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MORPHOLOGICAL AND QUANTITATIVE ANALYSIS OF THE NEURONS OF THE MYENTERIC PLEXUS OF THE CECUM OF STREPTOZOTOCIN-INDUCED DIABETIC RATS

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ABSTRACT-The purpose of this work was to study the neurons of the myenteric plexus of the cecum of rats with chronic streptozotocin-induced diabetes. We used four experimental groups of animals. In groups D2 and D8 animals were killed two and eight months, respectively, after diabetes induction and groups C2 and C8 were used as controls. We carried out whole-mount preparations stanied with Giemsa and NADH-diaphorase. We verified that the diabetes did not alter the shape and disposition of the myenteric ganglia; it provoked decrease on the neuronal density and increase on the incidence of weakly basophilic neurons. The effects of streptozotocin caused dilatation of the cecum still evidenced two months after induction, but no more observed on the eight months after induction. The smaller incidence of neurons in group D8 relative to group C8 was due to the early loss related to the drug toxicity and later to the aging in diabetic condition.

KEY WORDS: myenteric neurons, diabetes mellitus, myenteric plexus.

Análise morfológica e quatitativa dos neurônios do plexo mientérico do ceco de ratos diabéticos induzidos por estreptozootocina

RESUMO - O objetivo deste trabalho foi estudar os neurônios do plexo mientérico do ceco de ratos, com diabetes mellitus crônico, induzido por estreptozootocina. Utilizamos quatro grupos de animais. Nos grupos D2 e D8, os animais foram mortos, dois e oito meses, respectivamente, após à indução do diabetes e os grupos C2 e C8 foram controles dos grupos experimentais. Realizamos preparados de membrana corados pelo método de Giemsa e NADH-diaforase. Verificamos que o diabetes não alterava a forma e a disposição dos gânglios do plexo mientérico; provocou redução da densidade neuronal e aumento da frequência de neurônios fracamente basofílicos. Os efeitos da estreptozootocina provocavam dilatação do ceco ainda evidenciada dois meses após a indução, porém não mais verificada no oitavo mês após indução. A menor frequência de neurônios no grupo D8 em relação ao grupo C8 deve-se a perda inicial relacionada à toxicidade da droga e posteriormente ao envelhecimento em condição de diabetes.

PALAVRAS-CHAVE: neurônios mientéricos, diabetes mellitus, plexo mientérico.

Diabetes mellitus produces on the digestive tract a number of alterations[19,26], such as decrease on the gastric emptying[1,7,9], anorexia, sickness, vomits, diarrhea and constipation[18,20]. These manifestations are related to metabolic disturbances of neurons and nerves[6]. One of these is the increase on the blood glucose, that activates enzymes participating on the sorbitol formation, which generates an osmotic effect and causes edema and posterior rupture of the myelin sheath; another factor that interferes with the impulse conduction is the decrease on the biosynthesis of lipids by the Schwann cell[6].

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Considering that the myenteric plexus constitutes a mesh of nerve tissue functionally and structurally highly specialised[11], it is to be expected that its neurons and nerves are going to be involved on the pathophysiological manifestations of diabetes.

We verified on the literature experimental works concerning the acute or mid-term effects of diabetes induced by streptozotocin on the myenteric plexus of the ileum and colon of rats, with observations of the differential effects of diabetes on individual intestinal segments[3]. Information on chronic diabetes are scarce, which prompted us to carry out this research with the purpose of studying the morphological and quantitative aspects of the neurons of the myenteric plexus of the cecum of rats with chronic streptozotocin-induced diabetes mellitus for periods of two and eight months.

MATERIAL AND METHODS

Material

The present work, was carried out with the body of cecum of thirty two male rats (*Rattus norvegicus*), Wistar strain, weighing about 250 g (75 days) from the Central Biotery of the State University of Maringá.

Methods

The animals, sorted out after fourteen hours of fast in metabolic cages, received 35 mg/kg of streptozotocin endovenously.

The animals were divided on the following groups: D2-animals killed two months after diabetes induction; C2-non-diabetic animals, control of group D2; D8-animals killed eight months after diabetes induction; C8-non-diabetic animals, control of group D8.

