Effects of ascorbic acid supplementation in ileum myenteric neurons of streptozotocin-induced diabetic rats¹

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The exacerbation of the oxidative stress and of the polyol pathway which impair damage myenteric plexus are metabolic characteristics of diabetes. The ascorbic acid (AA) is an antioxidant and an aldose reductase inhibitor, which may act as neuroprotector. The effects of AA supplementation on the density and cellular body profile area (CP) of myenteric neurons in STZ-induced diabetes in rats were assessed. Four groups with five animals each were formed: normoglycemic (C); diabetic (D); AA-treated diabetic (DS) and AA-treated normoglycemic (CS). Dosagen of 50mg of AA were given, three times a week, for each animal (group DS and CS). Ninety days later and after euthanasia, the ileum was collected and processed for the NADPH-diaphorase technique. There were no differences (P>0.05) in the neuronal density among the groups. The CP area was lower (P<0.05) in the DS and CS groups, with a higher incidence of neurons with a CP area exceeding 200μm² for groups C and D. The AA had no influence on the neuronal density in the ileum but had a neuroprotective effect, preventing the increase in the CP area and allowing a higher number of neurons with a CP area with less than 200μm².

INDEX TERMS: Vitamin C, diabetes, intestine, myenteric plexus.
Systemic nitric oxide (NO) is produced by nitric oxide synthase during the transformation of L-arginine into L-citrulline. Once formed, the NO takes part in the relaxation or inhibition of the smooth muscle contraction, thus, acting in the regulation of gastrointestinal motility (Kurjak et al. 2001).

The relationship between hyperglycemia and diabetic neuropathy has already been demonstrated in several studies and, among the theories proposed to its etiology, the oxidative stress stands out (Giuliano et al. 1996, Afzaal et al. 2002). The oxidative stress occurs when there is an imbalance between the production of oxidants and the body’s defense system leading to the increase of molecules reactive to oxygen (free radicals) within the cells (Parthiban et al. 1995, Obrosova et al. 2002). Biological systems have non-enzymatic and enzymatic mechanisms which form the first line of defense against the reactive oxygen species (Rigo & Guterres 2002). In the diabetes neuropathy and some other complications, the oxidative stress is intensified by the reduction in the levels of enzymes participating in the antioxidant defense system (Parthiban et al. 1995, Obrosova et al. 2002) as well as by the reduction in the antioxidants levels such as ascorbic acid (AA), glutathione and vitamin E (Young et al. 1992, Lee & Chung 1999).

The NOS enzyme stains neurons through the reduction of nitro-blue tetrazolium in the presence of beta-nicotinamide adenine dinucleotide phosphate reduced (NADPH-reduced), indicating that the nadh-diaphorase histochemistry can be used as a NO marker (Santer 1994).

Quantitative changes in the nitrergic neurons from the myenteric plexus and a reduction in the expression and activity of NOS have been reported in diabetic animals (Wrzos et al. 1997, Spangéus et al. 2000, Watkins et al. 2000, Surendran & Kondapaka 2005), reinforcing the findings of the impairment of the myenteric plexus in diabetic autonomic neuropathy.

Multiple therapeutic strategies have shown that the treatment with antioxidants may prevent or reverse peripheral nerve dysfunction in STZ-induced diabetic rats (Cameron et al. 1993, Cameron & Cotter 1999). The AA supplementation reduces the capillary fragility and the sorbitol cellular concentration (Cunningham 1998), which suggests a neuroprotective role for this substance.

Changes in neurons of the myenteric plexus have been described in different conditions and experimental models which have tried to elucidate the mechanisms that lead to functional impairment of the digestive tube (Fregonesi et al. 2001, Sant’Ana et al. 2001, Furlan et al. 2002, Molinari et al. 2002, Zanoni et al. 2003, Fontes et al. 2004, Alves et al. 2006, Araújo et al. 2006, Silverio et al. 2008, Silva et al. 2008).

Diabetes mellitus (DM) is a syndrome of multiple etiologies characterized by chronic hyperglycemia with changes in the metabolism of carbohydrates, lipids and proteins leading to, among other complications, peripheral and autonomic neuropathies. Among the consequences of the diabetic neuropathy there are reports of stomach hypotonia and dilation with a slow gastric emptying (Block et al. 2002), intestinal involvement with episodes of diarrhea with night exacerbation followed by fecal incontinence alternating with episodes of constipation (Watkins et al. 2000). In part, the gastrointestinal disorders associated with DM are correlated to changes in the quantity and size of myenteric neurons (Diane et al. 1979).

