Study of the myenteric and submucous plexuses after BAC treatment in the intestine of rats

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ABSTRACT: A morphological and quantitative study in the ileal and colonic myenteric and submucous plexuses of rats after BAC denervation was performed. Four groups were employed: SI – ileum control; CBI – denervated ileum; SC – colon control; and CBC – denervated colon. We used the Myosin-V immunohistochemistry technique to study the myenteric and submucous plexuses. In the submucous plexus of the ileum and colon there was not a significant decrease in the number of neurons/mm^2 and of ganglia/mm^2. The denervation of the myenteric plexus in the group CBI was 44.7% and in the group CBC, 68.3%. In the myenteric plexus there was also a significant decrease in the number of ganglia/mm^2 (13.8% in group CBI and 52.14% in group CBC) and in the number of neurons/ganglion (33.9% in group CBI and 39.6% in group CBC). The morphological analyses showed that there was an alteration in the shape of the ganglia of the ileal and colonic myenteric plexus. The area of the cell bodies had a significant increase both in the myenteric and the submucous plexus in groups CBI and CBC. These data demonstrate that the BAC treatment causes morphologic and quantitative changes in the myenteric plexus and quantitative changes in the cell body area of the submucous plexus.

Introduction

The enteric nervous system has been used to study the neuronal plasticity due to its easy access. Besides, it is also used to study the role that nervous plexuses play in their different intestinal functions, as observed in neuronal degenerative diseases (Qualman et al., 1984; Rohrmann et al., 1984; Knusel et al., 1990; Jost and Schimrigk, 1993; Shankle et al., 1993).

A model of intestinal aganglionosis used to study the neuronal plasticity and the myenteric plexus functions is the chemical denervation by serosal application of Benzalkonium chloride (BAC), a cationic surfactant (Sakata et al., 1979; Fox et al., 1983; See et al., 1988, 1990; Zucoloto et al., 1988, 1997; Cracco and Filogamo, 1997; Hernandes et al. 2000). The cytotoxicity of BAC and others detergents in this model are apparently related to their ability to disrupt cell membranes (Fox et al., 1983; Herman and Bass, 1989).

The serosal application of BAC in any segment of the intestine eliminates most of the myenteric neurons (Fox et al., 1983), destroys completely the longitudinal cells and partially the circular cells in the muscle layer (Holle, 1998), without affecting the number of submucosal neurons (Fox et al., 1983; See et al., 1988). It also eliminates the sympathetic innervation of the muscle layer, and mucosal and submucosal plexuses by destroying the extrinsic nerves (See et al., 1990).

This experimental model contributes significantly to the assessment of the role played by the myenteric plexus on several intestinal functions. Although there

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are other models of intestinal aganglionosis, such as Hirschprung's disease (Howard and Nixon, 1968) and Chagas' disease (Köberle, 1963), the denervation model by BAC displays several advantages: (i) in the intrinsic neurons a selective elimination takes place only in the myenteric plexus; (ii) it can be applied to a specific intestinal segment; (iii) it can be performed in genetically intact animals; (iv) it gives the researcher the opportunity to investigate the changes induced by BAC application on several cellular populations (Cracco and Filogamo, 1997).

Within the works performed using this model only a few studied the myenteric plexus influence on the submucous plexus morphology. See *et al.* (1990) studied only the VIP-immunoreactive neurons of the rat jejunum submucous plexus, and Cracco and Filogamo (1997) the NADH-diaphorase-positive population in the ileum of rats. However, no work has attempted to assess the morphology of the overall neuron population.

The purpose of our work was to observe the denervation effect on the neuronal profile in the submucous and myenteric plexuses of the ileum and colon of rats, by using the immunohistochemistry technique for myosin-V. This technique allows the identification of all the neuronal population (Drengk *et al.*, 2000).