On the day of the experiment, the animals were anesthetized with ethilic ether, and subjected to laparotomy, after a sample of blood was obtained from the inferior cava vein and the cecum was removed. Diabetic condition was confirmed by serum determination of glucose (glucose oxidase method).

Morphological and quantitative study of the myenteric plexus neurons

The body of the cecum from five animals of each group was employed for whole-mount preparations with the Giemsa method, according to Barbosa[2]. Samples others three animals of each group were stained with NADH-diaphorase, according to Gabella[10].

In five animals of each group we carried out the quantification of the myenteric neurons through the counting of cells on the Giemsa-stained whole-mount preparations. Each whole-mount preparation was divided into four quadrants, and in each one, 10 fields were randomly selected, yielding 40 fields. Using a microscope with a 40X lens, all the neurons of each field were counted, half-seen neurons were ignored in a field and considered in another. The area of each microscopic field was 0.173mm^2.

The morphology of 300 neurons of each animal group and the major longitudinal and transverse axes of the cell bodies were measured (Fig 1) and studied, using the Giemsa-stained whole-mount preparations. The microscope used was equiped with a WF 10X lens coupled with micrometer disc and 40X objetive.

The study of the ganglion morphology was carried out with the NADH-diaphorase-stained membranes.

To classify the neurons as small, medium or large, the mean and standard deviation of data resulting from the addition of the major longitudinal and transverse axes of 300 neurons from group C2 was calculated. It was considered as medium neurons those whose addition of major longitudinal and transverse axes resulted in values within the confidence interval of the mean. Neurons whose addition of major longitudinal and transverse axes resulted in values inferior to the mean minus its standard deviation, were considered as small. Large neurons were considered those whose addition of longitudinal and transverse axes resulted in values greater than the sum of the mean and the standard deviation.

The photographic documentation was performed with the aid of a microscope Olympus B X50 and PM 10AK photographic equipment.

In addition we observed the cytoplasmic basophily, the nucleus position, the cell shape and the number of nucleoli in each neuron.

Statistical Analysis

The mean, standard deviation and the variation coefficient of the number of neurons found in each group, i.e., 200 fields per group, were calculated. The analysis of variance and Student's "t" test were applied to compare the difference among the average of the studied variables.

The Chi-square (χ^2) test was applied to analysis the significance of data referring to the frequency of small, medium and large neurons in the different groups of animals studied. The level of significance used for all testes was 5%.

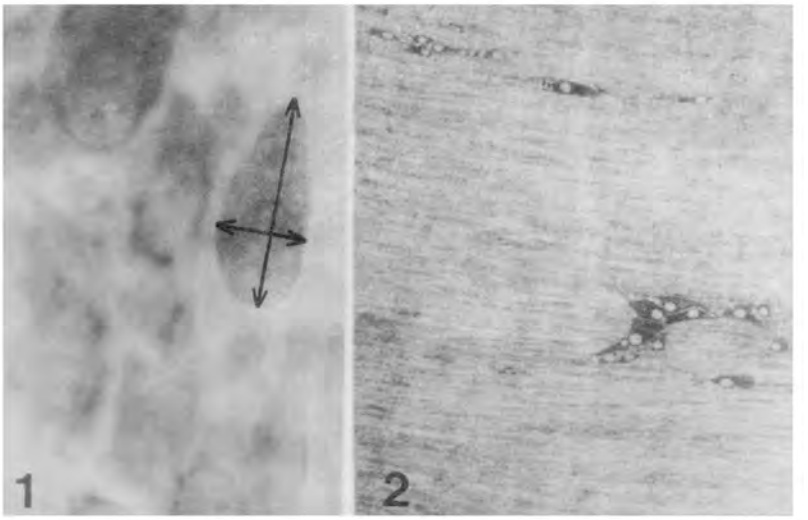


Fig 1. Giemsa-stained whole-mount preparation evidencing the major longitudinal and transverse axes of a neuron cell body. 900X. Green filter.
Fig 2. NADH-diaphorase-stained whole-mount preparation evidencing the arrangement of the myenteric plexus ganglia. 90.91X. Green filter.

