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Abstract: Serosal application of BAC (benzalkonium chloride) to the pyloric antrum of the rat led to a complete loss of the intrinsic innervation of the treated region and also extensive loss of extrinsic sensory projections in the treated and untreated regions of the stomach. However, no changes were seen in the numbers of primary afferents projecting to the stomach, or expression of neuropeptides or neuronal proteins in the neurons. This finding is in marked contrast to the changes reported in sensory cell bodies after sectioning of the sciatic nerve, and suggested that visceral and somatic afferents may respond differently to axonal destruction.

Key Words: BAC (benzalkonium chloride), intrinsic innervation, primary afferents, stomach, rat.

Mide'nin İnnervasyonunda BAC'nin Etkisi

Özet: Ratların pylorik antrum serozasına BAC (benzalkonium chloride) nin uygulanmasından sonra, BAC ile muamele edilen bölgede intrinsik innervasyonun tamamen kaybolduğu, ve BAC ile muamele edilmiş ve edilmemiş bölgede de ekstrinsik sensorik uzantıların kaybolduğu gözlendi. Bununla beraber mide'ye uzantı gönderen primary afferent nöronların sayısında veya bu noronlardaki nöronal protein veya nöropeptidlerin varlığında herhangi bir değişime rastlanmadı. Bu sonucun, siyatik sinirlerin kesilmesinden sonra, sensorik hücre gövdelerinde rastlanılan değişikliklerden farklı olması, viseral ve somatik afferentlerin axonal yıkıma farklı cevaplar vereceği fikrini ileri sürmektedir.

Anahtar Sözcükler: BAC (benzalkonium chloride), intrinsik innervasyon, primary afferent, mide, rat.

Introduction

Benzalkonium chloride (BAC) is a cationic detergent that disrupts cell membranes by solubilizing the lipid bilayer (1, 2). It has been demonstrated that application of BAC to the serosal surface of rat intestine leads to rapid degeneration of the superficial layers, that is, serosa, longitudinal muscle, myenteric plexus and, to a limited extent, circular muscle, and that, with the exception of myenteric neurons, these layers are rapidly reestablished (1, 3, 4). Thus, BAC can be used to selectively ablate myenteric neurons, and this technique has been used extensively in studies on the role of the myenteric plexus in regulating intestinal function (2, 5-11).

The effect of BAC on the innervation of the stomach has only recently been examined (12, 13). By radioimmunoassay, Higham et al. (13) demonstrated that serosal application of BAC to the pyloric antrum of rats resulted in depletion of gastrin releasing peptide, which is present in intrinsic gastric neurons. This depletion was detected in the antrum but not in the corpus, indicating destruction of antral myenteric neurons. In addition, levels of gastric CGRP were greatly reduced. Since gastric CGRP is derived solely from extrinsic afferents (14, 15), the data indicate that BAC treatment also destroys extrinsic sensory terminals in the stomach.

It is now well established that expression of peptides in primary afferent neurons changed in response to peripheral nerve injury. For example, transection of the sciatic nerve causes a decrease in substance P (16-19), calcitonin gene related peptide (20), cholecystokinin (17, 18) and somatostatin (17, 18) in lumbar dorsal root ganglia and dorsal horn, which is due to down regulation of peptide synthesis (19-21). In contrast, there is up regulation of synthesis of other neuropeptides, such as vasoactive intestinal polypeptide (18, 19, 22, 23), galanin (19, 21) and neuropeptide Y (21, 24). The apparent destruction of gastric CGRP fibres by BAC (13) raises the possibility that this treatment may also alter neuropeptide expression in gastric afferents.

The aims of the study described here were to establish the effects of BAC on the intrinsic innervation and extrinsic sensory projections to the stomach using morphological techniques.

Materials and Methods

Experimental methods

Six Wistar rats of both sexes were deeply anaesthetised with Hypnorm (0.3 ml/kg, i.m.) and Diazepam (2.5 mg/kg, i.p.), the stomach exposed and 0.5% BAC in saline (w/v) (n = 3) or saline (controls, n = 3) was painted onto the serosal surface of the pyloric antrum. The rats were sacrificed seven days later.

