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Sheila J. Adad · Cristiane G. Cançado
Renata M. Etchebehere · Vicente P. A. Teixeira
Uilho A. Gomes · Edmundo Chapadeiro
Edison R. Lopes

Neuron count reevaluation in the myenteric plexus of chagasic megacolon after morphometric neuron analysis

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Abstract This study was made with the objective of re-evaluating the colon denervation in chronic Chagas' disease. The diameters of neuron perikaryons of the myenteric plexus were measured on paraffin sections in a ring from the sigmoid in Chagas' disease patients, 17 with and 10 without megacolon and in 10 non-chagasic controls. All neurons were counted in ten en-echelon sections. Neuron hypertrophy only occurred in the group with megacolon, and the average increase in diameter was 69.3%. This could generate an error factor in the neuron count by increasing the probability of neurons being seen in a greater number of histological sections. The original result of the neuron count gave medians of 1264, 1961, and 2665 in the groups of chagasic patients with megacolon, without megacolon, and in the control, respectively. The denervation was greater than 55% in only seven megacolon cases (41.2%). After applying a correction factor, the median in the group with megacolon was 746, and the denervation was greater than 55% in 13 cases (76.5%). This occurrence demonstrates the need to apply a correction factor when the neuron count in chagasic megacolon is being evaluated and in the other pathologies where neuron hypertrophy may be found.

Keywords *Trypanosoma cruzi* · American trypanosomiasis · Megacolon · Enteric nervous system · Morphometric neuron analysis

S.J. Adad (✉) · C.G. Cançado
Discipline of Special Pathology,
Patologia Especial – Hospital Escola – Faculdade de Medicina do Triângulo Mineiro; Rua Getúlio Guaritá 130,
Uberaba – MG, 38025-440, Brazil
e-mail: pe_fmtm@mednet.com.br
Tel.: +34-3318-5152, Fax: +55-3312-6640

R.M. Etchebehere
Discipline of Surgical Pathology,
Faculdade de Medicina do Triângulo Mineiro,
Uberaba – MG, Brazil

V.P. A. Teixeira
Discipline of General Pathology,
Faculdade de Medicina do Triângulo Mineiro,
Uberaba – MG, Brazil

U.A. Gomes · E. Chapadeiro · E.R. Lopes
Postgraduate course in pathology,
Faculdade de Medicina do Triângulo Mineiro,
Uberaba – MG, Brazil

Introduction

Among the various aspects of the pathogenesis and physiopathology of megacolons with chagasic etiology that need clarification, is the extent of the numerical reduction in neurons that must be attained before the morphofunctional alterations characteristic of this enteromegaly appear. According to Köberle [11], the dilatation and hypertrophy of the colon are initiated when the reduction in ganglion cells surpasses a critical limit of 55%. On the basis of measurements of neuron diameters in the gastrointestinal tract, which are on average 45 µm and never more than 50 µm [10, 11], Köberle introduced a method for counting neurons that would permit evaluation of the degree of denervation by comparing the number of nerve cells in the segment being considered in Chagas' disease patients and controls [8, 9, 10].

Having studied the autonomous nervous system of the colon and other viscera in chronic Chagas' disease patients for years, our attention has been drawn to the increase in neuron volume, confirming findings referred to by other authors [2, 15]. In the light of these facts and considering that those few existing morphological studies on neurons of the myenteric plexus were not done using image analysis systems, it appeared interesting to us to determine whether there would be an increase in neuron size in chagasic megacolon (CM), suggesting hypertrophy. This could interfere in the evaluation of the extent of denervation, not only in CM, but also in other diseases of the digestive tract, when study methods such as that proposed by Köberle are used [8, 9, 10].

