ISSN- 0102-9010

EVALUATION OF THE NITRERGIC MYENTERIC NEURONS IN THE DISTAL COLON OF DIABETIC RATS TREATED WITH ACETYL-L-CARNITINE

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ABSTRACT

In this work, we evaluated the effect of acetyl-L-carnitine supplementation on the presence of NADPH-diaphorase positive myenteric neurons in the distal colon of rats with diabetes mellitus induced by streptozotocin. Rats 105 days old were divided into four groups: normoglycemic, normoglycemic supplemented with acetyl-L-carnitine, diabetic and diabetic supplemented with acetyl-L-carnitine. Diabetes was induced by the administration of streptozotocin (35 mg/kg, i.v.). Supplementation with acetyl-L-carnitine was done for 105 days. The neuronal density was similar in all groups. In diabetic rats, the area of the neuronal cell body profile was greater ($p<0.05$) than in normoglycemic rats, whereas in diabetic rats receiving acetyl-L-carnitine the areas were smaller than in the non-supplemented diabetic rats ($p<0.05$). The increase in the colonic area of diabetic rats was greater than in diabetic rats treated with acetyl-L-carnitine ($p<0.05$), indicating that the increment in the population of these neurons was higher in treated diabetic rats. These results suggest that the beneficial effect of carnitine is restricted to preventing an excessive increase in neuronal area. The greater dilatation of the distal colon seen in diabetic rats supplemented with acetyl-L-carnitine probably represents an adverse effect of this compound.

Key words: Acetyl-L-carnitine, diabetes mellitus, distal colon, myenteric neurons, NADPH-diaphorase

INTRODUCTION

Diabetes mellitus (DM) is a high-prevalence disease [29]. With the discovery of insulin in 1921 [39], and its therapeutic use in 1922 [18], the life expectancy of diabetic patients improved significantly because of a reduction in acute complications, especially cetoacidosis [11]. Although additional advances in the diagnosis and treatment of DM have prolonged the lifespan of these patients, ironically they continue to be affected by late chronic-degenerative complications [11,18], includind macroangiopathies, microangiopathies (retinopathy and nephropathy) and neuropathies [8], all of which have been increasing in prevalence [13] and are frequently observed 10 - 15 years after the beginning of the disease [8].

Although diabetic neuropathies are extremely common and represent one of the major complications of DM, the mechanisms producing the nerve lesion still remain poorly understood and investigated [2].

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Several hypotheses have been proposed to explain the physiopathological mechanisms that trigger the neuropathies, and include hyperactivity of the polyol pathway, non-enzymatic glycation of structural proteins, impairment in the action of trophic and nerve growth factors [2,34,36], vascular changes and endoneural hypoxia [34,36,42], immune mechanisms [31], oxidative stress [2,17,34] and lipid metabolism [2,28,34,36], particularly alterations in the levels of carnitine [16,20,22,27,38].

The onset of diabetic gastrointestinal autonomic neuropathy has been investigated by studying the behavior of myenteric plexus neurons in rats with streptozotocin-induced diabetes. In addition to decreased neuronal activity and the development of chromatolysis followed by degenerative changes [24], there is also a reduction in the density of neurons in the stomach [12], duodenum [5], ileum [19,41], cecum [40] and proximal colon [14,30], all of which indicate a variable response in the gastrointestinal tract [3].

In view of the association between decreased levels of serum and tissue carnitine and the pathogenesis of diabetic neuropathy, in this study, we examined the morphometric and quantitative alterations in NADPH-

diaphorase (NADPH-d) positive neurons of the myenteric plexus in the distal colon of rats with streptozotocin-induced diabetes and assessed whether supplementation with acetyl-L-carnitine (ALC) could prevent the changes.

MATERIAL AND METHODS

The experimental protocols used in this study are in accordance with the ethical principles in animal research followed by the Brazilian College of Animals Experimentation (COBEA).

