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MORPHOQUANTITATIVE EFFECTS OF ACUTE DIABETES ON THE MYENTERIC NEURONS OF THE PROXIMAL COLON OF ADULT RATS

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ABSTRACT - The effects of acute diabetes on the density and size of the myenteric neurons of the proximal colon of adult rats were investigated. The injection of streptozotocin was followed by a period of observation of seven days, during which the diabetic animals showed weight loss, excessive food and water intake, large urinary debt and hyperglicemia. The whole-mounts from the proximal colon were stained with the techniques of Giemsa and of the NADH-diaphorase, and the employment of these techniques made it possible to verify a decrease on the neuronal density and on the cell body size of the myenteric neurons in the colon of the diabetic rats. These observations were discussed in terms of the pathophysiology of the diabetes and the experimental protocol.

KEY WORDS: acute diabetes, myenteric neurons, proximal colon.

Efeitos morfoquantitativos do diabetes agudo sobre os neurônios mioentéricos do colo proximal de ratos adultos

RESUMO - Foram investigados os efeitos do diabetes agudo sobre a densidade e o tamanho dos neurônios mioentéricos do colo proximal de ratos adultos. À injeção de estreptozootocina seguiu-se um período de observação de sete dias, durante os quais os animais diabéticos apresentaram perda de peso, ingestão excessiva de alimento e água, grande débito urinário e hiperglicemia. Os preparados de membrana do colo proximal foram corados pelas técnicas de Giemsa e da NADH-diaforase. A aplicação dessas técnicas permitiu constatar uma redução da densidade neuronal e do tamanho do corpo celular dos neurônios mioentéricos no colo dos ratos diabéticos. Essas observações foram discutidas em termos da patofisiologia do diabetes e do protocolo experimental.

PALAVRAS-CHAVE: diabetes agudo, neurônios mioentéricos, colo proximal.

The research on the changes induced by experimental diabetes on the several tissues and organs of laboratory animals is quite large. Among those systems under investigation are the gastrointestinal tract and its intrinsic enteric nervous system, the neuronal network responsible for the control of the activities of the bowel. It is reported, for instance, that the myenteric neurons of the stomach, duodenum and cecum are numerically reduced in diabetes[1-3], and that specific neurochemical groups show response patterns to diabetes which depend on the intestinal segment and the duration of the diabetic state[4-7]. These neuronal changes, as well as those associated to the autonomic innervation of the gut[8-10], stand among the responsible by the clinical gastrointestinal symptoms of diabetes[11-13].

Recently, we described an increase in the NADH-diaphorase positive myenteric neuronal population in the duodenum of rats subjected to acute diabetes, although the total number of neurons was not affected[14]. Aiming at evaluating the response of the neurons of the proximal colon to this same condition, we subjected adult rats to streptozotocin-induced diabetes for a period of seven days. The number of neurons stained with Giemsa and NADH-diaphorase (NADH-diaphorase) was assessed, and the neuronal sizes in the proximal colon were measured as well.

METHOD

Male Wistar rats (*Rattus norvegicus*) aging seven months were used. These were divided at random in two groups, control and diabetic. After overnight fast, the animals of the diabetic group received a single i.v. injection of streptozotocin (35 mg/kg body weight) dissolved in citrate buffer, pH 4.5. The rats from the control group received only vehicle. All the animals were then transferred to indi-

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vidual metabolic cages, where they were kept during seven days with daily supply of ration (NUVILAB®) and water *ad libitum*. The cages were maintained under controlled conditions of temperature (22°C) and light/dark cycles (12/12 hr) for the whole experimental period. Food and water intake and urinary debt of each animal were recorded daily.

On the day before killing, the rats were weighted and subjected to overnight fast. The purpose of this fasting was to reduce the volume of the intestinal material so as to ease cleaning of the collected segments and return the colon to its resting dimensions.