Materials and Methods

Antibodies

Antitail polyclonal antibody, generated against a chicken myosin-V recombinant protein has been characterized previously (Espreafico *et al.*, 1992). A cDNA fragment (corresponding to amino acids 899 to 1830) of a myosin-V-tail clone from a chicken brain was subcloned into pGEX vector to generate a Glutatione transferase (GST)/myosin medial tail (corresponding to amino acids 1117 to 1435) fusion protein in XL1Blue bacteria. This GST/myosin-V medial tail was purified over glutatione resin. This fusion protein was used as an antigen for antibody production in rabbits. The immune serum was purified over a PLM/Myosin-V tail protein affinity column to enrich for IgG antibodies directed against the myosin-V medial tail. Secondary an-

tibodies peroxidase-conjugated Goat antirabbit IgG were used (PIERCE, Rockford, USA).

Experimental design

Animals used in this study were treated in accordance with the guidelines of the Committee on Care and use of Laboratory Animals of the National Research Council. Male Wistar rats weighting about 90 g were anaesthetized with Ketalar® and Rompum®, laparatomized and the proximal colon or ileum brought outside the peritoneal cavity. The proximal colon or ileum were wrapped with cotton soaked in a 2 mM solution of benzyldimethyltetradecylammonium (benzalkonium chloride BAC, Sigma Chemical Co) in saline for 30 min, or with saline only, in the controls. After treatment, the intestinal segment and the entire peritoneal cavity were rinsed with 0.9% saline at 36ºC, followed by closure of the abdomen (Sakata *et al.*, 1979). After surgery, animals were allowed to recover, housed in plastic cages with five animals under controlled temperature conditions and allowed free access to water and laboratory rat chow. The animals were allocated to four groups of five animals each: Group **SC** in which the colon was treated with saline solution; Group **CBC** in which the colon was treated with BAC; Group **SI**, in which the ileum was treated with saline solution; and Group **CBI**, in which the ileum was treated with BAC. The animals were kept in cages during 15 days and then killed.

Immunohistochemistry of the Myenteric and Submucous Plexuses

The animals were perfused with 1 ml per g body weight of saline solution followed by perfusion with 1 ml/g/body weight of fixation solution containing 10 mM sodium periodate, 75 mM lysine, 1% paraformaldehyde in phosphate buffer 37 mM, pH 7.4 (McLean and Nakane, 1974). Immediately after perfusion, one treated fragment of colon or ileum was removed, flushed with fixative solution, immersed in the same fixative and 15 min later opened and left in this solution for 1 h. Samples were dehydrated through graded alcohols, cleared in xylol, rehydrated back through the ethanol steps to 70%