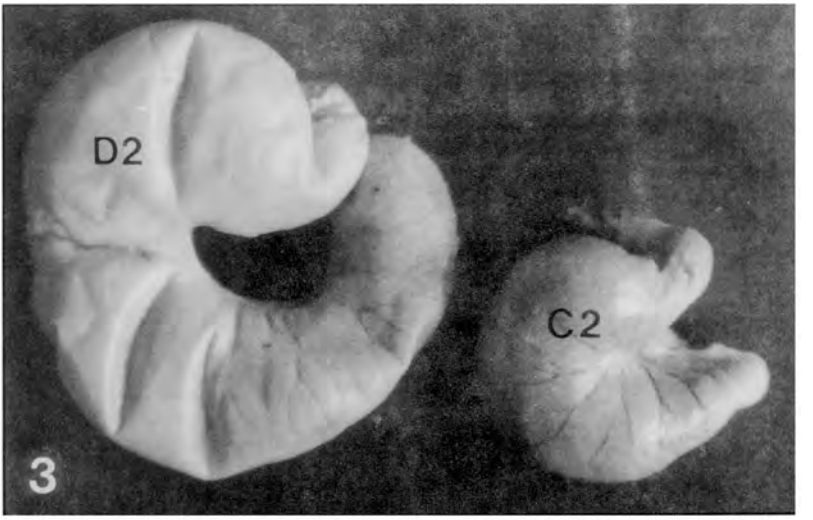
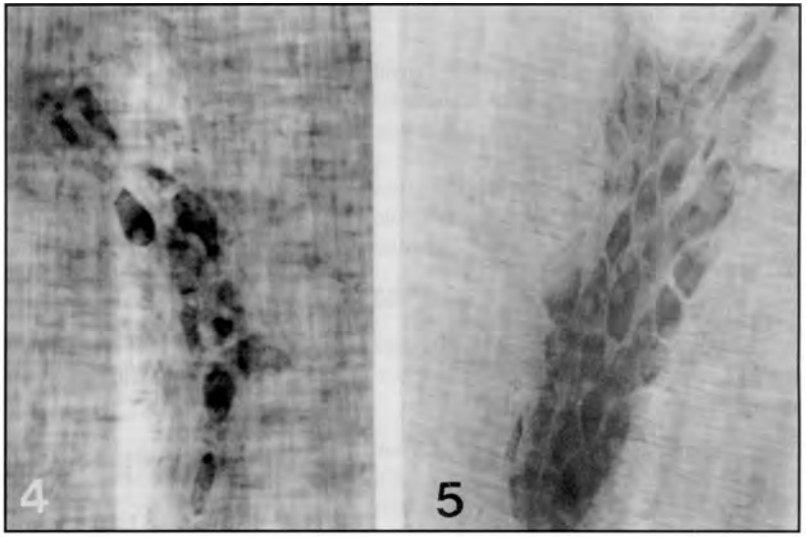


Fig 3. Photograph of the cecum of an animal of group D2 and of an animal of group C2.

Table 1. Mean body weights and blood glucose verified in eight animals of each studied group.

	C2	D2	C8	D8
Body wt (g)	372.33±40.52	270.71±54.25	436.57±17.23	246.86±38.84
Blood glucose (mg/dl)	100.79±25.40	405±101.19	143±23.56	458.38±116.80



Figs 4 and 5. Giemsa-stained whole preparation, evidencing myenteric plexus ganglia, from groups: D2 (Fig 4), C2 (Fig 5). 183.67X. Green filter.

RESULTS

All the diabetic rats used on the present study were hyperglicemic and showed body weight inferior to their controls (Table 1).

The cecum of diabetic animals of group D2 was larger than on the other groups, while on animals of group D8 (Fig 3) it showed similar size to that of control animals.

Morphology of myenteric neurons and ganglia

Both on the control and on the diabetic groups triangular and polygonal ganglia were found, but most of the ganglia were elongated (Fig 2).

The ganglia were constituted of neurons of varied sizes and elongate and oval shapes (Figs 4 and 5). Most of the neurons showed eccentric nucleus, with well-defined nucleoli in number of one to three; some did not exhibit nucleolus but chromatin clusters on the nucleoplasm.

Table 2. Values obtained by the Student's "t" test when comparing the average number of neurons in 6.92 mm² of cecum in each studied group.