The animals were killed by neck dislocation[15,16]. Blood samples were collected for the evaluation of blood glucose. The colon was removed, washed, measured and weighted. Washing and distension were made with the appropriate solution and ligature of the extremities. Those segments destined to Giemsa staining were washed and filled with acetic formaldehyde and then processed according to the description[17]. The segments selected to the technique of the NADH-d were washed and filled with Krebs solution, pH 7.3, and treated as described[18]. Times were set as follows: the samples were washed twice (10 min each) in Krebs, kept in 0.3% Triton X-100 for 5 min, again washed twice in Krebs and transferred to incubation medium, were they remained for 45 min, as established in the protocol of our laboratory. Reaction was interrupted by immersion in 10% buffered formalin.

The whole-mounts were made under stereomicroscope. Only the mucosa and the submucosa were completely dissected out so as to avoid removal of myenteric neurons, adhered to the circular smooth muscle. Next the whole-mounts were mounted in slide according to standard histological procedures.

Neurons were counted under a BX40 microscope with 40X objective. Eighty microscopic fields (17.68 mm^2), equally distributed on the intermediate and antimesocolic regions of the proximal colon circumference, had their neurons counted. Half-seen neurons were counted in alternate fields. The counts were carried out in the whole-mounts stained with Giemsa and those stained with the NADH-d.

The Giemsa-stained myenteric neurons were measured under microscope with 40X objective coupled to a computerized image analyzer (ImagePro Plus). Due to the proximity of the cell bodies in the myenteric ganglia, the cell body and nucleus profiles were drawn manually. The areas of 65 neurons and their nuclei of each circumferential region in each whole-mount were measured, yielding 520 neurons per group.

The results were statistically analyzed by the test *t* of Student with significance level of 5%. The values presented are the mean ± SEM of each set of data and the number of data considered is indicated in each case.

RESULTS

During the days the rats were in the cages, the diabetic animals exhibited more irritability than the controls, making their handling difficult. None of the animals of any group died during this period or was discarded because of any alterations. Also, control animals injected with vehicle had no changes, when compared to non-injected rats (data not shown). Table 1 presents the parameters recorded during the one-week period; the means of food and water intake and urinary debt are presented by 100 g of mean body weight to make comparisons easier. Table 2 shows the measures of the colon obtained in both groups.

Table 1. Physiological features of the control and diabetic rats.

	Controls (n=8)	Diabetics (n=8)
Initial body weight (g)	451.40±7.60	429.70±6.79
Final body weight (g)	448.40±12.06*	360.50±7.54*
Ingested food/100 g body weight (g)	6.22±0.29**	7.74±0.48**
Ingested water/100 g body weight (g)	10.40±0.67*	23.59±1.99*
Urinary debt/100 g body weight (ml)	1.32±0.16*	10.82±1.24*
Blood glucose (mg/dl)	125.00±3.49*	218.00±11.10*

*Values differ significantly between controls and diabetics ($p<0.01$)
**Values differ significantly between controls and diabetics ($p<0.05$)

Table 2. Measures of length, weight, circumference and area of the colon of control and diabetic rats.

	Controls (*n*=8)	Diabetics (*n*=8)
Length (cm)	16.33±1.57	14.49±0.61
Weight (g)	2.90±0.42	2.51±0.11
Circumference (cm)	2.60±0.15*	2.05±0.10*
Area (cm^2)	42.58±5.04*	29.91±2.27*

*Values differ significantly between controls and diabetics ($p<0.05$)

At the microscope, most of the myenteric neurons were found clustered in ganglia, with isolated neurons being scarce. Although the fibers of the plexus were not stained by either technique, the course of the thick primary connectives could be followed with relative ease in the whole-mounts stained with NADH-d. Most of the myenteric ganglia were polygonal and often fused with their neighbours or extended towards them. Their predominant orientation was parallel to the circular smooth muscle layer, but some had extensions in other directions, forming a patchy pattern.