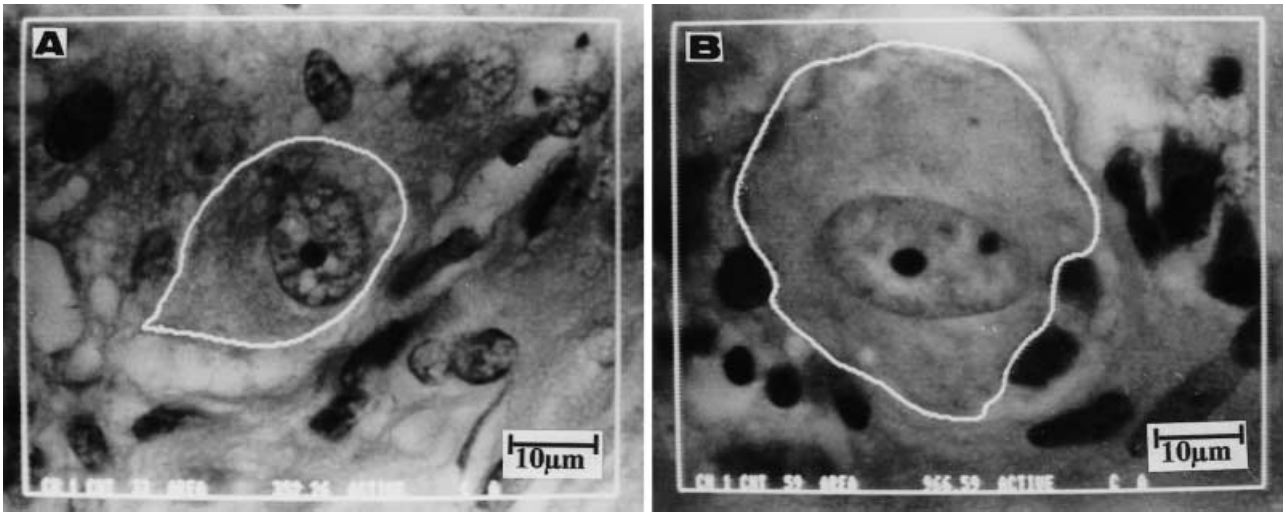

Fig. 1 Images from morphometry equipment showing neuron of the myenteric plexus from the middle portion of the sigmoid with delineated perikaryon, from which the maximum diameter may be obtained (Giemsa; ×1000 magnification). **A** Neuron from non-chagasic control. **B** Neuron from chagasic patient with megacolon. Note the increase in size of the megacolon

Materials and methods

Colons from 37 adults were analyzed: 17 with CM, 10 from chagasic patients without megacolon (CXM), and 10 from non-chagasic individuals (NC), whose ages were statistically similar (CM=57.1±13.8, CXM=51.8±15.7, and NC=56.6±20.5 years of age).

The diagnosis of Chagas' disease infection was based on positivity to at least two of the following three blood and/or pericardiac fluid reactions: fixation of complement, passive hemagglutination, and indirect immunofluorescence for *Trypanosoma cruzi*. The diagnosis of megacolon was established from clinical data (intestinal constipation frequently accompanied by complications, such as fecaloma and volvulus) and radiological data (opaque enema showing dilatation and loss of haustrations, possibly accompanied by lengthening of the colon, and/or single X-ray of the abdomen showing up fecaloma and intestinal dilatation).

A ring from the middle portion of the sigmoid was extracted, fixed in 4% formaldehyde, and processed for inclusion in a paraffin block. A series of 70 histological sections of 7-µm thickness were cut, and every seventh section starting from the first one, i.e., ten en-echelon sections, were stained using Giemsa's technique. All myenteric plexus neurons were counted on these ten en-echelon sections for each of the 37 cases (17 CM, 10 CXM, and 10 NC). In the morphometric study, the maximum diameters of the perikaryons of 30 myenteric plexus neurons from the first histological section from each case were measured consecutively, whose cytoplasm presented clear limits and evident nuclei and nucleoles (Fig. 1).

The measurements were made using an optical microscope, with the 100× objective coupled to a video camera that sent images to a high-resolution monitor. The image obtained on the monitor was integrated with a cursor that could be moved across a graphic measuring table. This table was in turn connected to a semi-automatic image analyzing system (MOP; Videoplan; Kontran Eletronik; Germany), and the measurements were expressed in microns. The ideal sample size for the number of neurons to be measured in each case and for the number of histological sections whose neurons would be counted was obtained from the calculation of accumulated averages for appropriate statistical quantities [16]. The expression "neuron diameter" will be used henceforth as the average of the 30 measurements of maximum diameter of the perikaryons of neurons in each case.

After obtaining the results of the morphometric analysis of the neurons, a correction factor was applied to the results of the neuron count for the CM group, bearing in mind the occurrence of

Table 1 Perikaryon diameters of neurons in the myenteric plexus, in µm, from the middle portion of the sigmoid. *CM* chagasic patient with megacolon; *CXM* chagasic patient without megacolon; *NC* non-chagasic control

Case	Group	Perikaryon
1	CM	35.40
2	CM	25.69
3	CM	23.28
4	CM	29.84
5	CM	40.37
6	CM	35.51
7	CM	38.95
8	CM	33.17
9	CM	37.59
10	CM	27.60
11	CM	28.21
12	CM	35.40
13	CM	32.01
14	CM	36.30
15	CM	31.96
16	CM	32.43
17	CM	30.66
18	CXM	20.49
19	CXM	24.86
20	CXM	20.15
21	CXM	20.29
22	CXM	17.20
23	CXM	16.75
24	CXM	20.66
25	CXM	22.92
26	CXM	24.93
27	CXM	16.18
28	NC	16.94
29	NC	20.34
30	NC	16.34
31	NC	22.28
32	NC	17.72
33	NC	21.38
34	NC	18.32
35	NC	23.48
36	NC	17.58
37	NC	18.18