There are sub-populations of myenteric neurons that respond differently to DM. Some neurons degenerate, others suffer changes in their neurotransmitters without undergoing degeneration, while others are not affected (Chandrasekharan & Srinivasan 2007). Similarly, the neurons containing the same types of neurotransmitters, but innervate different regions of the digestive tube, are affected differently in DM.

The nitrergic neurons are among the neurons that have been extensively studied within the Enteric Nervous System. Nitric oxide (NO) is produced by nitric oxide synthase during the transformation of L-arginine into L-citrulline. Once formed, the NO takes part in the relaxation or inhibition of the smooth muscle contraction, thus, acting in the regulation of gastrointestinal motility (Kurjak et al. 2001).

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Multiple therapeutic strategies have shown that the treatment with antioxidants may prevent or reverse peripheral nerve dysfunction in STZ-induced diabetic rats (Cameron et al. 1993, Cameron & Cotter 1999). The AA supplementation reduces the capillary fragility and the sorbitol cellular concentration (Cunningham 1998), which suggests a neuroprotective role for this substance. Therefore, the aim of this research...was to observe possible effects of AA-treatment in NADPH-diaphorase myenteric neurons present in the ileum of chronic STZ-induced diabetic rats through quantitative and morphometric analysis.

MATERIALS AND METHODS

This study was done within the guidelines of the COBEO (Brazilian College for Animal Experimentation) and was approved by the CEPPEA (Institutional Committee for Ethics in Animal Experimentation) from Unipar.

Twenty male rats (Rattus norvegicus) of Wistar lineage with 3 months of age, supplied by the Central Vivarium of the University of São Paulo, were used. The animals were distributed in four groups with five animals each (n=5) as follows: Group D (STZ-induced diabetic); Group C (normoglycemic control); Group
DS (STZ-induced diabetic AA-treated), and Group CS (AA-treated normoglycemic control).

The animals were housed in separate polypropylene cages, at room temperature (24±2°C) and controlled photoperiod (12 hours dark/light cycle) with access to food and water ad libitum. During the 90-days experiment period, the animals were weighed once a week.

**Streptozotocin-induced diabetes and ascorbic acid supplementation (AA)**

After 14 hours fasting, each animal of groups D and DS received a single dose of streptozotocin (35mg/kg of body weight) dissolved in sodium citrate buffer 10mm, pH 4.5, through intravenous injection. The animals from Group C and CS received the same dose of 10mm sodium citrate buffer. One week after the induction and confirmation of diabetes onset (blood glucose exams), every animal from Group CS and DS received 50mg of AA (through gavage), three times a week, during 90 days.

**Obtaining intestinal segments**

At the end of the experimental period, after fasting for 12 hours, the animals were euthanatized with a lethal dose of anaesthetic (Thiopental Abbout® 40mg/kg) given by intravenous injection. Blood was collected through cardiac puncture in order to measure the glucose levels. A laparotomy was then carried out to retrieve the ileum.

**NADPH-diaphorase histochemical technique (Scherer-Singer et al. 1983)**

The collected ileum was washed and filled with phosphate buffer (PB pH 7.4), and after had their extremities tied with suture thread, were fixed in 4% paraformaldehyde (Merk, Darmstadt, Germany) prepared in 0.1M phosphate buffer (PB; pH 7.4) for 30 minutes, immersed in 0.3% Triton X-100 (Sigma, St. Louis, USA) dissolved in phosphate-buffered saline (PBS, pH 7.4) for 10 minutes and then washed ten times (10 minutes each) in PBS. Subsequently, ileum was incubated in a broth containing 50mg of Nitro Blue Tetrazolium (Sigma®), 50mg of b-NADPH (Sigma®) and 0.3% Triton X-100 in buffered Tris-HCl drain plug (0.1M pH 6.0).

The histochemical reaction was controlled visually with the aid of stereomicroscope and lasted for 100 minutes. Then, the ileum was opened by cutting to suture extremities, washed three times in PBS for 5 minutes and then immersed in 4% paraformaldehyde solution for setting and storage.

**Obtaining the membrane whole-mounts**

The ileum was sectioned throughout the extension of the mesenteric edge and microdissected in a glass plate under the stereomicroscope with transillumination to remove the mucosal and e submucosal layers, preserving the muscular and serosal layers. Each membrane whole-mount was dehydrated in an ascending series of alcohols (90-100%) and clarified by three consecutive immersions in xylol, then placed between glass blade with Permoun® resin. The membrane whole-mount preparations were used for the quantification and morphometric analyses of the cellular body profile area of myenteric neurons.