Male Wistar rats (*Rattus norvegicus*) 105 days old were randomly allocated to four groups of five animals each: normoglycemic (C), normoglycemic supplemented with ALC (CC), diabetic (D) and diabetic supplemented with ALC (DC). The rats in groups D and DC were fasted for 14 h prior to the induction of diabetes with streptozotocin dissolved in citrate buffer, pH 4.5 (35 mg/kg, i.v.; Sigma, Chemical Co., St. Louis, MO, USA). The rats were housed in individual cages in a room with a controlled photoperiod (12 h light/dark cycle) and temperature (24 ± 2°C), and received balanced Nuvital® chow and water *ad libitum*. The rats in groups C and D were given tap water alone, while those in groups CC and DC received water containing acetyl-L-carnitine (Spfarma, São Paulo, Brazil) at a dose of 200 mg/kg body weight, prepared daily and supplied in opaque graduated flasks. The volume of water ingested by the rats was monitored daily, and the body weight was determined every 15 days. The amount of carnitine to be added to the water was calculated every two weeks based on the mean volume of water ingested in this period and on the weight of the rats on the day of the calculation. In each experimental group, the heaviest rat and that drinking the least water were used in the calculation to ensure that the rats ingested at least 200 mg of carnitine per kg of body weight. Since the diabetic rats (DC) drank more water (156.6 ± 8.54 ml/day; mean ± S.D.) than the controls (CC) (51.1 ± 1.87 ml/day), the two-week calculation of the amount of carnitine to be supplied was done separately for groups CC and DC.

At 210 days of age, the rats were fasted overnight and anesthetized with thiopental (40 mg/kg, i.p.) and underwent to laparotomy. Blood was collected by cardiac puncture to measure the levels of glucose and glycated hemoglobin, and the retroperitoneal and epididymal fat pads were removed. The distal colon was removed, washed in 0.01 M sodium phosphate buffer, pH 7.4, measured and weighed. The nose-to-tail length of the rats was also determined at this point. The intestinal segments were subsequently fixed for 30 min in 4% paraformaldehyde (Merck, Darmstadt, Germany) in 0.1 M PBS, pH 7.4, then immersed for 10 min in 0.3% Triton X-100® (Sigma) diluted in 0.01 M PBS, pH 7.4, and finally washed 10 times (10 min each) in 0.01 M PBS. Following this, the tissues were placed in incubation medium for 150 min to detect neuronal NADPH-d, according to Scherer-Singler *et al*. [32]. This medium contained 50 mg of nitro blue tetrazolium (NBT, Sigma), 100 mg of β-NADPH (Sigma) and 0.6 ml of 0.3% Triton X-100® in 200 ml of 0.1 M Tris-HCl, pH 7.6 (GibcoBRL, New York, USA). The reaction was interrupted by three 5 min washes in 0.01 M PBS, with the segments opened at both ends, followed by immersion in 4% paraformaldehyde solution in 0.1 M PBS.

After fixation, the distal colon was opened along the mesocolic margin and the circumference was measured in order to calculate the total area of the segments. Transverse sections about 1 cm wide were cut and microdissected to remove the mucosa and submucosa. This procedure yielded whole-mounts that were dehydrated, diaphanized and mounted on slides with a coverslip in synthetic resin (Permount, Fischer Chemical, USA). These transverse sections of the distal colon were divided into antimesocolic (120° to 240°), intermediate (60° to 120°; 240° to 300°) and mesocolic (0° to 60°; 300° to 360°) regions, with the mesocolic margin being 0° [23]. The myenteric neurons in the mesocolic and antimesocolic regions were counted in 80 random microscopic fields using a Leica DM RX light microscope with a 50X objective. The area of each field was 0.149 mm^2. The profiles of 500 neuronal cell bodies (250 from each of the mesocolic and antimesocolic regions) in each group were measured using a computerized image analyzer (ImagePro Plus 4.1) coupled to the microscope.

The mean and standard deviation of the profiles of the cell bodies of 500 neurons in the normoglycemic group (C) were used to classify the neurons of the remaining groups as small, medium and large. Medium neurons were those within the confidence interval of the mean, whereas neurons below and above this interval were classified as small and large, respectively.

