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Effect of the Ascorbic Acid Treatment on the NADHd-Positive Myenteric Neurons of Diabetic Rats Proximal Colon

Jacqueline Nelisis Zanoni*, Renata Virginia Fernandes Pereira and Priscila de Freitas
Departamento de Ciências Morfofisiológicas; Universidade Estadual de Maringá; jnzanoni@uem.br; Av. Colombo, 5790; 87020-900; Maringá - PR - Brasil

ABSTRACT

The aim of this work was to study the effect of the ascorbic acid on the myenteric neurons of diabetic rats proximal colon. Fifteen rats (90 days old) were divided into three groups: control, untreated diabetic and treated diabetic with ascorbic acid (DA). After 120 days of daily treatment with ascorbic acid, the intestinal segments were submitted to the NADH-diaphorase (NADHd) histochemistry technique to expose the myenteric neurons. The group DA showed a higher neuronal density (33.4 %) when compared to the untreated diabetic animals ($p < 0.05$). Cellular body area of neurons was significantly larger in group DA (17.3 %) when compared to the untreated diabetics ($p < 0.05$). It could be concluded that the ascorbic acid promoted a neuroprotective effect on the NADHd myenteric neurons of the proximal colon of diabetic rats.

Key words: Ascorbic acid, colon, diabetes, myenteric neurons

INTRODUCTION

Diabetes mellitus is a syndrome characterized by chronic hyperglycaemia, with disorders in carbohydrates, lipids, and proteins metabolism. There are several changes caused by the diabetes mellitus, such as the neuropathies (Afzaal et al., 2002). The neuronal damage due to diabetes mellitus has been attributed mainly to sorbitol - a polyol synthesized from the glucose reduction by the aldose reductase (Vinson et al., 1989) due to the increase of the glucose concentration in the neurons cytoplasm. The sorbitol is transformed into fructose and they both, in high concentrations, cause an increase of intracellular osmolality, with edema formation, neuronal lesion and a consequent reduction of the nervous conduction speed (Silva and Teixeira, 1999).

The free radicals are also responsible for provoking neuronal damage due to the increase of the oxidative stress (Kuyvenhoven and Meinders, 1999). Free radicals are energetically unstable molecules, capable to react with other biological substances, unchaining a series of reactions of electrons transference (redox) (Kuyvenhoven and Meinders, 1999). Therefore, they are involved in reactions that can cause irreversible damages to the cells such as loss of cellular function and death by necrosis or apoptosis (Imai and Nakagawa, 2003). The amount of free radicals formed in diabetes mellitus is larger due to an increase of the oxidative glycosylation and of the metabolic stress, as well as alterations in the stages of sorbitol formation and in the level of responsible mediators for unchaining inflammatory processes (Baynes, 1991).

* Author for correspondence

Due to the sorbitol action, free radicals and/or non-enzymatic glycosylation, the gastrointestinal tract neurons are heavily attacked in the diabetes mellitus, with a large number of degenerative alterations being observed (Monckton and Pehowich, 1980). The reduction in the number of neurons demonstrates that the diabetes mellitus causes the death of these cells. Besides, alterations had also been observed in its morphology, in cases of chronic diabetes, in different intestinal segments (Romano et al., 1996; Zanoni et al., 1997; Hernandes et al., 2000; Fregonesi et al., 2001; Zanoni et al., 2002; Zanoni et al., 2003).

The ascorbic acid is a substance that acts as antioxidant and also promotes the reduction on sorbitol plasmic concentration, inhibiting the aldose reductase (Vinson et al., 1989). This may play an important role in the treatment of neurological complications of diabetes mellitus, which explains the reason of studying this drug as a treatment of this neuropathology.

The aim of this work was to investigate the effect of the ascorbic acid supplementation in diabetic rats on the number and size of myenteric neurons NADH-diaphorase (NADHd) positive of the proximal colon of rats with diabetes mellitus.

MATERIALS AND METHODS

Treatment of rats

All the experiments were reviewed and approved by the State University of Maringá Animal Uses Committee. Fifteen male Wistar rats (*Rattus norvegicus*), 90 days of age, were used. The rats were divided into three groups: control, untreated diabetic and ascorbic acid treated diabetic (DA). Diabetes was induced through the administration of 35 $mg \cdot kg^{-1}$ of corporal weight of endovenous streptozotocin (Sigma, St. Louis, MO, USA), freshly dissolved in 10 $mmol \cdot l^{-1}$ citrate buffer (pH 4.5), after a fourteen-hour fast. The diabetes was confirmed by the presence of glycosuria and weight loss.