Groups	Student's "t" test
C2xD2	4.42
C2xC8	1.68
C2xD8	3.67
D2xC8	3.31
D2xD8	2.71
C8xD8	2.71

cv: 2.31. C2, non-diabetic animals, control of group D2; D2, animals killed two months after diabetes induction; C8, non- diabetic animals, control of group D8; D8, animals killed eight months after diabetes induction.

Incidence of neurons of the myenteric plexus

On the forty fields studied, that is, on an area of 6.92 mm², there were found on group C2 331.80±100.50, on group D2 120.60±22.48, on group C8 237.40±75.62 and on group D8 155± 38.66 neurons. Through the variance analysis it was found a difference between the studied groups (F=10 for a critical value=3.24). When applying Student's "t" test we did not find significant differences only between groups C2xC8 (Table 2).

Morphometry of cell body of myenteric neurons

According to the employed methodology small neurons were considered those measuring from 19.70 um to 28.89 um, medium those from 28.90 um to 49.89 um and large those above 49.90 um.

Table 3 shows the relative and absolute incidences of small, medium and large neurons on the different groups studied.

Applying the Chi-square test we did not find significant differences on the incidence of small, medium and large neurons only between groups C8xD8 (Table 4). We observed that the medium neurons predominate in all groups studied, however it is verified a decrease on the number of small and medium neurons on groups D2 and D8.

Concerning the affinity for the staining, strongly basophilic neurons predominate on group C2 and those of intermediate basophily predominate on groups D2, C8 and D8 (Table 5). Using the Chi-square test we did not find significant differences only between groups C8xD8 (Table 4).

DISCUSSION

On the animals studied we observed triangular and polygonal ganglia, however most of them were elongated, resembling those observed by other authors[5,17], with a variation of ganglionar shape among species[14].

Small, medium and large neurons were found, with the second type predominating in all groups, in agreement with literature data[1]. In none of the groups neurons were found whose dimensions allowed their classification as giant, a fact that is observed in sympatic neurons of diabetic humans[16].

Table 3. Incidence of small, medium and large neurons obtained from a sample of 300 neurons of each experimental group, projected to the number of neurons found on an area of 6.92mm².

	C2		D2		C8		D8	
	N	%	N	%	N	%	N	%
Small	57.50	17.33	13.27	11	40.36	17	21.19	13.67
Medium	232.26	70	63.92	53	154.31	65	102.81	66.33
Large	42.04	12.67	43.41	36	42.73	18	31	20
Total	331.80	100	120.60	100	237.40	100	155	100

C2, non-diabetic animals, control of group D2; D2, animals killed two months after diabetes induction; C8, non-diabetics animals, control of group D8; D8, animals killed eight months after diabetes induction.

Table 4. Values obtained by "Chi-square" ($\chi 2$) test referring to the comparasion of classes of neurons regarding to size and basophilic affinity.

Groups	Values of $\chi 2$ to frequency of neurons	
	Small, medium and large	Slightly, intermediate and strongly basolphilic affinity
C2xD2	51.17	42.42
C2xC8	19.17	19.53
C2xD8	16.18	17.75
D2xC8	39.52	27.41
D2xD8	36.53	25.63
C8xD8	4.53	2.73

α=5%; cv=5.99. C2, non-diabetic animals, control of group D2; D2, animals killed two months after diabetes induction; C8, non-diabetic animals, control of group D8; D8, animals killed eight months after diabetes induction.

Table 5. Incidence of slightly, intermediate, strongly basophilic affinity from a sample of 300 neurons of each experimental group, projeted to the number of neurons found on an área of 6.92 mm².

Basophilic affinity	C2		D2		C8		D8	
	N	%	N	%	N	%	N	%
Slightly	39.82	12	31.76	26.33	33.24	14	24.28	15.67
Intermediate	139.35	42	59.50	49.33	121.86	51.33	76.98	49.67
Strongly	152.63	46	29.34	24.33	82.30	34.67	53.74	34.67
Total	331.80	100	120.60	100	237.40	100	155	100

C2, non-diabetic animals, control of group D2; D2, animals killed two months after diabetes induction; C8, non-diabetic animals, control of group D8; D8, animals killed eight months after diabetes induction.