The counts of the neurons stained with Giemsa and NADH-d in the intermediate and antimesocolic regions of the proximal colon of both groups are pre-

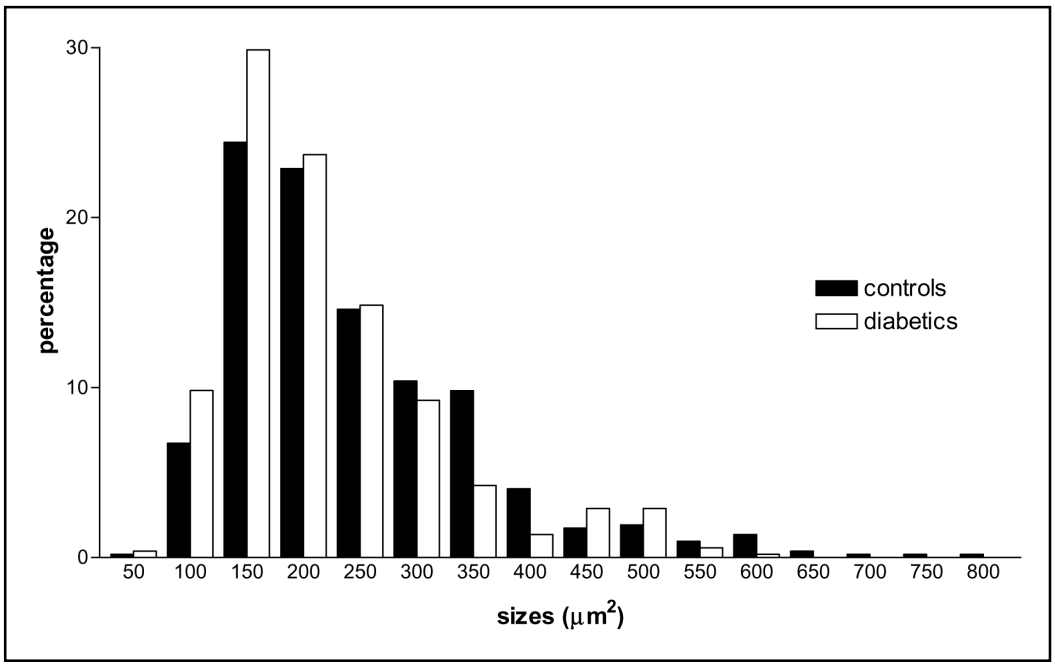

Fig 1. Percent distribution of the areas of cell body profiles of the myenteric neurons in the proximal colon of the control (n=520) and diabetic (n=520) rats.

sented in Table 3. The number of neurons stained with Giemsa was not different between the circumferential regions in either group. The same applied to the NADH-d positive myenteric neurons. However, with both techniques, there was a trend towards more neurons in the intermediate regions of the colon. This larger neuronal density could be visually observed in some whole-mounts.

There was no significant change on the neuronal density of the diabetic group relative to the control.

Figure 1 shows the percent distribution of the areas of the neuronal profiles in the studied groups. The general pattern for both groups was very similar, with the largest percentages between 100 and 250 μm^2. But it was observed that neurons with cell body profile above 600 μm^2 were found only in the control animals. Accordingly, the size range of the neurons of the diabetic animals was narrower than that of the controls (from 49.43 to 756.63 μm^2 in the control group and from 38.61 to 589.14 μm^2 in the diabetic group).

On average, the areas of the neuronal profiles of the diabetic rats were smaller than those of the controls (Table 4). The decrease was more pronounced in the total area of the cell body than on the area of the nucleus, so that the percentage of the total profile occupied by the nucleus had a significant increase.

DISCUSSION

The experimental diabetes induced by streptozotocin is currently the most used tool to evaluate the effects of diabetes mellitus under controlled conditions. Streptozotocin is a diabetogenic agent of quite stable action and dose-response relationship[19]; it allows the investigator to follow organic alterations for predetermined periods of time. In experiments carried out by our research group, the dose of 35 mg/kg body weight has had efficacy in the diabetes induction. It is also the minimal single dose capable of triggering a consistent diabetic state in the rat[19].

The diabetic rats of the present work showed features which are typical of the diabetic state: weight loss, excessive food and water intake, large urinary debt and hyperglicemia (Table 1). The impaired insulin secretion in these animals, caused by streptozotocin, increases lipolysis, proteolysis, glicogenolysis and neoglicogenesis, leading to above-normal blood glucose levels and reduced body mass[20]. Despite this abundant blood supply of nutrients, there is paradoxically a state of "cellular fast", once glucose uptake by most of the body cells is insulin-dependent. As a compensatory response, the diabetic animals eat more. However, there is a reduced food conversion, as increased food ingestion is not translated into increased body weight.