Ascorbic acid (Sigma, St. Louis, MO, USA) was added daily to the animal's water from DA group for four months (1 $g \cdot l^{-1}$) (Young et al., 1992). The daily average of water intake was 133.6 ml. The animals were kept in individual metabolic cages in a room with a maintained photoperiod (6:00 a.m. - 6:00 p.m.) and room temperature (RT) (24ºC ± 2ºC). Water was given *ad libitum* and Nuvital® lab chow served as the diet. At the 210th day of age, the rats were anaesthetized with thiopental (40 $mg \cdot kg^{-1}$) and sacrificed. Blood was collected by heart puncture for measuring the glucose (Bergmeyer and Bernet, 1974), glycated hemoglobin (Koenig et al., 1976) and ascorbic acid (Henry et al., 1980).

Histochemical technique for NADHd (Gabella, 1969)

After a laparotomy, segments from the proximal colon of each animal were collected, washed, and filled with Krebs solution without stretching the organ. Then, they were immersed for five minutes in a Triton X-100 0.3 % solution (Sigma, St. Louis, MO, USA) and then they were washed again in Krebs. The segments were immerged in a medium containing β-NADH (Sigma, St. Louis, MO, USA) and Nitro Blue Tetrazolium (Sigma, St. Louis, MO, USA) for 45 minutes. The reaction was interrupted with buffered formol. Later on, they were micro dissected under stereomicroscopy in order to obtain whole mount preparations of the muscular tunica. They were then dehydrated, diaphanized and mounted between lamina and cover glass. The NADHd product reaction appears as a blue/purple coloration of different shades.

Quantitative and morphometric analysis of myenteric neurons

Number of cells: The quantitative analysis was performed at the intermediate region (60° - 120°; 240° - 300°) of the intestinal circumference, considering the mesenteric insertion as 0° (Zanoni et al., 2005). The myenteric neurons were counted on an Olympus BX-40 microscope under 40 X lenses. Sixty-three microscopic fields were counted in each intestinal segment. The area of each field was 0.224 mm^2. The number of quantified fields was previously determined, in accordance with the calculated variation coefficient.

Area of the cellular profile: In order to perform the morphometric analyses, the images were taken with the high-resolution camera, transmitted to a personal computer, and recorded into a compact disc. The area (μm^2) of 100 cell bodies from each animal's colon was measured through an image-analysis software (Image-Pro-Plus 3.0.1), in a total of 500 neurons/group. The micrographs were prepared using the Adobe Photoshop 5.5 Software.

Statistical analysis

Comparisons between groups were made using one-way ANOVA. All the quantitative results underwent the Tukey test, and the morphometric data underwent the test t-Student. The security level significance was 5 %. The results were expressed as mean (M) ± standard error (SE). (n = number of rats).

RESULTS

All the diabetic rats used in the study were severely hyperglycaemic (Table 1). No differences were observed in the glycate hemoglobin level between the diabetic animals and those from group DA ($p > 0.05$) (Table 1). The plasmic level of ascorbic acid decreased in the untreated diabetic animals when compared to the controls ($p < 0.05$). The supplementation increased the levels of ascorbic acid by 25.7 % in group DA compared to the controls ($p > 0.05$) and 61.9 % compared to the untreated diabetic animals ($p < 0.05$) (Table 1).

Table 1 - Glycaemia (GLI), glycated hemoglobin (GHb) and ascorbic acid (AA) for animals from groups: control (C), untreated diabetic (D) and AA-treated diabetic (DA). All results are shown as M ± SE. n = 5 rats per group.