In all the groups studied there was a predominance of eccentric nuclei. Our results. as well as those of other authors[4,12,25] demonstrate that the eccentric nucleus is not indicative of degenerative processes, once in myenteric neurons of healthy animals this feature is common.

We observed significant difference when we compared the number of neurons of group C2 (331.80±100.05 neurons/6.92 mm²) with that of group D2 (120.60±22.48 neurons/6.92 mm²), with a decrease of 63.65% on the neuronal density in group D2, which is a value even smaller than the neuronal density on group D8 (155±38.66 neurons/6.92 mm²). We believe that this decrease is attributable to the toxic effects of streptozotocin on the cecum neurons, which at first would suffer changes on their functional capacity; this would result, among other things, in decrease of the tonus of the muscular fibers from the cecum wall, causing it to dilate, effect that was evidenced through macroscopical observations. The strikingly smaller neuronal density on the two-month group would have as a cause, more than junt neuronal loss, the increase in distance between neurons due to the colon dilatation.

The neuronal changes on the streptozotocin-induced diabetes are reported as beginning from 1 to 3 days after induction with the development of chromatolysis[22], with processes of regeneration observed by the sixth week[22,23]. This contributed to explain the fact that the cecum of animals with eight-months diabetes had a neuronal density greater than that of two-months diabetics, because on this period many neurons which suffered with the drug toxicity, even on the presence of diabetes could regenerate and recover their regulatory function of the intestinal motility, causing the cecum to come to its normal dimensions, similar to those of non-diabetic animals; as a result the neurons became less sparse.

One of the indications that many animals from group D2 showed cells in stage of chromatolysis is the fact that 26.33% of their neurons were weakly basophilic, in contrast to their controls, where only 12% of the neurons are weakly basophilic. On the other hand on group D8 the percentage of weakly basophilic neurons falls to 15.67% without a corresponding decrease on the total number of neurons, demonstrating therefore that many neurons that underwent chromatolysis recovered. This is also evidenced when the incidence of strongly basophilic neurons on group D2 (24.33%) is compared with that of groups D8 (34.67%).

With aging, cellular decay, and increase in lipofuscine inside nerve cells, as well as the changes on the processes of biosynthesis, the affinity of neurons for dyes is altered, making them less basophilic, which we believe is the cause of animals of group C8 to have less cells with strong basophily (about 12%) than animals of group C2. In sympatic neurons of diabetic humans it was observed increase on the number of lipid-rich lisossomes and dilatation of the endoplasmic reticulum[8].

The fact that animals of group D8 had neuronal density about 34.60% smaller than animals from group C8, despite the similar dimension of their cecum, takes us to two hypotheses: first, part of the neurons were lost by the time diabetes was induced, a fact verified on the rat duodenum[5], where after a week after induction 18% of the neurons had been lost; second, the aging period of eight months in a diabetic condition would cause neuronal loss due to the pathophysiology of diabetes,

which is pointed as the cause of a series of impairing and pathogenic phenomena that speed the aging process[21]. Aging, in turn, is directly related to the decrease on the number of neurons of the myenteric plexus[10,13,15,24].

Although animals of group C8 have less neurons than those of group C2 statistical analysis did not reveal significance at the level of 5%, demonstrating that the neuronal loss on the cecum of animals that aged for six months in supposed normal conditions, is not as intense as that of diabetic animals.

Our results evidenced that the absolute incidence of large neurons, on the four groups studied, almost did not changed, while the incidence of small and medium neurons showed their highest values on the control groups and lowest values on the diabetic groups, demonstrating that the large neurons are less frequently lost, while small and medium neurons were lost in greatest amounts.

We conclude that the streptozotocin-induced diabetes does not alter the shape and disposition of the ganglia and neurons of the myenteric plexus of the cecum of rats. It causes decrease on the neuronal density and changes on the amount of polirribossomes, increasing the incidence of weakly basophilic neurons. The effects of streptozotocin are seen also as cecum dilatation, which can be evidenced after the second month of induction. The smaller incidence of neurons on animals of groups D8 relative to group C8 is due to the early loss related to the drug toxicity and to the continual loss related to the aging process in diabetic condition.

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