Effects of the ascorbic acid supplementation on NADH-diaphorase myenteric neurons in the duodenum of diabetic rats

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ABSTRACT: We assessed the ascorbic acid (AA) supplementation on the myenteric neurons in the duodenum of rats. Fifteen rats with 90 days of age were divided into three groups: control (C), diabetics (D) and ascorbic acid treated diabetics (DA). After 120 days of daily treatment with AA, the duodenum was submitted to the NADH-diaphorase (NADH-d) histochemical technique, which allowed us to evaluate the neuronal density in an area of 8.96 mm^2 for each duodenum, and also to measure the cellular profile area of 500 neurons *per* group. The supplementation promoted an increase on AA levels. The neuronal density (p <0.05) was higher in the group DA when compared to group D. There were no significant differences in the neuronal areas, when we compared groups C (204 ± 16.5) and D (146.3 ± 35.84) to groups D and DA (184.5 ± 5.6) (p> 0.05). The AA-supplementation avoided the density reduction of the NADHd myenteric neurons in the duodenum of diabetic rats.

Introduction

Diabetes mellitus (DM) is a syndrome of multiple etiologies, due to the lack of insulin and/or to the insulin incapacity of performing appropriately (American Diabetes Association, 1997). The complete clinical characteristics of DM, besides the complex alterations on the metabolism of carbohydrates, fats and proteins, include late manifestations, which will appear 10 to 15 years after the disease onset. Among them are the micro-angiopathies, atherosclerosis, nephropathy and diabetic neuropathy (Nathan, 1993; Cotran *et al*., 1996; Arduino, 1980).

The neuronal damage due to DM has been attributed mainly to sorbitol. This substance is produced by the glucose reduction in the reaction catalyzed by the aldose reductase enzyme (Vinson *et al*., 1989). The increase of its concentration causes an increase in the intracellular osmolality, with edema formation, neuronal lesion and a consequent reduction of the velocity of nerve conduction (Hosking *et al*., 1978). These changes will cause the long-term complications in the diabetes (Lindsay *et al*., 1998), which are called diabetic neuropathies. The ascorbic acid (vitamin C) has been studied for the treatment of this disease.

Li *et al.* (2003) showed in their studies of neuronal cell culture that these cells concentrate the ascorbate and the intracellular ascorbate eliminates the free radicals that oxidize the alpha-tocopherol, preserving and protecting these neurons against the lipid peroxidation. Cotter *et al*. (1995) studied the effectiveness of natural antioxidants, such as the AA, vitamin E, and beta-caro-

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tene, in preventing the blood irrigation decrease and the reduction on nerve conduction in streptozotocin-induced diabetic rats. The ascorbic acid, the vitamin E, and the beta-carotene have a neuroprotector effect and the association of ascorbic acid with the vitamin E has an additive effect in the prevention of nerve dysfunction (Cotter *et al*., 1995). In people with DM, the tissue concentrations of ascorbic acid may be reduced since its transport during the hyperglycemia is inhibited, as well as its renal reabsorption (Cunningham, 1998). It has also been suggested that patients may present a reduction on the AA-concentration since they are more likely to be exposed to the oxidative stress (Young *et al*., 1992). The oxidative stress is due to an increase of the non-enzymatic glycolization, increase of auto-oxidation, increase of the metabolic stress, and changes in the sorbitol formation stages (with a consequent increase of its concentration) (Baynes, 1991), and also due to the frequent inflammatory processes that happen in the diabetes.

The compared literature reveals that the ascorbic acid has a neuroprotector role, since it reduces the capillary fragility, the oxidative stress and the sorbitol concentration, through the inhibition of the aldose reductase (Yue *et al*., 1989; Cunningham *et al*., 1994; Cunningham, 1998; Will and Byers, 1996, Darko *et al*., 2002) and it also decreases the extracellular citotoxicity.

Recent works, carried out at the Department of Morphophysiological Sciences of the State University of Maringá, Paraná, Brazil inferred that, after long periods of DM, the myenteric neurons died, as demonstrated by the reduction in their number and also by changes in the size of these cells in several intestinal segments (Romano *et al*., 1996; Hernandes *et al.,* 2000;

Fregonesi *et al*., 2001; Furlan *et al*., 2002; Zanoni *et al*., 2003).