FIGURE 1. Higher magnification of myenteric plexus: SI group (A), SC group (B), CBI group (C), CBC group (D) ganglia and fibres with the myosin-V medial tail antibody and submucous plexus: SI group (E), SC group (F), CBI group (G) and CBC group (H).
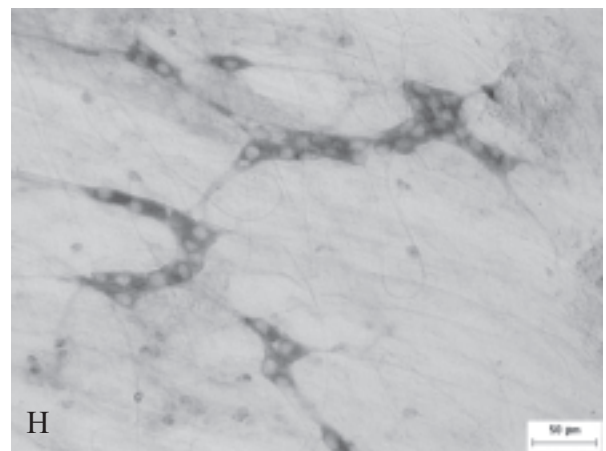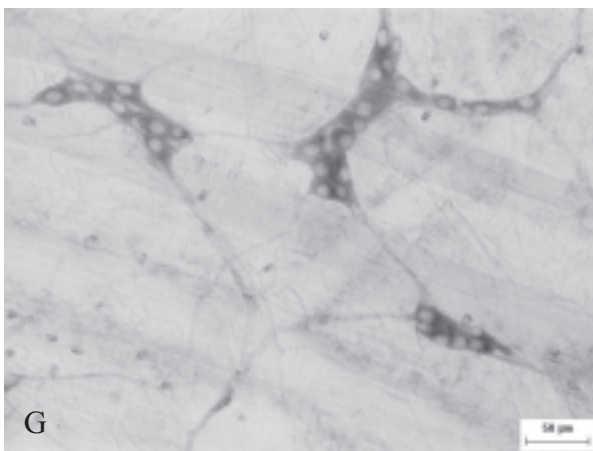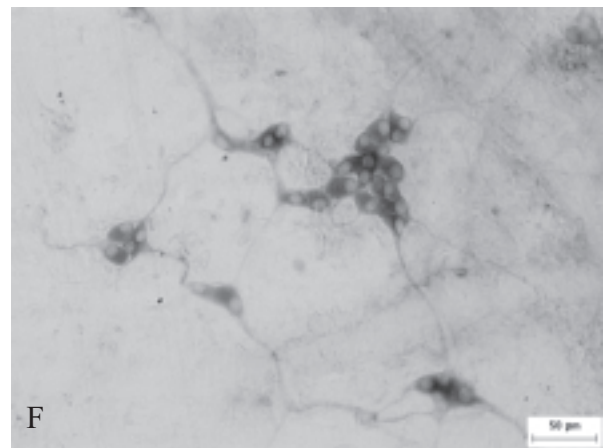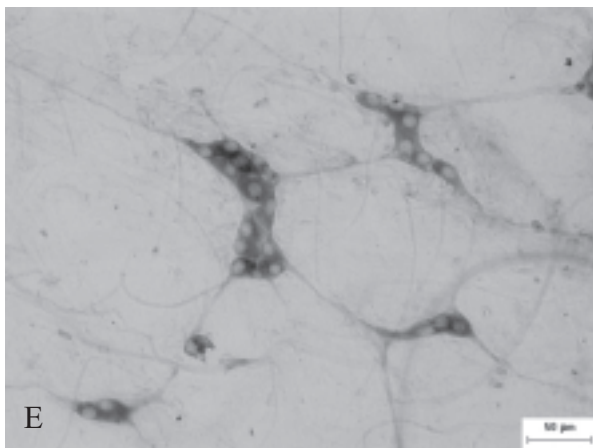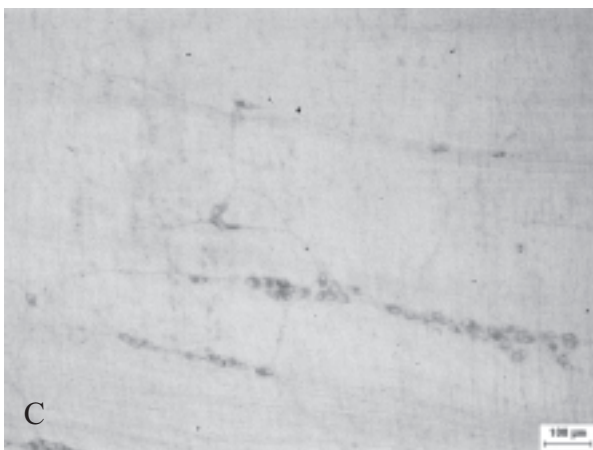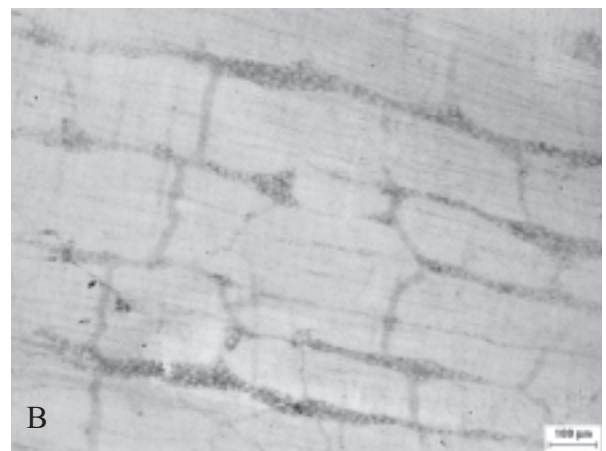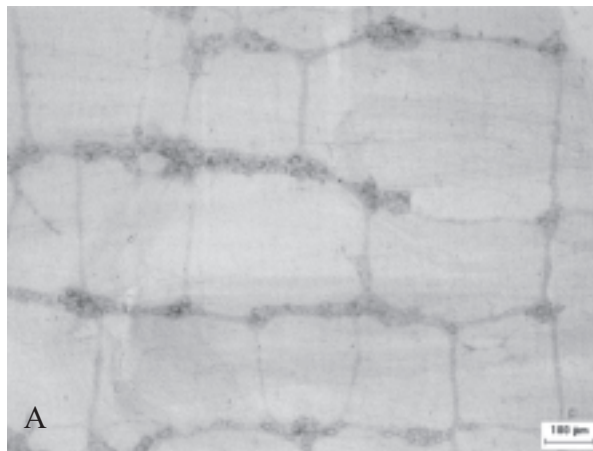
where the tissue could be stored. At this step colon or ileum fragments were dissected separating the muscle wall containing the myenteric plexus from the submucosal layer. The submucosal layer was separated from the mucosal layer by a wooden spatula. Dissection was followed by hydration resumed through 60% and 50% ethanol to PBS. The tissue was washed twice in PBS and blocked for two h with PBS containing 2% BSA, 2% goat serum and 0.5% Triton-X-100 at room temperature. Immuno-staining was proceeded by incubating the tissue fragments in 0.89 μg/ml of affinity-purified antibody specific to the myosin-V medial tail domain diluted in PBS containing 1% BSA, 2% goat serum and 0.1% Triton-X-100, under agitation during 48 h at room temperature. Following incubation, the fragments were washed in PBS containing 0.1% Triton-X-100 and then in PBS with 0.05% Tween-20. They were incubated with 10 μg/ml secondary antibodies conjugated to peroxidase in PBS for 24 h at room temperature under agitation, washed four times for 15 min in PBS containing 0.05% Tween-20. Immunoreaction with peroxidase-conjugated antibodies was developed by incubation with 0.75 mg/ml Diaminebenzidine in PBS and 0.03% H_2O_2 for 10 min at room temperature under agitation. Samples were mounted in a gel mounting media containing 50% glycerol, 0.07 g/ml gelatin in PBS and 2 μl/ml phenol.

