The present study shows the myenteric neurons and intestinal mucosa of diabetic rats after AA supplementation. This present study is an interesting trial.

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