Based on these previous researches, our purpose was to study the possible neuroprotector effect of the ascorbic acid supplementation on myenteric neurons in the duodenum of streptozotocin-induced diabetic rats.

Materials and Methods

Fifteen albino, male, Wistar rats (*Rattus norvegicus*), with 90 days of age, were divided into three groups. Five animals were kept normoglycemic as a control group (C); five animals received an intravenous streptozotocin injection (penial vein) (35mg/kg, Sigma, USA), in order to induce diabetes (group D); the remaining five animals also received an intravenous streptozotocin injection (35mg/kg) and were supplemented with ascorbic acid (1g/L/dia) throughout the experiment period (group DA). Animals from groups D and DA were submitted to a previous fourteen-hour-fast before being injected with streptozotocin. The animals were kept in individual cages and received water and food (Nuvital ® lab chow) *ad libitum*.

The animals were sacrificed after 120 days of the experiment treatment. On the sacrifice day, they were weighed and anesthetized with thiopental (40mg/kg/ body weight). Blood was collected by heart puncture in order to measure glycemia (glucose oxidase method) and the glycated hemoglobin. The duodenum was sectioned immediately after the pylorus and proximal to the duodenojejunal plica. The segments were washed and submitted to the histochemical technique to stain

TABLE 1.

Initial body weight in grams (IBW), Glycemia (GYL), hemoglobin glycated (GHb), plasma ascorbic acid (AA) and final body weight (FBW) in the animals from groups: control (C), diabetic (D) and ascorbic acid treated diabetic (DA). All results were expressed as mean $\pm$ SE. n = 5 rats *per* group.

	IBW (g)	GYL/mg . dl^{-1}	GHb/%	AA/μg.ml^{-1}	FBW (g)
C	339.4 $\pm$ 12.36[a]	129$\pm$3.9[a]	4.1 $\pm$ 0.3[a]	24.58 $\pm$ 5.5[a]	456.2 $\pm$ 14.57[a]
D	329.6 $\pm$ 8.49 a	466.4$\pm$24.6[b]	8.1 $\pm$ 0.2[b]	12.6 $\pm$ 1.9[ab]	318.6 $\pm$ 8.22[b]
DA	339.0 $\pm$ 12.45[a]	493.0$\pm$10.1[b]	7.9 $\pm$ 0.5[b]	33.1 $\pm$ 2.5[ac]	285.0 $\pm$ 20.29[b]

Means followed by different letters in the same column are different by Tukey test. (p<0.05)

the nerve cells through the activity of the NADH-diaphorase (NADH-d) enzyme (Gabella 1969). To do so, the duodenums were washed and filled with Krebs solution, without stretching the organ. Then, they were immersed in a Triton X-100 solution, and washed in Krebs solution. The segments were immersed in a broth containing NADH and Nitro Blue Tetrazolium (NBT) for 45 min. The reaction was interrupted with buffered formol. Later on, they were micro-dissected under a stereomicroscope in order to obtain whole mounts of the muscular tunica. They were then dehydrated, cleared and set up between lamina and cover glass. The NADH-d reaction product was seen as a blue/purple coloration of different shades.

The quantitative analysis the NADH-d myenteric neurons was performed at the intermediate region (60° - 120°; 240° - 300°) of the intestinal circumference, considering the mesenteric insertion as 0°. The count was made under a light microscope (Leica DM RX), with a 40X objective, and all neurons of each field were counted in a total of forty randomly microscopic fields. The field area was 0.224 mm^2, in a total of 8.96 mm^2. We also measured the cellular body area of the NADH-d neurons. The images were taken by a high resolution camera, transferred to a personal computer, and recorded in a compact disc. We used the Image Pro Plus 3.01 software to measure the area (μm^2) of 100 neurons per segment in a total of 500 cellular bodies per group.

The data obtained were analyzed statistically employing the variance analysis. We used the test of Tukey for the quantitative data and the test t of Student for the morphometric data. The significance level was 5%. The results were expressed as mean (M) ± standard error (SE). (n = number of rats).