The results were expressed as the mean ± S.D. and were compared statistically using Student's t-test or ANOVA followed by the Tukey test. A value of $p<0.05$ indicated significance.

RESULTS

Rats with streptozotocin-induced diabetes (groups D and DC) showed increased water ingestion, hyperglycemia and higher levels of glycated hemoglobin ($p<0.05$), in addition to a reduced body weight and smaller retroperitoneal and epididymal fat pads ($p<0.05$), compared to control (C and CC) rats; there were no alterations in body size compared to groups C and CC (Table 1). Except for the water ingestion and fasting glucose levels, which were lower ($p<0.05$) in group DC rats compared to group D rats, and for the retroperitoneal fat pad, which was reduced ($p<0.05$) in group CC rats compared to group C, the other parameters did not vary significantly among the four groups of rats.

Table 2 shows that there was a marked increase ($p<0.05$) in the total area of the distal colon in groups D and DC when compared to group C (53% and 84.6%, respectively). The total area of the distal colon in group DC was 20.6% greater than in group D rats.

The neurons in a total of 160 microscopic fields (total area of 23.84 mm^2 per rat) were counted. The mean number of neurons was 894 ± 115.1 in group C, 1.017 ± 96.65 in group CC, 777 ± 115.1 in group D, and 781 ± 60.75 in group DC. The differences in the neuronal density between group C and the other groups and between groups D and DC were not

Table 1. Initial body weight, final body weight, nose-to-tail length, epididymal fat, retroperitoneal fat, daily water ingestion, glycemia and glycated hemoglobin in rats studied.

Parameters	Groups			
	C	CC	D	DC
IBW (g)	362 ± 39.9[a]	328.4 ± 14.1[a]	363.6 ± 7.7[a]	356.2 ± 19.2[a]
FBW (g)	472 ± 53.3[a]	452.6 ± 29.2[a]	311.8 ± 15.6[b]	343.8 ± 36[b]
NTL (cm)	44.6 ± 2.19[a]	46.3 ± 1.2[a]	45.4 ± 0.82[a]	45.6 ± 0.96[a]
EF (g)	4.54 ± 0.38[a]	3.46 ± 1.15[a]	1.1 ± 0.44[b]	1.4 ± 0.51[b]
RF (g)	4.83 ± 1.31[a]	3.07 ± 0.59[b]	0.32 ± 0.33[c]	0.74 ± 0.84[c]
DWI (ml)	62.13 ± 1.72[a]	51.1 ± 1.87[a]	173.4 ± 8.94[b]	156.6 ± 8.54[c]
Gl ($mg.dl^{-1}$)	105.4 ± 25[a]	99.8 ± 13.1[a]	357.4 ± 40.3[b]	247 ± 56.5[c]
GHb (%)	3.9 ± 0.42[a]	3.9 ± 0.51[a]	6.6 ± 0.5[b]	6.7 ± 0.57[b]

The results are expressed as the mean ± standard deviation of 5 rats per group. Means followed by different letters in the same row are significantly different ($p<0.05$, Tukey test). **IBW**- initial body weight at 105 days of age, **FBW** - final body weight at 210 days of age, **NTL** - nose-to-tail length, **EF** - epididymal fat, **RF** - retroperitoneal fat, **DWI** - daily water ingestion, **Gl** - glycemia, **GHb** - glycated hemoglobin, **C** - normoglycemic, **CC** - normoglycemic supplemented with acetyl-L-carnitine, **D** - diabetic, **DC** - diabetic supplemented with acetyl-L-carnitine.

Table 2. Length, circumference and area of the distal colon of rats in the four groups studied.