Table 2 Average and SD of perikaryon diameter of neurons in the myenteric plexus, in microns, from the middle portion of the sigmoid, for each group in the study. Test: variance analysis. *CM* chagasic patient with megacolon; *CXM* chagasic patient without megacolon; *NC* non-chagasic

	Non-chagasic	Chagasic patients	
		Without megacolon	With megacolon
Average	19.26	20.44	32.61
SD	2.44	3.13	4.71
No. of cases	10	10	17
NC × CXM × CM	NC × CXM	NC × CM	CXM × CM
$P<0.0001$	$P=0.6403$	$P<0.0001$	$P<0.0001$

Table 3 Number of neurons in the myenteric plexus from the middle portion of the sigmoid for the three groups in the study. *CM* chagasic patient with megacolon; *CXM* chagasic patient without megacolon; *NC* non- chagasic

Case	Group	Number of neurons	
		Original count	Modified value after correction for neuron hypertrophy
1	CM	942	556
2	CM	2873	1695
3	CM	1682	992
4	CM	2129	1256
5	CM	614	362
6	CM	1999	1179
7	CM	360	212
8	CM	1242	733
9	CM	2575	1519
10	CM	258	152
11	CM	2550	1505
12	CM	940	555
13	CM	721	425
14	CM	1264	746
15	CM	1823	1076
16	CM	2725	1608
17	CM	330	195
18	CXM	3125	–
19	CXM	3067	–
20	CXM	1802	–
21	CXM	2241	–
22	CXM	1567	–
23	CXM	2149	–
24	CXM	1733	–
25	CXM	1778	–
26	CXM	1801	–
27	CXM	2120	–
28	NC	3038	–
29	NC	2338	–
30	NC	2780	–
31	NC	2290	–
32	NC	2792	–
33	NC	2520	–
34	NC	2791	–
35	NC	3459	–
36	NC	1819	–
37	NC	2550	–

neuronal hypertrophy in this group. This was calculated by dividing the average of the ganglion cell diameters for the NC group by the average for the CM group. This factor was then multiplied by the number of neurons for each case in the CM group.

Informed consent was obtained from all individuals and/or their parents or guardian prior to their inclusion in this study. The institution approved the protocol for this investigation involving humans and all experimentation was conducted in conformity with ethical and human principles of research.

Results

Table 1 presents the myenteric plexus neuron diameters for each case, and Table 2 presents the average and SD for the groups in the study. In the CM group, the neuron diameters were significantly greater than in the CXM and NC groups, which were not different from each other. The average increase in diameter for the CM group was 69.3% in relation to the NC group (Fig. 1A, B).

The results of the myenteric plexus neuron count for each case in the groups in the study, and also the modified values for the CM group after applying the correction factor for neuron hypertrophy, are presented in Table 3. The median, minimum, and maximum values are found in Table 4.

In the original results of the myenteric plexus neuron count (without correction factor), the number of neurons was significantly less in the CM and CXM groups than in the NC group, although there was no significant difference between CM and CXM. The degree of denervation was greater than 55% in only seven cases of CM (41.2%). After applying the correction factor to the CM group, due to the methodological fault related to neuron hypertrophy, the denervation became greater than 55% in 13 cases of this group (76.5%). Moreover, after the correction, a statistically significant difference between the CM and CXM groups was noted.

Discussion

Our data related to myenteric plexus neuron hypertrophy in CM agree with that of Banhos [2], who evaluated the area of nuclei and nucleoles of these cells. They also agree with that of Tafuri [15], who related that “the neurons of the Auerbach plexus in megaesophagus and megacolon undergo a compensation process and probably increase the volume of the nucleoli”. Nevertheless, the measurements of neuron diameters obtained by us in all the groups are much smaller than those reported by Köberle [10, 11], who did not specify the group analyzed. This leads us to suppose that the methodology used by that author and also used by others who have evaluated the degree of denervation in CM [3, 4, 5] does not count all the neurons present in the segment examined. Our findings also provide evidence for the existence of an error factor in the neuron count when the groups with and without megacolon are compared, caused by the neuron hypertrophy observed in CM. This

Table 4 Median, minimum, and maximum values for the number of neurons in the myenteric plexus from the middle portion of the sigmoid. Kruskal-Wallis test. *NC* non-chagasic controls; *CXM* chagasic patients without megacolon; *CM* chagasic patients with megacolon; *CM mod* modified value from the correction factor for neuron hypertrophy

	NC	CXM	CM	CM mod	
Median	2665	1961	1264	746	
Minimum value	1819	1567	258	152	
Maximum value	3459	3125	2873	1695	
No. of cases	10	10	17	17	
NC × CXM × CM *P*<0.0001	NC × CXM *P*=0.0284	NC × CM *P*=0.0028	CXM × CM *P*=0.0707	NC × CM mod *P*<0.0001	CXM × CM mod *P*<0.0001