**Quantification of NADPH-diaphorase positive neurons**

The ileum membrane whole-mount was visualized by light microscope in order to perform the neuronal quantification area (mm²). The image seen in the microscope was captured by a high resolution digital camera and transferred to a computer. The neurons were quantified by means of a system test adapted to each membrane whole-mount consisting of 60 fields. Based on the width and length of the membrane whole-mount preparations, the 60 fields were distributed as ten columns with six lines each, sampling all regions (mesenteric, intermediate and anti-mesenteric).

**Measurement of the cell body profile (CP) area of NADPH-diaphorase positive neurons**

The measurement of the CP area of NADPH-diaphorase positive (NADPH-dp) myenteric neurons was carried through image-analyses software (Image-Pro-Plus 3.0.1). The images of the neurons captured for digital chamber for the quantification of the neuronal density had been used. The CP (in mm²) of all the neurons quantified previously in the membrane whole-mount preparations of each animal for group had been measured. The neurons had been grouped by class intervals of 100im².

**Statistical analysis**

All the results were expressed as mean ± standard error. The data from different groups were compared by the Tukey’s test. The adopted significance level for all groups was P<0.05.

**RESULTS**

The glucose blood concentration of animals in groups D and DS were higher (P<0.05) than those found for the animals in Group C and CS (Table 1). There were no differences (P>0.05) in blood glucose between animals of Group D and DS (Table 1).

Throughout the trial period (90 days), the animals in all groups showed an increase in their body weight (Table 1). Weight gain was lower for animals in Group D and DS when compared to animals in Group C and CS, but the difference between them were not significant (P>0.05).

The light microscopy analysis of the ileum membrane whole-mounts showed no changes in the myenteric plexus organization and arrangement among the groups. The

<table>
<thead>
<tr>
<th>Groups</th>
<th>Initial glucose (mg dl⁻¹)</th>
<th>Final glucose (mg dl⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (n = 5)</td>
<td>101.3± ± 5.26</td>
<td>101.3± ± 3.54</td>
</tr>
<tr>
<td>CS (n = 5)</td>
<td>97.25± ± 4.73</td>
<td>95± ± 2.85</td>
</tr>
<tr>
<td>D (n = 5)</td>
<td>387± ± 16.97</td>
<td>446.5± ± 5.33</td>
</tr>
<tr>
<td>DS (n = 5)</td>
<td>395.5± ± 4.62</td>
<td>436.5± ± 1.37</td>
</tr>
</tbody>
</table>

Means followed by different letters in the same line and column are different (P<0.05) by the Tukey’s test.
NADPH-dp myenteric neurons were gathered predominantly in ganglia interconnected by nerve fibers bundles (Fig.1A) and distributed homogeneously between the mesenteric, intermediate and antimesenteric regions of the ileum circumference. Isolated neurons, especially in the nerve fibers path, were also found (Fig.1B). Neurons inside the ganglia had a scattered arrangement, closer to the ganglia peripheral region (Fig.1C,D,E,F).

The density of NADPH-dp neurons present in 13.33 mm² of ileum (area equivalent to 60 fields viewed under light microscopy with a 40x lens) did not differ (P>0.05) among the studied groups (Table 2).

The neuron PC area ranged 73.1-761.50 µm² in Group D, 64.49-837.31 µm² in Group C, 87.09-641.81 µm² for Group DS, and 61.47-449.93 µm² in Group CS.

The neuron PC mean area measured in groups D (289.8±5.5 µm²) and C (289.7±5.62 µm²) were equivalent, but differed (P<0.05) from the neurons PC area in the ileum of groups DS (253.1±4.22 µm²) and CS (210.1±3.14 µm²) (Table 2). Treatment with AA reduced the neurons PC area.

Table 2. Means and standard error (SE) of: the NADPH-diaphorase stained neurons present in 13.33 mm² of ileum whole-mount preparations and respective cellular profile area (CP) from animals in group C (control-normoglycemic), CS (AA-treated control normoglycemic), D (STZ-induced diabetes) and DS (STZ-induced diabetes and AA-treated)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Neuron density/13.33 mm²</th>
<th>CP area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (n = 5)</td>
<td>97.8 ± 4.54</td>
<td>289.7 ± 5.62</td>
</tr>
<tr>
<td>CS (n = 5)</td>
<td>100.8 ± 5.4</td>
<td>210.1 ± 3.14</td>
</tr>
<tr>
<td>D (n = 5)</td>
<td>94.2 ± 5.44</td>
<td>289.8 ± 5.5</td>
</tr>
<tr>
<td>DS (n = 5)</td>
<td>104.2 ± 8.75</td>
<td>253.1 ± 4.22</td>
</tr>
</tbody>
</table>