Parameters	Groups			
	C	CC	D	DC
L (cm)	8.2 ± 0.44[a]	8.4 ± 0.54[a]	10.7 ± 10.7[b]	12.3 ± 12.3[c]
Ci (cm)	3.06 ± 0.13[a]	2.76 ± 0.16[b]	3.58 ± 0.23[c]	3.76 ± 0.05[c]
A (cm^2)	25.06 ± 0.98[a]	23.12 ± 0.75[a]	38.35 ± 5.13[b]	46.26 ± 3.89[c]

The results are expressed as the mean ± standard deviation of 5 rats per group. Means followed by different letters in the same row are significantly different ($p<0.05$, Tukey test). **L** - length, **Ci** - circumference, **A** - area, **C** - normoglycemic, **CC** - normoglycemic supplemented with acetyl-L-carnitine, **D** - diabetic, **DC** - diabetic supplemented with acetyl-L-carnitine.

significant. Table 3, shows that there was an increase ($p<0.05$) in the area of the neuronal cell bodies in diabetic rats, and that this increase was attenuated in DC rats (Fig. 1). Medium-size neurons predominated in groups C and CC whereas large neurons predominated in groups D and DC (Table 4); DC rats had fewer large and more medium-size neurons compared to group D rats.

Table 3. Area of the cellular profiles (500 neurons per group) of NADPH-diaphorase positive myenteric neurons in the distal colon of rats in the four groups studied.

Groups	Area (μm^2)
C	282.1 ± 81[a]
CC	244.6 ± 70[a]
D	463 ± 123[b]
DC	395.7 ± 107[c]

The results are expressed as the mean ± standard deviation of 5 rats per group. Means followed by different letters are significantly different ($p<0.05$, Tukey test). **C** - normoglycemic, **CC** - normoglycemic supplemented with acetyl-L-carnitine, **D** - diabetic, **DC** - diabetic supplemented with acetyl-L-carnitine.

DISCUSSION

The greater water ingestion, fasting glucose concentrations and glycated hemoglobin levels, as well as the lower body weight and decreased fat pads ($p<0.05$) relative to groups C and CC, confirmed the diabetic condition of groups D and DC. Group DC showed a reduced water ingestion (10%) and lower fasting glucose level (31%) relative to group D. This reduced glycemia is probably not relevant, since the values for glycated hemoglobin, a parameter that reflects the mean concentration of blood glucose to which erythrocytes are exposed [7], were similar in both groups. Our results agree with those of Sima *et al.* [35] who observed no significant changes in the body weight and glycated hemoglobin level in diabetic rats that received daily doses of 90-100 mg of ALC/kg administered through a catheter.

The length and circumference of the distal colon were greater in diabetic rats, and resulted in a significant increase in the total area of the distal colon in these animals. In diabetes, this alteration is related to an increase in the amount of food ingested [26] and to a reduction in the number of myenteric neurons

Table 4. Distribution of NADPH-diaphorase positive myenteric neurons according to their size (small, medium and large) in the distal colon of rats in the four groups studied.

Groups	Size			Total
	S	M	L	
C	67 (13.4)	352 (70.4)	81 (16.2)	500 (100)
CC	148 (29.6)	322 (64.4)	30 (6.0)	500 (100)
D	0 (0.0)	104 (20.8)	396 (79.2)	500 (100)
DC	5 (1.0)	216 (43.2)	279 (55.8)	500 (100)

The values indicate the number of neurons of each size in five rats from each of the experimental groups. The percentages are shown in parentheses. **S** - small (≤ 200.8 μm^2), **M** - medium (200.9 – 363.3 μm^2), **L** - large (≥ 363.4 μm^2), **C** - normoglycemic, **CC** - normoglycemic supplemented with acetyl-L-carnitine, **D** - diabetic, **DC** - diabetic supplemented with acetyl-L-carnitine.