Quantitative analysis of submucous and myenteric plexuses

a) Neuron counts: The quantification of the submucous and myenteric plexus neurons was obtained through the counting of cells on the Myosin-V immunohistochemistry stained whole-mount. These whole-mounts had 40 fields randomly selected for counting. An Olympus microscope with 40X objective was used. The total area of the 40 microscopic fields was 6.36 μm^2. The results were expressed as number of neurons per mm^2.

b) Perikarion area: Neuronal cell profile area measurements were made on 50 randomly chosen cells in each animal (250 neurons per group). For these measurements, images were captured from an Olympus BX50 microscope with 40X objective and processed with an image analysis software (Image-Pro Plus version 4.5.1.22 (2002) of Media Cybernetics, Inc.). The results were expressed in μm^2.

Statistical analysis

Student's *t* test was used to compare the number of neurons and neuronal cell profile between each experimental group and its control. Significance level was set at $p<0.05$.

TABLE 1.

Effect of BAC on the number of neurons per area, number of ganglia per area, number of neurons per ganglia and mean perikarion area on the myenteric plexus of the four groups studied.

	SI	CBI	SC	CBC
Neurons/mm2	158.1±12.99	87.42±9.96*	78.38±2.71	24.84± 2.10*
Ganglia/mm2	15.53±0.98	13.38±1.84*	8.61±0.36	4.49±0.08*
Neurons/ganglia	10.18±0.52	6.73±0.44*	9.15±0.47	5.53±0.51*
Mean area (μm2)	275.50±6.21	312.50±8.78*	263.70±9.76	449.50±11.58*

SI –ileum control
CBI – ileum treated with benzalkonium chloride (BAC)
SC – colon control
CBC – colon treated with BAC
*Significantly different when compared to respective control ($p<0.05$). All results are expressed as mean ± SEM, n=5 rats for groups.

Results

Qualitative observations of the ganglia and neurons

A regular and uniform pattern was observed in the ileal myenteric plexus of animals in SI group (Fig. 1a). However, there was an irregular distribution in the CBI groups, with regions with no neurons (Fig. 1c). In the ileum, the ganglia shape in the myenteric plexus varied. Elongated, round, triangular, square and rectangular ganglia were observed. Most of the myenteric neurons were elongated, although many round neurons were seen in the ileum.

In the submucous plexus, the ganglia in the SI and CBI groups were nodular and showed a regular pattern of distribution (Fig. 1b, 1d). The ganglia were polygonal or triangular; some were elongated in shape and distinctly separated. Neurons were generally smaller, with elongated, roundish or pear-like profiles. Few neurons were as elongated as those in the myenteric plexus.

In the proximal colon, we observed that the myenteric plexus had the same pattern as in the ileum. In the SC group, the pattern of distribution was regular and uniform (Fig. 1b). The CBC group showed an irregular distribution, with absence of neurons in some areas (Fig. 1d). The neurons had the same morphology as those in the ileal myenteric plexus.

We noticed a different distribution pattern in the colonic submucous plexus, with ganglion accumulation in colonic folds both in the SC and CBC groups. The morphology of the colonic submucous plexus neurons was identical to the ileal submucous plexus neurons.

Neuron size

The ranges of perikaryon size, expressed as perikaryon area in all plexuses examined, are shown in Table 1 and 2. All the analyzed areas presented significant differences when compared with their respective controls.

Neurons counts

All the analyzed parameters in the myenteric plexus of ileum and colon showed significant differences (Table 1).

There was a significant difference in the number of neurons per ganglia in the ileal submucous plexus (Table 2). As for the colonic submucous plexus, no significant differences in any of the analyzed parameters were seen (Table 2).

Discussion

Few quantitative and morphometric studies on the myenteric and submucous plexuses have been done employing the BAC denervation model. Most of the works deal only with the degree of denervation in the myenteric plexus after BAC treatment (Sakata *et al.*,

TABLE 2.

Effect of BAC on the number of neurons per area, number of ganglia per area, number of neurons per ganglia and mean perikarion area on the submucous plexus of the four groups studied.

	SI	CBI	SC	CBC
Neurons/mm2	94.12±9.19	82.28±5.56	74.72±7.02	71.16±6.95
Ganglia/mm2	19.66±1.88	20.76±1.20	11.51±0.83	12.01±0.44
Neurons/ganglia	4.79±0.14	3.97±0.20*	6.50±0.46	5.88±0.44
Mean area (mm2)	246.90±5.03	275.80±6.04*	320.40±5.53	339.60±6.10*

SI –ileum control
CBI – ileum treated with benzalkonium chloride (BAC)
SC – colon control
CBC – colon treated with BAC
*Significantly different when compared to respective control ($p<0.05$). All results are expressed as mean ± SEM, n=5 rats for groups.