The water alterations are also related to the metabolic imbalances caused by the partial or total lack of insulin[20]. The hyperglicemia is accompanied by urinary loss of glucose, once the renal tubular mechanisms of glucose reabsorption get saturated with

Table 3. Number of neurons stained by the techniques of Giemsa and NADH-d in the antimesocolic and intermediate regions of the proximal colon of control and diabetic rats (n=4 for each technique per group).

		Giemsa	NADH-d
Controls	Antimesocolic	2240±159.70	1458±103.60
	Intermediate	2882±221.10	1475±15.59
	Total	5122±361.30	2933±88.05
Diabetics	Antimesocolic	2278±74.33	1471±138.10
	Intermediate	2316±33.77	1588±174.80
	Total	4594±107.50	3059±267.70

Table 4. Areas of cell body and nucleus profiles of the myenteric neurons of the proximal colon of control and diabetic rats.

	Controls (*n*=520)	Diabetics (*n*=520)
Total profile of the cell body (μm^2)	219.20±4.99*	193.60±4.32*
Nucleus profile (μm^2)	81.88±1.57**	76.77±1.31**
% of the profile occupied by the nucleus	39.89±0.46*	42.28±0.42*

*Values differ significantly between controls and diabetics ($p<0.001$).
**Values differ significantly between controls and diabetics ($p<0.05$).

the large glucose load filtered by the renal glomerulli. Being an osmotically active compound, the glucose which is filtered and not reabsorbed causes excretion of an increased amount of water, building up the urinary debt. To compensate for this loss of volume, neural mechanisms are put into action, which increase water intake. In this way, the most characteristic clinical and behavioural signs of diabetes mellitus were observed early in this work.

Although significantly high, the blood glucose level was inferior to that recorded in other investigations of our laboratory[2], and this deserves some consideration. Streptozotocin has as one of its distinctive features the fact that the dose has a good correlation with the severity of the resulting diabetes, regardless of this being assessed by blood glucose levels, plasma insulin content or pancreatic insulin content[19]. However, as our standard dose is always 35 mg/kg body weight, the possibility that different doses could have caused different degrees of severity (assessed by the blood glucose level) can be discarded. Other factors then could account for this variation. In those early studies, diabetes induction was made in rats aging 2.5 months and the animals were killed after several weeks, while the present work employed seven-month old rats that were kept diabetic for only a week. These differences could mean, for instance, differences in the sensitivity of the pancreatic β cells to the cytotoxic effects of streptozotocin, then less destruction of these cells and less pronounced decrease in insulin secretion. As far as we were able to investigate, there are no systematic data on the literature which can throw some light on this issue.

The seven-days period could also have been too short to allow a wide destruction of the β cells, so that a residual release of insulin could be present. Progressive morphological alterations were observed in the pancreatic islets of mice from 5 to 18 days after streptozotocin injection[21], and the plasma glucose levels also changed during this time, with the highest levels being observed after 10 days.

The rats of the control group did not gain weight during the experimental period. This can be an inherent feature of the animals at this age, but the physical restrain imposed by the size of the metabolic cages should be considered as a preclusion to the weight gain.

The dimensions of the colon of the diabetics were inferior to those of the controls, with circumference and total area reaching statistical significance (Table 2). This decrease can probably be explained by the smaller body weight of the diabetic animals, which was accompanied, although not proportionately, by

a diminishment of the internal organs: in percentual terms, the diabetics had a body weight loss of 16.10% relative to their initial weight, while the colon showed a weight decrease of 13.45% as compared to that of the controls. Smaller intestinal segments were frequently observed in our laboratory, specially in animals losing weight due to protein desnutrition[22,23]. Insulin deficiency is also an explanation for the reduced dimensions observed here, once insulin is an anabolic hormone and, as such, a promoter of cellular development. On the other hand, there are reports that rats subjected to experimental diabetes have dimensional and weight increases of the small and large intestines[2,4,6,7,12]. The absence of hypertrophy of the proximal colon in this study could be related to factors such as the duration of the experimental period, the age of the animals and the moderate diabetic state. Also, there must be myenteric neurons still functionally capable of maintaining the tonus (and hence the dimensions) of this intestinal segment.