	GLI/mg·dl^{-1}	**GHb/%**	**AA/µg·ml^{-1}**
C	129 ± 3.9 [a]	4.1 ± 0.3 [a]	24.58 ± 5.5 [a]
D	466.4 ± 24.6 [b]	8.1 ± 0.2 [b]	12.6 ± 1.9 [ab]
DA	493.0 ± 10.1 [b]	7.9 ± 0.5 [b]	33.1 ± 2.5 [ac]

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

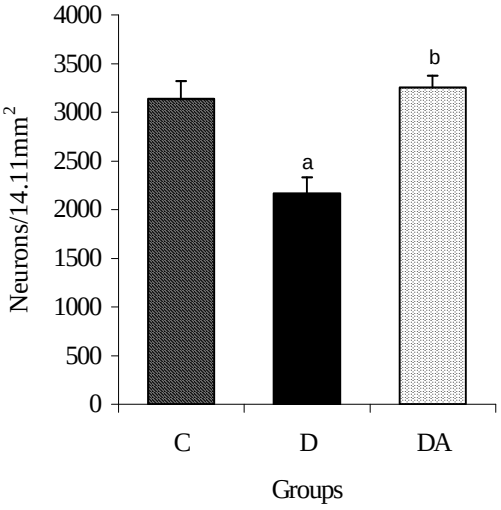

Figure 1 - Number of NADHd-positive myenteric neurons in 14.11 mm^2 found in the proximal colon of animals from groups: control (C), untreated diabetic (D) and diabetic treated with ascorbic acid (DA). n = 5 animals per group.
[a] $p < 0.05$ when compared to the group C
[b] $p < 0.05$ when compared to the group D

Quantification results for NADHd-positive myenteric neurons of proximal colon

We observed a reduction in the neuronal density in the diabetic animals when compared to the controls ($p < 0.05$). On the other hand, on the AA-supplemented animals (group DA) the number of neurons was 33.4 % larger ($p < 0.05$) when compared to the diabetics. The data regarding the neuronal quantification are shown on Fig. 1.

Morphometric results of the proximal colon

No significant difference was observed when comparing the neuronal profile results between the control and diabetic animals groups ($p > 0.05$). On the other hand, an increase in the cellular profile of the AA-supplemented diabetic animals (17.3 %) was observed when compared to the diabetic animals ($p < 0.05$) (Fig. 2). Representative micrographs of NADHd-positive neurons of proximal colon in myenteric plexus whole mount from all the animal groups studied are shown in Fig. 3.

DISCUSSION

The glycated hemoglobin results showed that both the diabetic animals and ascorbic acid-treated diabetics (DA) were diabetic during the whole experiment. The daily medium ingestion of ascorbic acid by animals from group DA was 133.6 ± 5.4 mg. The plasmatic level of ascorbic acid suffered a reduction in the diabetic animals, when compared to the controls. It was likely that this reduction happened due to the oxidative stress, which promoted an increase on the free radicals frequency and a decrease on the substances responsible for fighting them, the antioxidants (Young et al., 1992). The tissue concentrations of ascorbic acid also reduced because its transport was inhibited during hyperglycaemia, as well as its renal reabsorption (Cunningham, 1998). The ascorbic acid supplementation proved to be effective since it increased the levels of ascorbic acid in animals from group DA in 61.9 % when compared to the diabetic ones.

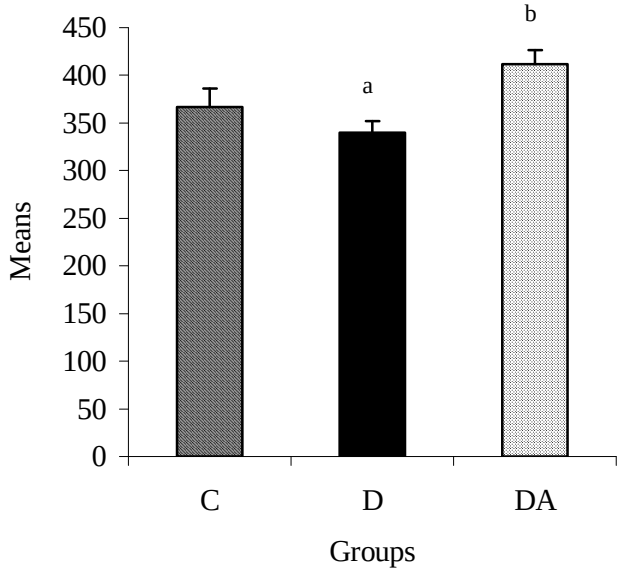

Figure 2 - Means of areas of NADHd-positive cell bodies observed in the proximal colon of animals from groups: control (C), untreated diabetic (D) and diabetic treated with ascorbic acid (DA). n = 5 animals per group.
[a] $p > 0.05$ when compared to the group C
[b] $p < 0.05$ when compared to the group D