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

Table 3. Relative frequency of NADPH-diaphorase neurons positives of groups C (control-normoglycemic), D (STZ-induced diabetes), CS (control normoglycemic AA-treated) and SD (STZ-induced diabetes and AA-treated) according to class intervals of cell body profile (PC) area (µm²)

<table>
<thead>
<tr>
<th>PC (µm²)</th>
<th>Relative frequency of neurons (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group C (n=5)</td>
</tr>
<tr>
<td>&lt;100</td>
<td>1</td>
</tr>
<tr>
<td>100-200</td>
<td>23</td>
</tr>
<tr>
<td>200-300</td>
<td>39</td>
</tr>
<tr>
<td>300-400</td>
<td>21</td>
</tr>
<tr>
<td>400-500</td>
<td>10</td>
</tr>
<tr>
<td>&gt;500</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
</tr>
</tbody>
</table>
of Group DS and CS, 12.7% and 27.5% when compared to Group C and D, respectively.

An increase in the number of neurons with a PC area lower to less than 200μm² in the AA-treated group (41% for Group DS and 52% for Group CS) was verified when compared to Group C (24%) and D (19%); Group C and D showed a relative frequency of neurons with a PC area higher than 200μm² (Table 3). Thus, the AA-supplementation for diabetic and non-diabetic animals changed the incidence of neurons with a PC area higher to 200μm² (C=76%; D=81%; SD=69% and CS=48%).

DISCUSSION

The STZ is a substance that induces the experimental diabetes and has been used to assess the behavior of neurons in the enteric nervous system of several gastrointestinal segments (Young et al. 1992, Büttow et al. 1997, Wroz et al. 1997, Zanoni et al. 2003, Clebis et al. 2004, Fregonesi et al. 2005). In order to be effective in the diabetogenic activity it should be given in doses higher than 25mg/kg of body weight (Junod et al. 1969).

The initial and final hyperglycemias in animals from Group C and DS (Table 1) showed blood glucose levels higher (P>0.05) than those in Group C and CS, confirming that the animals that received the STZ (35mg/kg) remained diabetic throughout the trial period (90 days).

Similar results of the diabetes onset induced by STZ were obtained by Zanoni et al. (2003), but with a resulting hyperglycemia higher than that observed in Group D and DS, probably due to the length of the trial period which was lower for Group D and DS (90 days).

The AA-supplementation of STZ-induced diabetic animals (Group DS) did not cause a significant decrease in the glucose concentration as also observed by Young et al. (1992).

The animals’ body weight were not different (P>0.05) among Group C (295±15.81g), D (283.3±11.24g), DS (314±12.25g) and CS (301.5±2.05g) at the beginning of the experiment (90 days). However, during the experiment, the animals from Group C and CS gained more weight (40% and 17%, respectively). However, when compared to each other (Group DS), the average weight gain was lower for Group D and DS (90 days).

Enhanced changes in the neuronal density can be better observed when a generalized neuronal population is stained regardless of their neurochemical phenotype. As described by Miranda-Neto et al. (2001).

In the long run, it is known that the metabolic changes taking place in the diabetes are responsible for chronic-degenerative diseases complications that lead to vascular and nerve impairment (Crawford & Cotran 1996, Squarzoni et al. 2007). Changes in the myenteric plexus are included in diabetic autonomic neuropathy (Araújo, 1996), whose etiopathology has not been clarified yet (Celis et al. 2003). The polyol pathway activity, among others, can be changed due to direct or indirect the intensification of the oxidative stress mediated by the hyperglycemia (Davison et al. 2002, Feldman 2003). In diabetes, the AA administration, an antioxidant substance and aldose reductase inhibitor [a fundamental enzyme to the polyol pathway] (Levine 1996, Will & Byers 1996) is used in an attempt to minimize or prevent the deleterious consequences arising from diabetes. Thus, to verify the effects of AA-supplementation in STZ-induced diabetes in rats, the nitricergic neurons (NADPH-dp neurons) of the ileum myenteric plexus were measured and quantified.