In a second group of rats, suspension of True Blue in distilled water (%5 w/v) was injected into either the pyloric antrum (n = 3) or the corpus (n = 2) with 10ml Hamilton microsyringe. A total of 20µl, in volumes of 1-2µl, was injected into each region. After each injection, the needle was left in place for up to 1 minute to reduce leakage of dye along the needle tract, and the injection site was then swabbed with saline. Viscera were replaced in the abdominal cavity, and the incision in the abdominal muscle, then the skin was sutured. Seven days later, the animals were re-anaesthetised, and treated with BAC as described above (n = 3). The remaining two animals, one from antral and one from the corpus True Blue injection groups, were treated with saline only.

Tissue processing

In the first experiment, the animals were decapitated, then the stomach was removed and fixed by immersion in Bouin's fluid. These samples were processed for wax sectioning and immunoperoxidase staining.

In the second experiment, the animals were terminally anaesthetised (overdose of sodium pentobarbitone, i.p.) and 0.1ml of heparin (5000 I.U per ml) was injected into the left ventricle of the heart. The animals were transcardically perfused with 0.1M PBS, pH 7.4, followed by 4% formaldehyde in sodium cacodylate buffer (0.1M, pH 7.4). Dorsal root ganglia from segments T_9 to T_{12} and stomach were dissected out. Tissues were placed in 0.1M sodium cacodylate, pH 7.4 containing 20% sucrose for at least 24 hours at 4°C. Samples were snap frozen in isopentane and liquid nitrogen, sectioned at 10-15µm and processed for immunofluorescence or immunoperoxidase staining.

Immunohistochemical staining

Prior to applying primary antiserum, cryostat sections were pretreated with ethanol (50% for 5 seconds, 70% for 20 minutes, 50% for 5 seconds) to increase antigenicity. For those wax or cryostat sections being stained by the immunoperoxidase technique, an additional incubation in 0.08% H_2O_2 in methanol for 5 minutes was included to reduced endogenous peroxidase.

The innervation of the stomach was examined using immunoperoxidase staining for a general neuronal marker, protein gene product 9.5 (PGP) (25), and CGRP. Sections were incubated for 14-16 hours at 4°C with rabbit polyclonal antibodies against PGP (Ultraclone Ltd, UK) or CGRP (CRB Ltd., UK) both diluted to 1:1500 in PBS containing 0.25% sodium azide and 2.5% bovine serum albumin. Sections were then incubated in goat anti-rabbit IgG (ICN Ltd), followed by rabbit peroxidase anti-peroxidase (Sigma), both at a dilution of 1:50 in PBS for 1 hour at room temperature. Sections were washed in PBS for 30 minutes after each incubation and finally immersed in GDN substrate (26) for 10 minutes. After washing in distilled water and counterstaining with eosin, sections were dehydrated and coverslips mounted with DPX.

Expression of peptides and proteins in afferents projecting to the stomach was examined by immunofluorescence staining of dorsal root ganglia. Sections were incubated with the following rabbit polyclonal antisera diluted to 1:200 in PBS containing 0.25% sodium azide and 2.5% bovine serum albumen: anti-neuropeptide Y (NPY), anti-galanin (both purchased from Peninsula Labs, UK), anti-CGRP (CRB Ltd., UK), anti-secretoneurin or with monoclonal antibodies against calbindin (Code 300, Swant, Switzerland). Incubations were performed at 4°C for 16-20 hours. Sections were then incubated in biotinylated goat anti-rabbit IgG (Sigma) at a dilution of 1:20 or biotinylated horse anti-mouse (Vector Labs, UK), followed by streptavidin-fluorescene isothiocyanate (FITC) complex diluted to 1:20 (Vector Labs, UK), both for 1 hour at room temperature. Sections were washed for 30 minutes in PBS after each incubation and mounted under coverslips in Vectashield mounting medium (Vector Labs, UK). Preparations were examined using a Leitz Dialux 20 microscope using separate filter packs to examine True Blue (pack A) and FITC fluorescence (pack 12) in the same field.