Results

The neurons stained by the activity of the NADH-d enzyme were gathered, forming ganglia with different shapes, disposed transversally around the intestinal wall, parallel to each other. Their cellular bodies had several sizes, an eccentric nucleus, and the majority did not present evident nucleolus. We did not observe evident differences in the neurons and ganglia morphology in the three experiment groups under light microscopy (Fig. 1).

The initial and final body weights of the animals in the three studied groups are shown in Table 1. Rats from
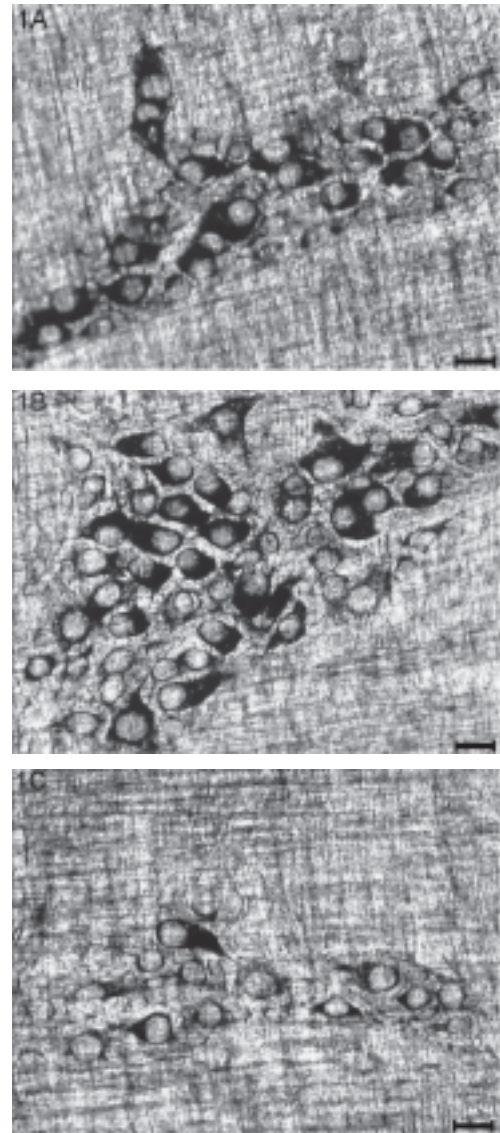

FIGURE 1. Membrane whole-mount of the intermediate region of the duodenum of rats. NADH-d positive diaphorase myenteric neurons. Bars calibrations: 20μm. Control (A) ascorbic acid treated diabetics (B) and diabetics (C). Calibration bar= 20μm.

group D and DA were hyperglycemic. There were no difference in the glycated hemoglobin between group D and DA (p > 0.05) (Table 1). The AA supplementation reduced the glycated hemoglobin in group DA. However, there were no significant differences (p >0.05) regarding the glycated hemoglobin between the groups of diabetic rats (Table 1).

The plasma level of ascorbic acid had a reduction of 48.73% in animals from group D, when compared to group C (p > 0.05). The supplementation raised the ascorbic acid level in 61.9% in DA when compared to D (p <0.05) and in 25.7% in DA when compared to C (p>0.05) (Table 1).