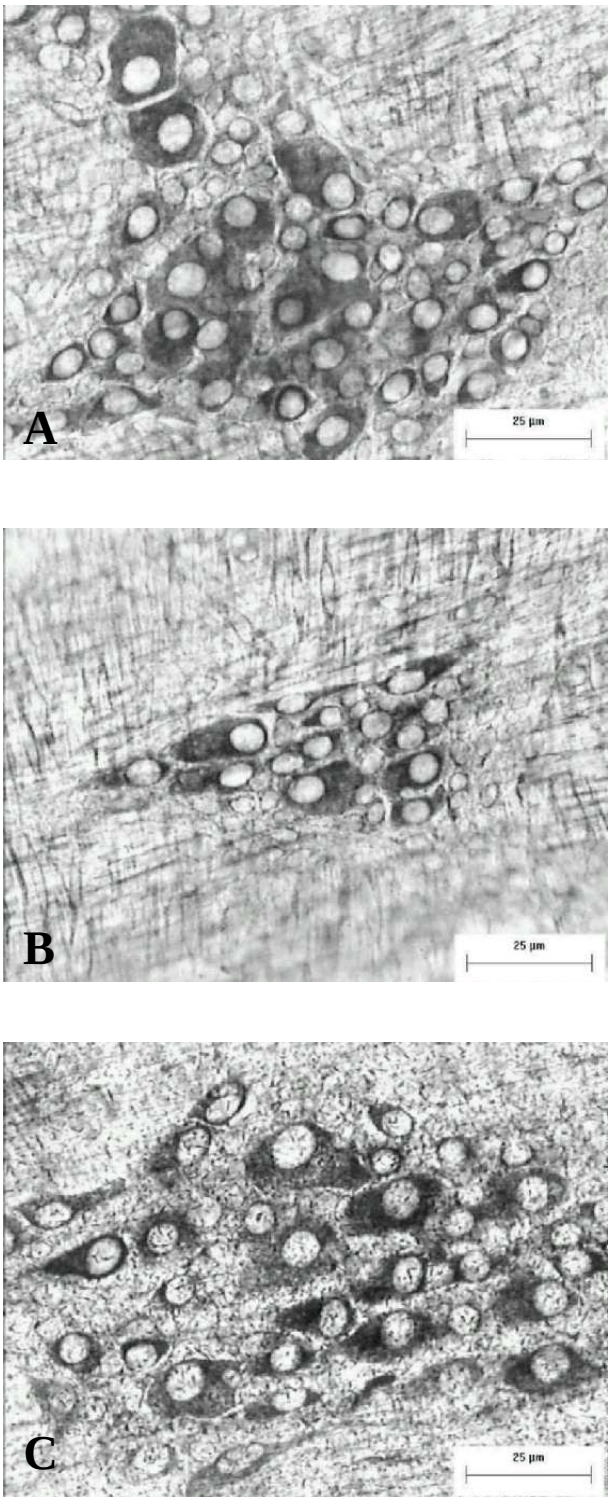

Figure 3 - NADHd-positive myenteric neurons of the proximal colon in ganglia of myenteric plexus of the: control rats (A), untreated diabetic rats (B) and diabetic rats treated with ascorbic acid (C). Calibration bar: 25 µm.

According to Miranda-Neto et al. (2005) the technique of neuronal evidenciation by the activity of the NADH-diaphorase enzyme stains those neurons of greater respiratory activity and not

necessarily the whole neuronal population. When the same staining conditions were provided, the reduction in the population of NADH-diaphorase positive in an experimental group, relative to its control, was suggestive of an intense metabolic decrease or even of the occurrence of cell death in part of this population. A reduction of 30.9 % in the neuronal density in the proximal colon of group diabetic was observed when compared to the control. Other authors have also observed a reduction on the number of neurons in the small intestine (Hernandes et al., 2000), in the proximal colon (Romano et al., 1996) and in the cecum (Zanoni et al., 1997) after long periods of diabetes. The oxidative stress may lead to neuronal death in a variety of circumstances. It happens when the equilibrium between the oxidative events and the antioxidant defences is unbalanced. This occurs due to a decrease of reducer agents or due to an increase on the oxidative species (Bains and Shaw, 1997). The oxidative stress increase is due to the increase of the non-enzymatic glycosylation, alterations in the stages of sorbitol formation (consequently elevation of its concentration), and also to the frequent inflammatory responses present in diabetes (Baynes, 1991). Bearing this in mind, the supplementation with antioxidant agents in the diabetes mellitus may be of extreme importance, since high levels of free radicals, sorbitol and the increase of non-enzymatic glycosylation are responsible for the neuropathy development in the diabetics. The reduction in the number of NADHd-positive neurons in the proximal colon of diabetic rats could be related to the neuronal death and/or to the reduction of the diaphorasis activity.