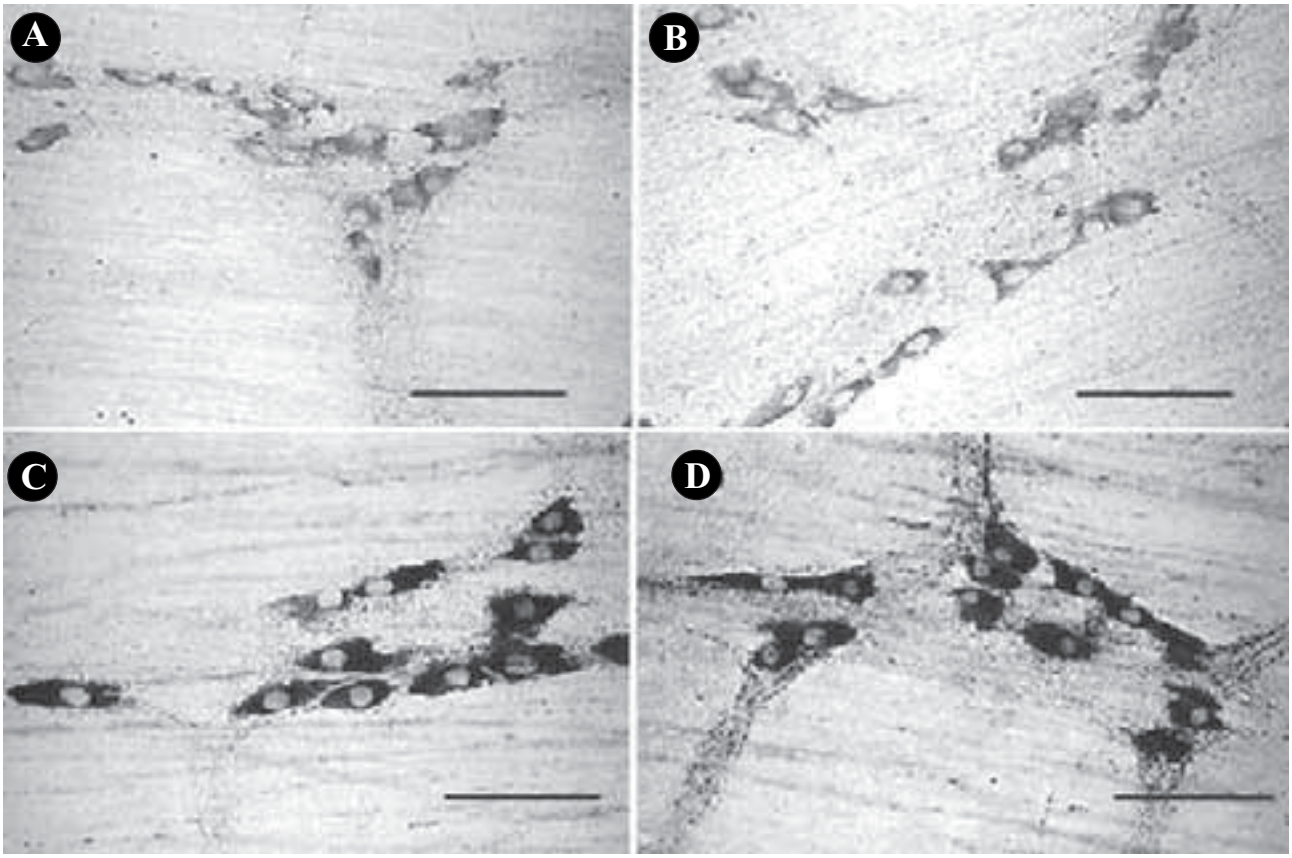

Figure 1. NADPH-diaphorase positive neurons in ganglia of the myenteric plexus of the distal colon of rats, **A**) Normoglycemic, **B**) Normoglycemic supplemented with acetyl-L-carnitine, **C**) Diabetic, **D**) Diabetic supplemented with acetyl-L-carnitine. Bar = 100 μm.

that leads to hyperplastic and hypertrophic changes in the intestinal cells [33]. The increase in the total area of the distal colon in group DC was 20.6% greater than in group D. This increase was related to a weakening of the colon wall, since tearing of the tissue was common during removal of the mucosa and submucosa, and this made it hard to prepare whole mounts.

Group CC had a greater neuronal density (13.8%) and a smaller colonic area (7.74%) relative to group D. Thus, an increase in the neuronal density related to the smaller dispersion of the neurons, and not necessarily to the effects of ALC, was to be expected. The rats in group D had a smaller neuronal density (13.1%) than those in group C, but an even greater decrease was expected since the area of the distal colon in the former animals was 53% greater. In group DC rats, the density of neurons was 12.6% lower than in group C, while the colonic area was 84.6% greater. These data indicate increases of 39.9% and 72% in

the population of neurons stained in groups D and DC, respectively.