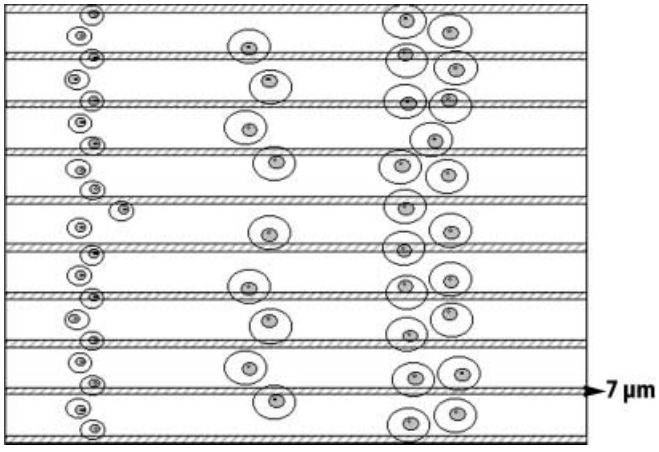

Fig. 2 Schema representing neurons of the myenteric plexus of the colon. The *left column* shows neurons of normal size in non-chagasic controls without any intestinal pathology. The *central column* presents hypertrophied neurons from a chagasic patient with megacolon, in which there is a reduction in the number of these cells. In the *right column*, hypertrophied neurons of the colon with surgical stenosis are represented, in which denervation has not occurred. The *striped bands* represent ten en-echelon histological sections, 7 µm in thickness, in which the neurons are counted. Note that a greater proportion of hypertrophied neurons than neurons of normal size can be detected in the histological sections, which makes it appear that there was an increase in the number of neurons in the experimental stenosis and creates an error factor in the evaluation of the degree of denervation in the chagasic megacolon

hypertrophy increases the probability of the neurons being seen in a greater number of histological sections in series or en-echelon, showing the need for studies to be done to reevaluate the extent of denervation required for the initiation of chagasic megacolon and megalies in general.

In the original neuron count, there was extensive matching between the CM and CXM groups regarding the degree of denervation, which was only greater than 55% in seven cases (41.2%). However, after applying the correction factor to the CM group, there was a significant difference between these groups, although some minor matching between the two groups persisted. In the CM group, the denervation in the middle portion of the sigmoid was greater than 55% in 13 (76.5%) of the 17 cases and, in the rest, it varied from 36% to 43.5%. This data appears at first sight to contradict Köberle's affirmation [11] that the "critical limit" of neuron destruction for the appearance of megacolon should be around 55%. Nevertheless, we believe new quantitative studies on the colon will be necessary, taking the error factor from neuron hypertrophy into consideration and evaluating the denervation in different regions of the colon, as the denervation may not be uniform throughout the large intestine.

With regard to experimental studies, there has so far not been any success in inducing the characteristic chagasic megalies, with documentation of muscle hypertrophy associated with the dilatation of the colon. Some authors have reported dilatation of the colon, although not describing hypertrophy of the wall [1, 13]. In one of these studies, a denervation was detected of up to 75% in the colon of mice with Chagas' disease that had dilatation and a denervation of up to 87% in those that did not have dilatation [12]. Experimental studies in which morphometric evaluations were made did not detect megacolon in rats, despite there being denervation [6, 14].

Studies of surgically induced intestinal stenosis in different animals have brought into evidence neuronal hypertrophy accompanying the muscle hypertrophy [7]. According to Gabella [7], "increases in the number of neurons counted, reported by Bernninghoff (1951), Filogamo and Vigliani (1954), and Okada and Okamoto (1971), are attributable to the fact that the large (hypertrophic) neurons were more easily detectable with the histological techniques employed than control neurons". We believe, however, that the error factor that we referred to earlier may explain the paradoxical apparent increase in the number of neurons in the intramural plexus described in these experiments, instead of the differentiation of reserve cells in neurons, as proposed by those authors, or the justification proposed by Gabella [7]. Our hypothesis is based on the following rationale. The increase in neuronal volume permits this neuron to be seen in a greater number of histological sections than the neurons of normal size and, for this reason, it gives the impression that the number of neurons has increased in relation to the normal number (Fig. 2). In the case of the chagasic patients, the number of neurons will not appear greater than in the control group, because there is significant denervation, although this generates an error factor that makes it difficult to evaluate the degree of denervation.

In conclusion, the occurrence of the error factor resulting from the neuron hypertrophy demonstrates the need to apply a correction factor when the neuron count in chagasic megacolon is being evaluated and in other pathologies where neuron hypertrophy may be found.

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