Figure 2 shows the density of NADH-d myenteric neurons in 8.96 mm^2 observed in the duodenum. The neuronal density of group C was 790.2 ± 45.53. The neuronal density for groups D and DA was 321.61 ± 39.56 and 1251± 69.45, respectively. The number of NADH-d neurons was reduced significantly in group D (p <0.05) when compared to C. We noticed an increase on the number of NADH-d neurons in animals of group DA, when compared to group D (p <0.05). The neuronal density in DA was higher than that found in C (p <0.05).
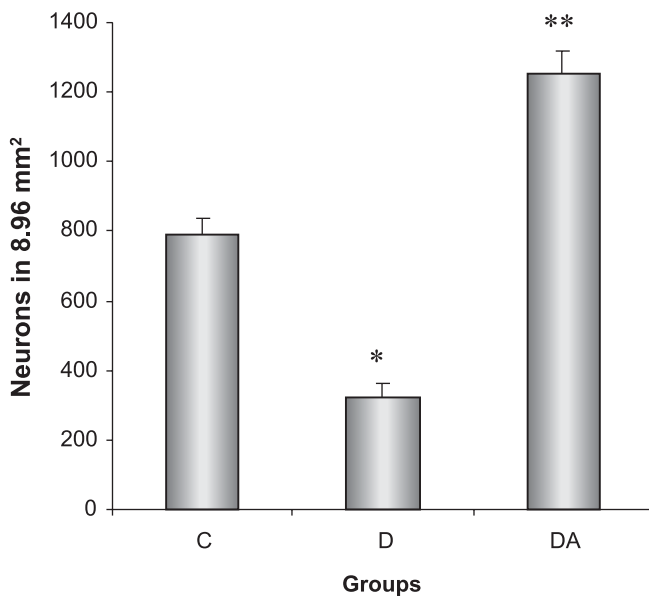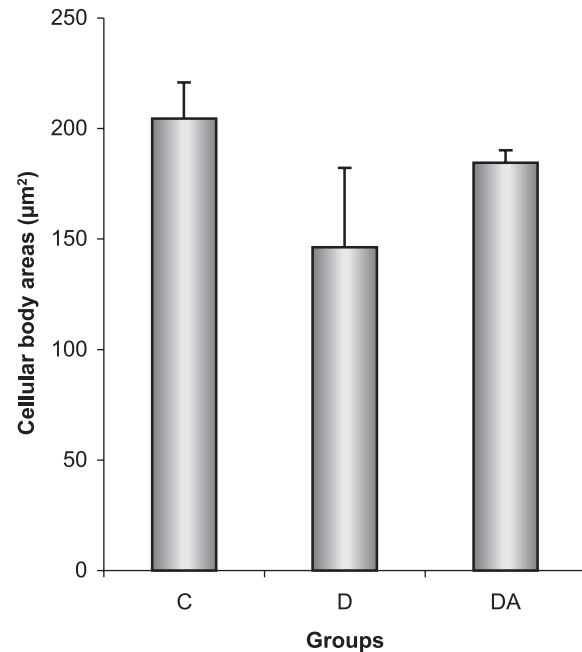

FIGURE 2. NADH-d stained myenteric neurons in 8.96 mm^2 observed in the duodenum of rats from groups: control (C), diabetic (D) and diabetic treated with ascorbic acid (DA). All results were expressed as means ± SE. n=5 rats *per* group.
 * p < 0.05 when compared to group C
** p < 0.05 when compared to group D.

Figure 3 shows the means of cellular body areas of neurons from the three experiment groups. There was no significant difference between the means of the cellular body areas of NADH-d neurons when comparing neurons from groups C and D (p>0.05) and the neurons from groups D and DA.

FIGURE 3. Cellular body areas of NADH-d myenteric neurons. Groups: control (C), diabetic (D) and diabetic treated with ascorbic acid. All results were expressed as means ± SE. p > 0.05 when all groups were compared.

Discussion

When examining the membrane whole mounts at the duodenum intermediate area, we observed that the myenteric plexus neurons are located between the circular and longitudinal stratum of the duodenal muscle tunica, forming ganglia with different shapes, with a predominance of the elongated ones and parallel to one another. This arrangement and position is not different from those already described in the literature for the duodenum of rats in different conditions of experimental treatment (Natali and Miranda Neto, 1996; Buttow *et al.*, 1997). It is also in accordance with Matsuo's observations (1934) in the duodenum of guinea pigs and with Karaosmanoglu *et al.* (1996) in the small intestine of guinea pigs. All authors used the membrane whole mounts stained by different techniques.

The diabetic rats showed a significant reduction on the blood levels of ascorbic acid when compared to the

controls. These data were similar to those described by Cunningham (1998) in humans with diabetes mellitus. He inferred the possibility of the ascorbic acid transportation, as well as, its renal absorption were inhibited in this disease. Besides, it has also been suggested that the largest exposition of those suffering from diabetes to the oxidative stress may contribute for the reduction on the ascorbic acid concentration (Young *et al*., 1992).