The increase in the population of NADPH-d positive enteric neurons in the distal colon of diabetic rats could lead to a decrease in muscle tonus and an enlarged intestinal lumen since these neurons are inhibitory. Thus, the overall decrease in the myenteric neuronal population often seen in diabetic rats, as shown by Giemsa staining of the duodenum [5], ileum [19], proximal colon [14,30] and cecum [40], could result in an increase in the relative proportion of inhibitory (nitrergic) neurons. The enhanced nitric oxide (NO) production caused by this increase in the relative proportion of nitrergic neurons may lead to reduced cell functions or cell death, especially in cells not producing NO [25]. The NO produced by these neurons could adversely affect other neuronal types. According to Dawson [9], an excess of NO can be neurotoxic, probably through its interaction with superoxide anion to yield oxidant compounds such as peroxynitrite. Nitrergic neurons are probably more resistant to cell death because of a possible antioxidant defensive mechanism [4].

The area of nitrergic neurons decreased by 13.3% in group CC, and increased 64.1% in group D and 40.3% in group DC, when compared to group C, with a predominance of medium-size neurons in normoglycemic rats and large neurons in diabetic rats. Similar results were also reported by Zanoni *et al*. [41] in the ileum of rats studied under similar conditions to those used here. These authors observed an increase in the area of NADPH-d positive neurons in diabetic rats and showed that ascorbic acid prevented this increase in these animals.

Diabetic rats showed an increased neuronal density and a marked increase in the area of the cell bodies. In diabetic rats, ALC partially prevented this increase in neuronal area, with a 37% reduction in the neuronal area and a 30% reduction in the number of large neurons relative to group D; however, ALC did not prevent the increase in neuronal density. These observations suggest that other factors could contribute to the increase in distal colon area since this was greater in group DC despite the reduction in the area of nitrergic neurons. The increased population of nitrergic neurons seen here could be linked to an increase in other inhibitory neurotransmitters and/or to a decrease in excitatory neurotransmitters. In agreement with this, Defani *et al*. [10] observed an increase in the profile area of the cell bodies of VIPergic neurons in the submucous plexus of the jejunum of diabetic and non-diabetic rats after supplementation with ALC (200 mg/kg).

Since acetylcholine stimulates NO release from endothelial and neural cells in the stomach and duodenum of diabetic rats [1], and since carnitine can increase intracellular acetylcholine [21,38], the greater number of NADPH-d positive neurons seen in diabetic rats receiving ALC could be related to an increase in the acetylcholine concentration induced by carnitine.

Several authors have reported gastrointestinal complications such as diarrhea [6], nausea and vomiting [15] in humans treated with high doses of carnitine. Although some authors have used ALC dosages higher than that used here (300 mg/kg) [27], others have observed benefits with lower doses of ALC (50 mg/kg), including normalization of the nerve conduction velocity [22,28,37], Na^+,K^+-ATPase activity [28,37] and myo-inositol concentration [22,27] in peripheral nerves of diabetic animals.

As shown here, diabetic rats treated with ALC had a more dilated colon, more fragile colon walls, a smaller cell body area for NADPH-d neurons and a greater proportion of these neurons than diabetic rats that did not receive ALC.

These results suggest that the beneficial action of ALC is restricted to preventing an excessive increase in the neuronal areas. The greater dilatation of the colon in diabetic rats receiving ALC may represent a side effect of the dose of ALC used in these animals (this effect was not seen in rats receiving only ALC; CC). Thus, caution is required in the use of ALC since its protective effect on enteric neurons of the digestive system may be attenuated by undesirable excessive dilatation of the colon.

ACKNOWLEDGMENTS

This work was supported by CAPES, Universidade Estadual Paulista (Presidente Prudente) and the Post-graduate Program in Cell Biology of the Universidade Estadual de Maringá.

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Received: February 17, 2004
Accepted: May 14, 2004