Counts of labelled dorsal root ganglion cells

The number of spinal afferents projecting to the antrum and corpus was estimated by counting True Blue-labelled cells in ganglia T_{10}-T_{12}. Counts were performed on alternate sections throughout each ganglion, and numbers pooled from right and left ganglion.

In sections stained for secretoneurin, calbindin or neuropeptides, True Blue-labelled cells containing immunoreactivity were counted and expressed as a percentage of the True Blue-labelled cells in those sections.

Results

Effect of BAC treatment on the innervation of the stomach

Staining of wax or cryostat sections for the general neuronal marker, PGP, revealed a rich innervation of all layers of the stomach wall and nerve cell bodies in the myenteric plexus in the antrum and corpus (Figs.1a,b & 2a,b). In animals treated with BAC, immunoreactivity was almost totally depleted from the antral wall, whereas the innervation of the adjacent corpus resembled that seen in control animals (Figs.1c,d & 2c,d).

CGRP-immunoreactivity was detected in cryostat but not wax sections. In control animals, immunoreactive nerve fibres were distributed in all layers of the wall of antrum and corpus (Figs.3a,b & 4a,b). No immunoreactive nerve cell bodies were detected. In BAC-treated animals, there was extensive loss of immunoreactivity in both the antrum and corpus, only rare fibres in the myenteric plexus and mucosa were labelled (Fig.3c,d & 4c,d).

Effect of BAC treatment on gastric afferents

In control animals, injection of True Blue into the wall of the stomach revealed a similar pattern of spinal projections to the corpus and antrum. Projections to both regions were highest bilaterally in ganglia from thoracic segments T_{10}-T_{12}, and were detected in similar numbers