1979; Fox *et al.*, 1983; See *et al.*, 1988, 1990; Zucoloto *et al.*, 1988, 1997; Cracco and Filogamo, 1997; Hernandes *et al.* 2000) or with specific populations of the submucous plexus neurons (Fox *et al.*, 1983; See *et al.*, 1990; Cracco and Filogamo, 1997).

The myenteric plexus morphology found in the ileum and colon show elongated ganglia parallel to the circular muscle, as mentioned by other authors (Gabella, 1987). The ganglion shape varies among species and they may be elongated, geometrical (polyedric) or round-shaped (Gabella, 1987). We found elongated, triangular and polygonal ganglia in the SI group, similar to Hernandes' (1994) findings in the ileum of rats. The nervous cell distribution pattern was very different in the CBI and CBC groups when compared with the SI and SC. Extensive areas devoid of myenteric innervation could be observed, while others display isolated, spindle-shaped ganglia made up of few neurons, organized in irregular networks. The neurons presented a spherical, pear-like or fusiform shape in the ileum and colon. The ganglia in the ileum were closely packed, whereas they were more widely spaced in the colon.

The submucous plexus ganglia were nodular and showed a regular pattern of distribution in the ileum while the submucous plexus in the colon showed ganglia with more neurons in pleat region. The neurons were mainly spherical, elongated or pear-like in shape in the ileum and colon as observed by Liberti *et al.* (1994) in the small intestine of guinea pigs.

The technique employed allowed us to observe that the ganglia are connected through bundles of nervous fibers (Fig. 1).

The BAC treatment caused an expressive increase in the perikaryon area of both the myenteric plexus cells and the submucous plexus cells (Table 1,2). Cracco and Filogamo (1997), using the NADH-diaphorase histochemistry in the rat ileum, observed a reduction in the perikaryon area in denervated animals (460 ± 110 μm^2 in controls; 285 ± 94 μm^2 in denervated animals). These results might be due to the technique employed, which stains only the neuronal population enizmatically active (Young *et al.*, 1993). There was also a significant increase in the perikaryon area in the submucous plexus in the denervated group, whit the SC showing a mean area of 320.4 ± 5.52 μm^2 and the CBC group 339.6 ± 6.10 μm^2. Barbosa (1973) studied the correlation between the increase in the cytoplasmic area and the smaller number of nervous cells, making a comparison between the colon and the cecum, knowing that the cecum has less neurons per area than the colon. See *et al.* (1990), when studying the VIP-immunoreactive neurons, observed an increase of 2.5% in the perikaryon

area 15 days after the denervation. Dahl *et al.* (1987) observed that there was a compensatory increase in the production of certain neurotransmitters by the surviving neuronal elements. This increase in the cell area of myenteric and submucous plexus neurons in the ileum and colon may be due to a compensatory neuronal hypertrophy, with the purpose of increasing neurotransmitter production, since the number of myenteric plexus neurons in these two segments decreased (Table 1,2). Other works (See *et al.*, 1988, 1990; Zucoloto *et al.*, 1988, 1997; Holle *et al.*, 1998) showed that the chemical denervation with BAC causes a hypertrophy in the circular and longitudinal muscle resulting an increase in the neurons cell profile area due to an increase in the area to be innervated by them. After inducing muscle hypertrophy in the guinea pig ileum, Gabella (1990) noticed an increase in the perikarion area in the myenteric and submucous plexus of these animals. Studies have proved that chemical ablation with BAC causes the denervation of the circular muscle soon after the treatment. However, 30 days later there is a re-innervation by neurons of the submucous plexus (Herman and Bass, 1990). Cracco and Filogamo (1997), using the NADH-diaphorase histochemistry, demonstrated a significant increase in the perikaryon area in the submucous plexus of the BAC-treated-group. These authors suggest that the submucosal neurons hypertrophy in the BAC-treated segment may be caused by the lack of inhibitory influence exerted by the neural input (both myenteric and extrinsic) on their growth and by the increased mass of the target tissue innervated (Gabella, 1990), i.e. increased volume of the mucosa (See *et al.*, 1990; Hernandes *et al.* 2000) and partial re-innervation of the thickened circular muscle layer (Luck *et al.* 1993). See *et al.* (1990) also describe enlarged cell bodies of the VIP-immunoreactive neurons of the submucosal plexus.