There were no significant differences in the 13.33mm² ileum total area of animals from Group C, D, DS and CS (P>0.05) among the density of NADPH-dp neurons (Table 2) as well as the NOS-stained neurons observed by Wroz et al. (1997). A decrease in the number of myenteric neurons in STZ-induced diabetes in rats was observed in the stomach (Fregonesi et al. 2001, Clebis et al. 2004), in the duodenum (Romano et al. 1996, Buttw et al. 1997, Furlan et al. 1999), in the ileum (Zanoni et al. 2003, Alves et al. 2006), in the small intestine as a whole (Hernandes et al. 2000), in the cecum (Zanoni et al. 2007), in the proximal colon (Furlan et al. 2002). We can justify the controversial results for the NADPH-dp neurons in Group D, C, CS and DS when compared to other reports: the diabetes maintenance period as well as the biochemical phenotype of the stained neurons was not similar. Enhanced changes in the neuronal density can be better observed when a generalized neuronal population is stained regardless of their neurochemical phenotype, as occurs, for example, with the use techniques such as the cuproline blue (Phillips et al. 2004), myosin V (Zanoni et al. 2003), NADH-diaphorase (Sant'Ana et al. 2001), Young et al. 1993, Molinari et al. 2002, Clebis et al. 2004, Zanoni et al. 2007, Silva et al. 2008), methylene blue (Hernandes et al. 2000), among others, which stain the whole neuronal population, including the nitricergic neurons. When analyzing specific populations, such as NADPH-dp neurons, more specific results were obtained. Vinson et al. (1989) reported that not all types of myenteric neurons are affected...
simultaneously with the same intensity and extent, since the diabetes causes selective changes in the myenteric plexus (LePard 2005). The deleterious processes that lead to neuronal loss do so according to the differentiated neurochemical phenotype and neurons location. Nitricergic neurons are more resistant to the consequences arising from diabetes, as well as neurons in the duodenum are impaired earlier than colon neurons towards aboral-anal. In that order, the ileum precedes the colon; however, it is more resistant than the duodenum and jejunum.

On average, the neurons PC area of Group D (289.8±5.5 lm²) and C (289.7±5.62 lm²) were equivalent. However, the AA treatment significantly reduced (P<0.05) the PC area of groups DS (253.1±4.22 lm²) and CS (210.1±3.14 lm²). The PC areas in Group DS and CS were 12.7% and 27.5% lower when compared to Group D and C, respectively (Table 2). A similar effect of the AA treatment on the PC area of diabetic animals is reported in the jejunum (Silverio et al. 2008) and in the ileum of rats that remained diabetic for 120 days (Zanoni et al. 2003).

There was an increase in the incidence of neurons with a PC area lower to 200 lm² in Group DS and CS (31% and 52%, respectively) and a decrease in the incidence of neurons with a PC area exceeding 200 lm² compared to the other groups (C=76%, D=81%, SD=69%, and CS=48%). Zanoni et al. (2003) and Silverio et al. (2008) have also found a decreased frequency of neurons in the PC area exceeding 200 lm² and an increased incidence of neurons with a PC area lower less than to 200 lm² in the ileum and jejunum of myenteric plexus of STZ-induced diabetic rats AA-treated, although with not with the same percentage.

The results suggest that, although the STZ-induced diabetes has not changed significantly the mean PC area when compared to Group C and D, the AA supplementation had a protective effect in diabetic neurons (Group DS) as well as in the non-diabetic with a higher intensity in the last one (Group CS) since the PC area in those groups was lower. Zanoni et al. (2003) mentioned that the AA treatment may reduce the NOS expression. This, together with the inhibition of the aldose-reductase, results in the reduction of the NADPH-dp neurons PC area of the rat’s ileum. As the treatment with AA proved to be effective in reducing the PC area in the diabetic animals (DS) and non-diabetic (CS), with an even more pronounced decrease in the non-diabetic animals (CS), the results reinforce the AA neuroprotective function over the diabetes development, and more intensely, over aging. Also is suggestive that the intensity of diabetes effects on the NADPH-dp neurons in the myenteric plexus is proportional to the diabetes time and the animal aging.

CONCLUSIONS

The results obtained in the stipulated conditions of this work allow the following conclusions regarding the myenteric plexus in the ileum of rats:

The intramural organization of the myenteric plexus and the number of NADPH-dp myenteric neurons in the ileum were neither altered by the AA supplementation nor by STZ-induced diabetes, suggesting a greater resistance of these neurons to the deleterious processes resulting from the STZ-induced diabetes;

The diabetes did not alter the mean PC area of NADPH-dp myenteric neurons, but the AA supplementation worked as a neuroprotector preventing the increase in neurons PC in the diabetic and non diabetic animals AA supplemented;

The AA supplementation was responsible for a higher number of NADPH-dp neurons with a PC area lower to less than 200 lm², with a more intense effect on the non diabetic rats, followed by the diabetic ones.

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REFERENCES


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