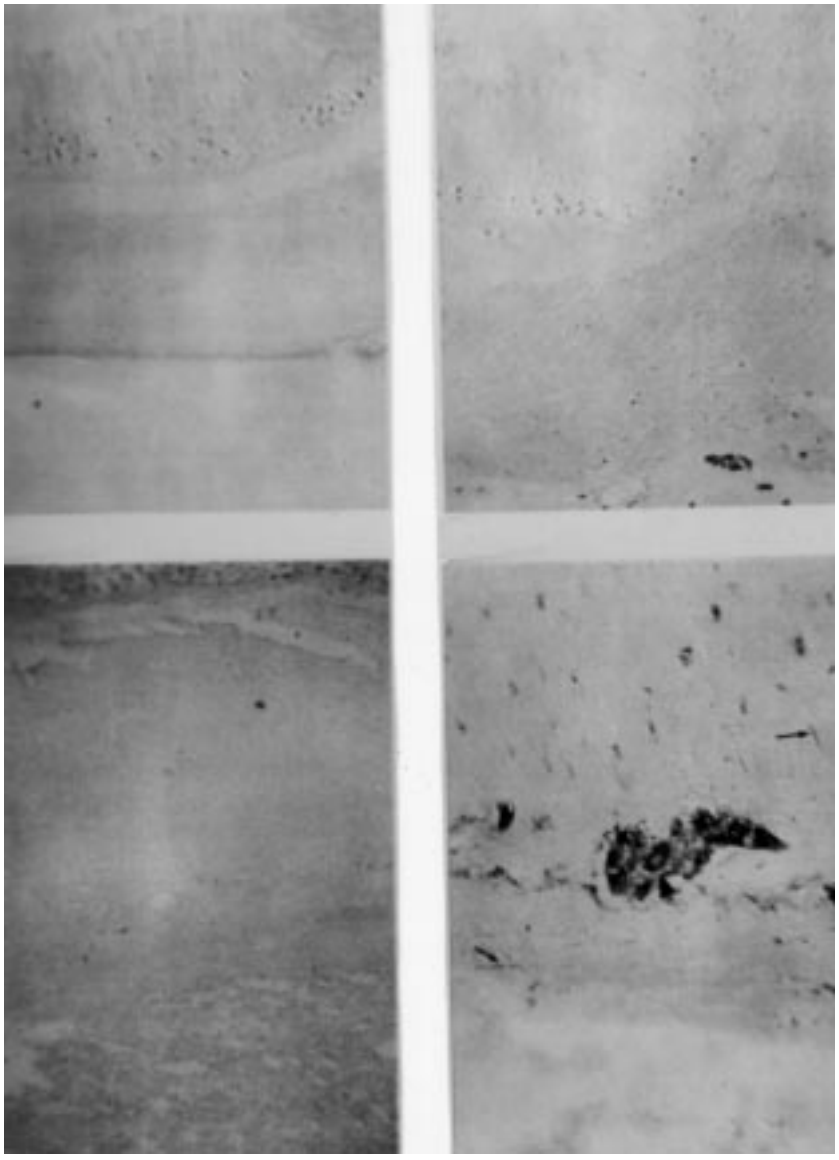

Fig. 1 a, b, c, d. Immunoperoxidase staining for PGP immunoreactivity in the antrum of control and BAC-treated rats.

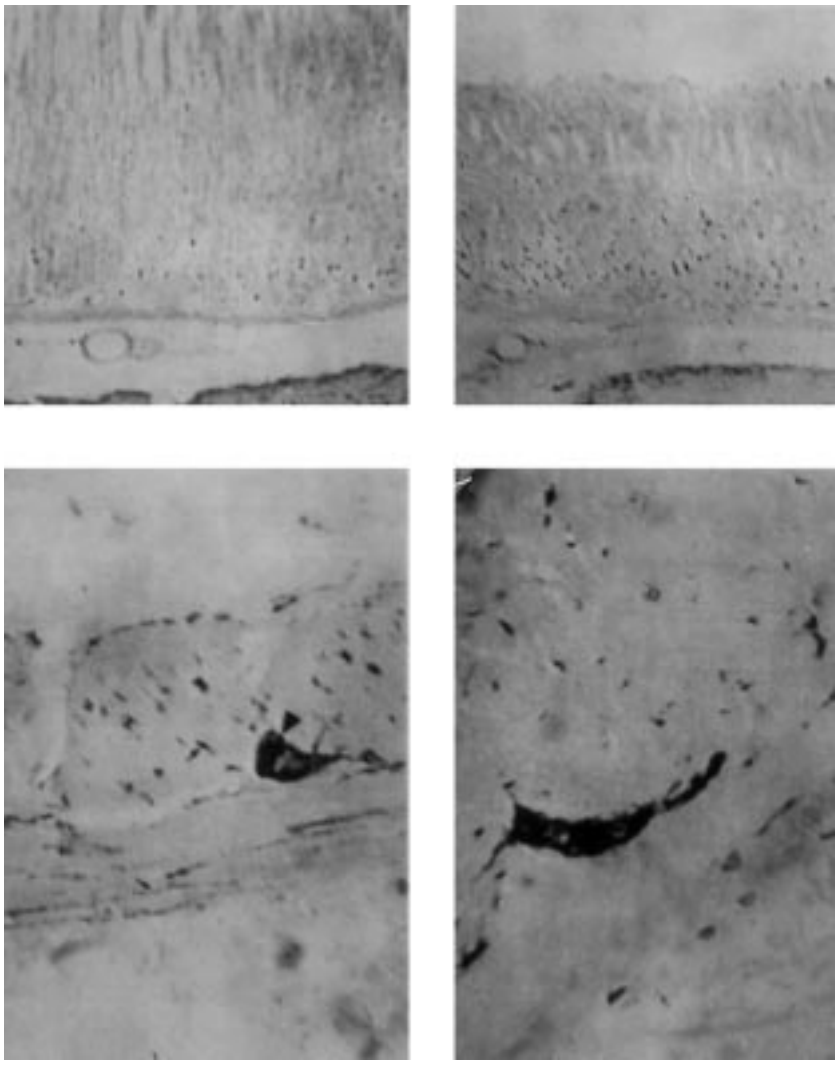

Fig. 2 a, b, c, d. Immunoperoxidase staining for PGP immunoreactivity in the corpus of control and BAC-treated rats.