The staining techniques employed here evidence larger numbers of neurons in the intermediate region of the proximal colon, in both groups. Although not attaining significance, these circumferential differences were observed in other instances[24], correlated to the fact that the intermediate region of the colon has a thicker layer of longitudinal smooth muscle, analogous to the human, which must require a larger neuronal population for its innervation and control[24]. This kind of regional variation was also observed in other segments of the bowel[25-29].

The Giemsa staining is easy to use and allows the visualization of the whole myenteric neuronal population in a given intestinal segment. The neuronal counts made with Giemsa yielded a neuronal density corresponding to 29,000 neurons/cm^2 of proximal colon, a value much similar to that found in other age-and weight-matched rats[28]. In younger rats, is was found a number of neurons/cm^2 larger than this[30], which can be related to the smaller intestinal area and correspondingly lesser neuronal dispersal. On the other hand, the technique of the NADH-d stains only a fraction of the myenteric neuronal population. Although there are reports that it stains almost all the myenteric neurons[27,31], this has not been the case in our research group[14,28]. In this study, the number of NADH-d positive neurons in the proximal colon was of 57-66% that found with Giemsa. As the dissection could have caused neuronal loss, all care was taken to avoid the removal of the muscular tunicas, to which the myenteric neurons are fixed. The incubation times adopted in these investigations are probably the most satisfactory explanation for the discrepancies; especially important are the periods during which the intestine is kept in the media containing the emulsifying agent and the substrates for the NADH-d activity, respectively. The longer are these periods – and they vary a lot from one study to another – the greater the possibility of staining cells of low enzymatic activity. To minimize these effects, the periods adopted in our laboratory in these media are those described in the section Methods. The neuronal counts with the NADH-d revealed a density of about 16,600 neurons/cm^2 of intestinal area.

The density of neurons in the proximal colon of the diabetics did not have significant changes. Nevertheless, when considering that the colon area was 30% smaller in this group, it would be expected that the neuronal density was 30% greater, because the smaller growth makes neuronal density larger. The Giemsa technique displayed 10.3% less neurons in the diabetics than in the controls, suggesting that there was a mean loss of 40.3% of the neurons. With the NADH-d, the neuronal density was 4.3% greater in the diabetics, and thus this neuronal subpopulation had a percentual loss of 25.7% relative to the controls. Alternatively, neuronal activity could only be reduced to the point that the neurons did not stain after 45 minutes. In the duodenum, on the other hand, there was an increase in the NADH-d positive neuronal population in conditions of acute diabetes[14]. This shows that the NADH-d positive neurons react differently from the general neuronal population to the diabetic condition and that the location of the myenteric neurons in the bowel length influences how they react to modifications in their environment[4,7].

Large neurons (above 600 μm^2) were found only in the control animals. The histogram of Figure 1 resembles those of other investigations[31,32], although neurons having areas above 800 μm^2 had not been recorded.

The comparisons of the neuronal areas between the two groups showed that the mean area of the neurons in the diabetics was significantly smaller than that in the controls. In other words, the myenteric neurons of the proximal colon of the diabetic rats reduced their size, despite the short duration of the experimental period. The smaller neuronal area was caused primarily by a reduction in the cytoplasmic area of the cell body. There are reports of changes in the nuclear and/or cytoplasmic areas in other regions of the nervous system in diabetics[9,33,34], which were attributed to changed rates of transcription and

translation of biomolecules and of electrical activity. The role of cellular dehydration to the reduced size also has to be taken into account.

In summary, it was verified that the acute diabetes induced by streptozotocin caused increased blood glucose levels, body weight loss, excessive food and water ingestion, and large urinary debt; it reduced the area of the colon and the sizes of the myenteric neurons and their nuclei and decreased the overall neuronal population.

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