Quantitative parameters of neurons in the myenteric and submucous plexuses were thoroughly studied in this work. Here, we studied the number of neurons per area, the number of ganglia per area and the number of neurons per ganglia. No other work using the BAC chemical denervation model has analyzed these last two parameters. A significant reduction was only found in the myenteric plexus when the number of neurons/mm^2 was analyzed, as reported by other authors (Sakata *et al.*, 1979; Fox *et al.*, 1983; See *et al.*, 1988, 1990; Zucoloto *et al.*, 1988, 1997; Cracco and Filogamo, 1997; Hernandes *et al.* 2000; Dahl *et al.*, 1987; Herman and Bass, 1989, 1990; Luck *et al.*, 1993). In our experiment, the denervation of the proximal colon after 15 days treatment was 68.31% and 55.29% in the ileum.

Our results were similar to those found by Buttow *et al*. (2003), who found a denervation degree of 69% in the proximal colon when using the immunohistochemistry technique for myosin-V in animals of the same age. There is a large variation on the denervation percentage in experiments using BAC; this may be a consequence of the animal age and also of the dose given at the beginning of the experiment (Hernandes *et al*., 2000; Herman and Bass, 1989). Garcia *et al*. (2002), studying the influence of aging on the number of neurons of the myenteric plexus, observed that the animals treated with BAC after 2, 6, 12 and 18 months, showed a greater neuronal survival when compared to controls, and suggest that these results are due to a neuroplasticity phenomenon. The colon denervation of young rats, weighing about 60 g, reached 78% of neurons of the myenteric plexus five months after 0.2% BAC treatment (Zucoloto *et al*., 1988). Zucoloto *et al*. (1997), induced the myenteric plexus ablation with 2 mM BAC in the distal colon of young rats weighting about 50 g, and presented a reduction around 57% in the number of neurons of the plexus, ten days after BAC treatment.

As it was demonstrated in other works, there was no significant reduction in the number of submucous plexus neurons (Fox *et al*., 1983; See *et al*., 1988, 1990; Cracco and Filogamo, 1997). According to Fox *et al*. (1983), for an unknown reason, the diffusibility of BAC through the circular muscle was hindered. It is also possible that the myenteric neurons are more sensitive to BAC than the submucosal neurons.

By analyzing the number of ganglia/mm^2, a significant reduction only in the ileal and colonic myenteric plexuses was observed. We found a significant reduction in the ileum and colon myenteric plexus, and in the ileum submucous plexus by observing the number of neurons per ganglia. The alterations seen in the myenteric plexus are the result of a reduction in the number of neurons; there is also a decrease in the number of ganglia per unit area and in the number of neurons per ganglion. No significant reduction in the number of ganglia per millimeter in the submucous plexus was observed; however, there was a significant reduction in the number of neurons per ganglion in the ileum of the CBI group. This may be a consequence of a small neuron dilution caused by the small dilatation seen in the treated segment.

This work allowed us to conclude that the serosal treatment with BAC in the ileum and colon causes a significant change in the quantitative parameters analyzed, especially in the myenteric plexus of the ileum, and that the dilatation seen in the treated segment may alter some parameter in the submucous plexus, with no denervation taking place. There were no morphological alterations in the neurons and ganglia shape in the submucous plexus when compared with the controls; however, the myenteric plexus ganglia showed an irregular pattern due to denervation. There was also a significant increase in the area of these cells as a result of the treatment in the myenteric as well as the submucous plexus.

Acknowledgemensts

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