(Table 1). In BAC-treated animals, there was no reduction in the number of True Blue-labelled cells in these ganglia, following injection into either the antrum or corpus (Table 1). Neither was there a reduction in the proportion of True Blue-labelled cells immunoreactive for CGRP (Table 2a,b), calbindin (Table 3a,b) or secretoneurin (Table 4a,b) in control and BAC-treated animals (Tables 2a,b to 4a,b). Galanin- and NPY-immunoreactive cells were not detected in cells in these ganglia in control or BAC-treated rats.

Discussion

The use of benzalkonium chloride (BAC) to selectively destroy intestinal myenteric neurons is well established (1-5, 7, 9, 11, 27, 28). However, the effect of BAC on gastric myenteric neurons has only recently been examined (12, 13). Radioimmunoassay data demonstrated a loss of neuropeptides present in myenteric neurons in the treated region of the stomach (13). In the present study, staining for the general neuronal marker, PGP, confirmed a loss of myenteric neurons, and also demonstrated a loss of nerve fibres from all layers of the wall in the BAC-treated region of the stomach, whereas there were no apparent changes in the innervation of adjacent untreated regions. Thus, the morphological data confirm that BAC can be used to ablate myenteric neurons in specific regions of the stomach. The extensive loss of nerve fibres indicates that BAC additionally destroys extrinsic projections to the stomach. The two previous studies also found a loss of extrinsic fibres. A reduction in parasympathetic fibres

Table 1. The number of True Blue-labelled cells in DRG from control rats and BAC-treated rats after injection of True Blue into either the corpus or the antrum (pooled counts from DRGs left and right T_{10}-T_{12}, 5 rats).

Animal	Injection site	Segment	TB cell	Treatment
Rat 1	corpus	T10	100	saline
Rat 2	corpus	T10	146	BAC
Rat 3	antrum	T10	110	saline
Rat 4	antrum	T10	124	BAC
Rat 5	antrum	T10	166	BAC
Rat 1	corpus	T11	225	saline
Rat 2	corpus	T11	229	BAC
Rat 3	antrum	T11	140	saline
Rat 4	antrum	T11	178	BAC
Rat 5	antrum	T11	194	BAC
Rat 1	corpus	T12	76	saline
Rat 2	corpus	T12	88	BAC
Rat 3	antrum	T12	45	saline
Rat 4	antrum	T12	76	BAC
Rat 5	antrum	T12	86	BAC

Table 2. (a) Percentage of gastric spinal afferents containing CGRP-immunoreactivity in control and BAC-treated rats after injecting TB into the corpus (pooled counts from DRGs left and right T_{11}, 2 rats).

Animal	Injection site	Treatment	TB cell	TB cell +CGRP	% of TB cell +CGRP
Rat 1	corpus	saline	46	22	47.8
Rat 2	corpus	BAC	27	13	48.1

(b) Percentage of gastric spinal afferents containing CGRP-immunoreactivity in control and BAC-treated rats after injecting TB into the antrum (pooled counts from DRGs left and right T_{11}, 3 rats).

Animal	Injection site	Treatment	TB cell	TB cell +CGRP	% of TB cell +CGRP
Rat 3	antrum	saline	57	18	31.5
Rat 4	antrum	BAC	44	14	31.8
Rat 5	antrum	BAC	62	18	29

projecting to the stomach was demonstrated morphologically Neuberger by et al. (12), whereas Higham and colleagues (13) found reduced levels of the sensory neuropeptide, CGRP. In the present study, a loss of sensory CGRP-immunoreactive fibres was demonstrated in both the antrum and in the untreated corpus. The fact that the loss of these fibres was not detected when staining for PGP is due to the density of the general innervation of the corpus. The widespread loss of CGPR-immunoreactive fibres confirms the previous finding of reduced levels of the peptide in both regions of the stomach after BAC application to the antrum (13), and suggests that CGRP-immunoreactive fibres projecting to the corpus and antrum enter the stomach via the pyloric antrum.