The neuronal density in our control was 790.296 neurons in an area of 8.96mm^2. When converting this result to 1 cm^2, we obtained 8.819 neurons which was similar to that obtained by Johnson *et al.* (1998) 9.940 neurons/cm^2, when quantifying the myenteric neurons on the small intestine of rats. They used the same technique used in our experiment.

The NADH-d technique allows the staining of neurons that had a higher activity of the enzyme, thus enabling us to evaluate if there has been an increase or reduction of the metabolism through the number of stained neurons. We agree with Furlan *et al*. (2002) and Miranda Neto *et al*. (2001) when they state that, in experiments involving comparison between animal groups submitted to different experimental conditions, it is necessary to be rigorous with the incubation period. This is true because a long exposure would allow that even low metabolism neurons to form enough amounts of formazan granules to be stained. In order to reduce this possible error in the experiment results, we used exactly the same incubation period for the three groups, and for all duodenum segments.

If we compare the neuronal density of the control to those found by Pereira *et al*. (2003) in the ileum and Buttow *et al*. (1997) in the duodenum in their controls – both stained by the Giemsa technique – it becomes evident that the NADH-d stains a sub-population of myenteric neurons while the Giemsa technique stains the overall number of neurons, since the final coloration results from the affinity of methyl blue by the polyribosomes. Smaller numbers of NADH-d stained neurons were also found in membrane whole mounts by Sant'Ana *et al.* (1997) in the colon, Miranda Neto *et al.* (2001) in the ileum and Molinari *et al*. (2002) in the stomach when compared to neurons stained by the Giemsa technique. Natali *et al*. (2003) observed the overall population of neurons (21757 neuron/cm^2) in the duodenum of rats aged 210 days stained by the Giemsa method. When we performed the relative proportion between the neurons of the controls (group C) and those verified by Natali *et al*. (2003), we obtained a proportion of 59.46% NADHd positive neurons.

Animals from group D had their neuronal density reduced in 59.30% when compared to the control. Our data are in accordance with several authors that also described changes in neurons from the myenteric plexus, which had their size decreased in several segments of the large and small intestine of diabetic rats (Romano *et al*., 1996; Zanoni *et al*., 1997; Hernandes *et al*., 2000; Fregonesi *et al*., 2001; Furlan *et al*., 2002; Zanoni *et al*., 2003).

The reduction in the number of NADHd stained neurons in the duodenum may be linked to neuronal death evidenced by the reduction of the diaphoresis activity. A possible factor could be the increase of the oxidative stress associated to the diabetes. The oxidative stress results from the additional creation of oxygen reactive species arising from several sources (Bains and Shaw, 1997). Among these sources we have the increase of non-enzymatic glycosilation, increase of autoxidation, increase of the metabolic stress, and alterations in the stages of sorbitol formation, with a consequent rise of its intracellular concentration (Baynes, 1991). The ascorbic acid supplementation increases the antioxidant defenses necessary to fight the oxidative events, preserving the neurons and protecting them against the lipid peroxidation (Li *et al*., 2003).

The ascorbic acid supplementation increased in 74.29% the number of NADHd stained neurons in group DA, when compared to group D. Group DA presented a higher number of neurons when compared to group C. These data lead us to believe that the ascorbic acid supplementation may have induced an increase on the activity of neuron sub-population, besides avoiding a reduction on their number.

The protection afforded by the ascorbic acid on the NADHd stained neurons of supplemented diabetic animals is supported by studies (Cotter *et al*., 1995; Garg and Bansal, 2000; Li *et al*., 2003). These studies state the benefits of the ascorbic acid therapy in eliminating free radicals, increasing the vitamin E levels, decreasing the levels of lipid and plasmatic peroxidation, besides increasing the activity of the glutathione peroxidase, with a consequent prevention of nervous dysfunction.

The area increase of neurons in DA, although not significant when compared to group D, can be attributed to an increase in the synthesis activity of NADH-d positive neurons. Further researches are necessary to clarify the neurotrophic action of the ascorbic acid on neurons.

This work allows us to conclude that the ascorbic acid supplementation has a neuroprotector effect on the population of NADH-d- positive neurons of dia-

betic rats, when compared to the non-supplemented diabetic animals.

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