The ascorbic acid supplementation increased in 33.4 % the density of the NADHd-positive neurons in the proximal colon of group DA when compared to group D. Apparently, the supplementation with this antioxidant had a positive effect on them. The values found in the proximal colon for group DA were even larger than those observed for the control group. This was evidence that besides having avoided the death of this neuronal sub-population, the ascorbic acid supplementation also promoted an increase on the neuronal activity. Zanoni et al. (2003) when used the myosin-V technique for staining the total population of myenteric neurons, did not observe significant differences between the number of neurons when comparing the groups of diabetic animals and the ascorbic acid supplemented. On the other hand, Zanoni et al. (2002) and Zanoni et al. (2003) reported that the ascorbic acid supplementation was positive on the VIP-ergic submucous and nitrergic neurons (NADPH-diaphorase histochemistry technique) of the ileum of diabetic rats, respectively.

The ascorbic acid supplementation is beneficial for diabetics since, according to Garg and Bansal (2000), it reduces the levels of lipidic and plasmatic peroxidation, regulates the plasmatic levels of vitamin C and increases the levels of vitamin E, besides increasing the glutathione peroxidase activity. The ascorbic acid supplementation presents little effect over the glycaemia concentration (Som et al., 1981; Young et al., 1992). However, it reduces the capillary fragility and also the cellular sorbitol concentration (Yue et al., 1989; Cunningham et al., 1994; Will and Byers, 1996; Cunningham, 1998), thus, suggesting a neuroprotective effect of this substance. Vinson et al. (1989) observed a 44.5 % reduction on the sorbitol concentration in erythrocytes of diabetes patients, when supplemented with 2 g of ascorbic acid during three weeks. When using a dose of 100 - 600 mg of ascorbic acid, Cunningham et al. (1994) found similar results. Cotter et al. (1995) studied the efficiency of naturals oxidants such as the ascorbic acid, vitamin E and β carotene not only on the prevention of decreasing blood irrigation but also in the nervous conduction in induced-streptozotocin diabetes mellitus rats. They verified a reduction of 19.1 % in the speed conduction of the sciatic nerve one month after the diabetes onset. Cotter et al. (1995) observed that the maximal protection using ascorbic acid was 36 %.

The areas of the NADHd-positive neurons from group DA were larger than those observed in the group of untreated diabetic animals. The increase in the area of these neurons may be a reflex of the increase in the neuronal activity, which may have forced the cell to increase its synthesis processes.

In conclusion, the supplementation with ascorbic acid showed a neuroprotective effect on the NADHd-positive myenteric neurons of the proximal colon of diabetic rats.

RESUMO

O efeito do ácido ascórbico sobre neurônios mioentéricos do colo proximal de ratos diabéticos foi avaliado. Quinze ratos com 90 dias foram

divididos nos grupos: controle, diabéticos e diabéticos tratados com ácido ascórbico (DA). Após 120 dias de tratamento diário com ácido ascórbico os segmentos intestinais foram submetidos à técnica histoquímica NADH-diaforase (NADHd). A densidade neuronal, em uma área de 14,11 mm^2 para cada segmento, e o perfil do corpo celular de neurônios (500 neurônios/grupo) foram avaliados. O grupo DA apresentou maior densidade neuronal (33.4 %) em relação aos animais diabéticos ($p < 0.05$). Da mesma forma, a área do corpo celular dos neurônios foi significativamente maior no grupo DA (17.3 %) quando comparado com o grupo diabético ($p < 0.05$). Concluímos que o acido ascórbico apresentou um efeito neuroprotetor sobre os neurônios mioentéricos NADHd do colo proximal de ratos diabéticos.

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