In contrast to the clear depletion of CGRP afferent fibres in the stomach of BAC-treated animals, no changes were detected in the cell bodies of these neurons. In this pilot study, a loss of cells or differences in the proportion of gastric afferents expressing neuropeptides, calbindin or secretoneurin, were not observed. Thus, the changes induced in dorsal root ganglia following nerve transection, that is, a decreased number of cells immunoreactive for CGRP, and an increased number

Animal	Injection site	Treatment	TB cell	TB cell +Calb	% of TB cell +Calb
Rat 1	corpus	saline	27	8	29.3
Rat 2	corpus	BAC	27	9	33.3

Table 3. (a) Percentage of gastric spinal afferents containing calbindin-immunoreactivity in control and BAC-treated rats after injecting TB into the corpus (pooled counts from DRGs left and right T_{11}, 2 rats).

Animal	Injection site	Treatment	TB cell	TB cell +CGRP	% of TB cell +CGRP
Rat 3	antrum	saline	20	9	45
Rat 4	antrum	BAC	18	6	33.3
Rat 5	antrum	BAC	13	6	46.2

(b) Percentage of gastric spinal afferents containing calbindin-immunoreactivity in control and BAC-treated experimental rats after injecting TB into the antrum (pooled counts from DRGs left and right T_{11}, 3 rats).

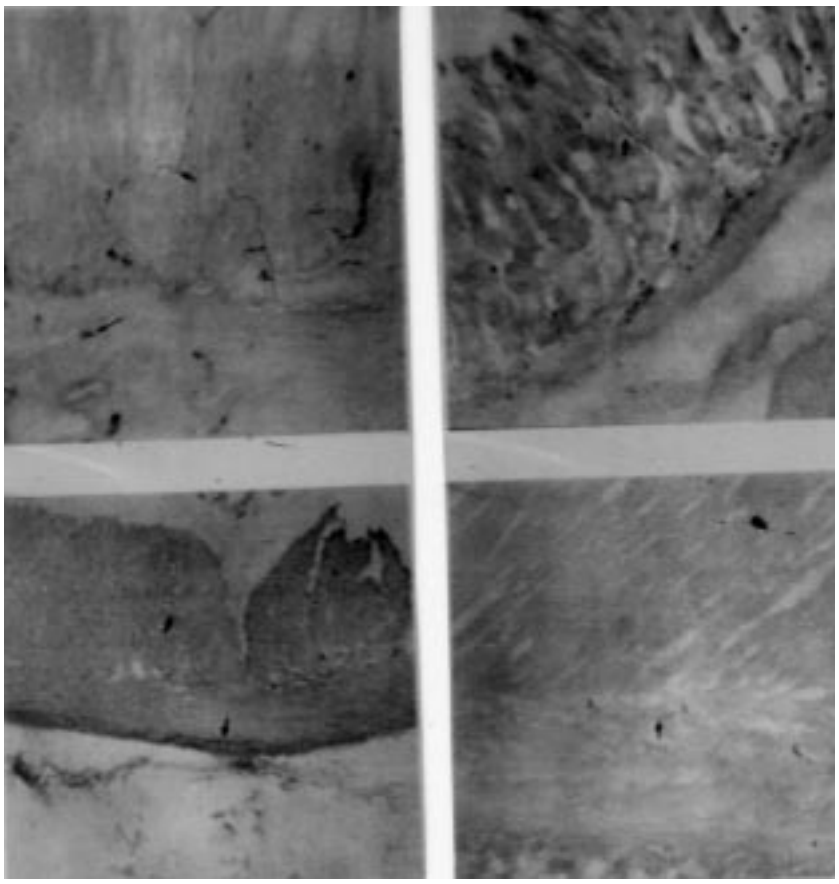

Fig. 3 a, b, c, d. Immuno-peroxidase staining for CGRP immunoreactivity in the antrum of control and BAC-treated rats.

immunoreactive for NPY and galanin (19-21, 24) do not appear to be induced by BAC destruction of axons. Although the methods of peripheral nerve injury are different, both result in a long term loss of peripheral projections. Loss of CGRP-immunoreactive afferent terminals was examined after only one week, but Neuberger et al. (12) have demonstrated a loss of parasympathetic projections to the stomach 9 months after BAC treatment. Both situations involve C-fibre afferents, unmyelinated afferents constituting around

Table 4. (a) Percentage of gastric spinal afferents containing secretoneurin-immunoreactivity in control and BAC-treated rats after injecting TB into the corpus (pooled counts from DRGs left and right T_{11}, 2 rats).

Animal	Injection site	Treatment	TB cell	TB cell +SN	% of TB cell +SN
Rat 1	corpus	saline	17	7	41.2
Rat 2	corpus	BAC	10	4	40

(b) Percentage of gastric spinal afferents containing secretoneurin-immunoreactivity in control and BAC-treated experimental rats after injecting TB into the antrum (pooled counts from DRGs left and right T_{11}, 3 rats).

Animal	Injection site	Treatment	TB cell	TB cell +SN	% of TB cell +SN
Rat 3	antrum	saline	10	4	40
Rat 4	antrum	BAC	22	9	40.9
Rat 5	antrum	BAC	17	6	35.3

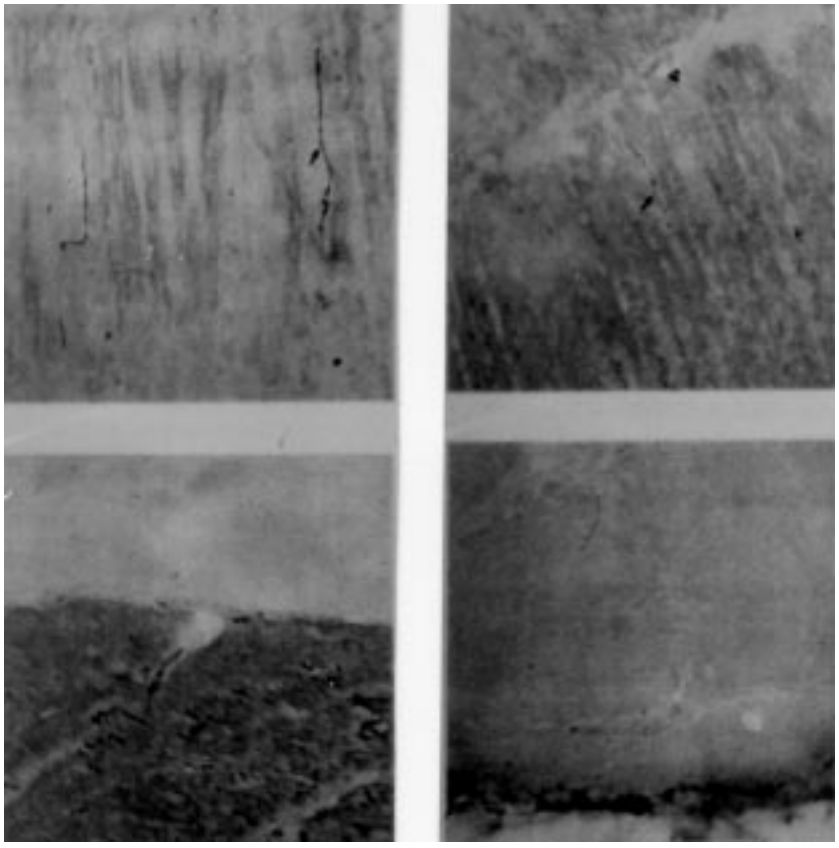

Fig. 4 a, b, c, d. Immunoperoxidase staining for CGRP immunoreactivity in the corpus of control and BAC-treated rats.

48% of the axons in the rat sciatic nerve (29), and both situations involve a large proportion of CGRP afferents (15, 20, 30). However, those in the sciatic nerve are somatic afferents in contrast to the visceral afferents examined in the present study. Thus, it is possible that somatic and visceral C-fibre afferents respond differently to axonal damage.

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