J. Pharm. Pharmacol. 1990, 42: 123–125
Communicated May 19, 1989

Resistance of myenteric neurons in the rat's colon to depletion by 1,8-dihydroxyanthraquinone

E. A. HEINICKE, J. A. KIERNAN, *Department of Anatomy, The University of Western Ontario, London, Ontario N6A 5C1, Canada*

Abstract—Earlier reports have suggested that anthraquinone purgatives in excessive amounts cause degeneration of neurons in the enteric nervous system. Danthron (1,8-dihydroxyanthraquinone) was administered to rats in their drinking water for four months. The effects of the drug on the total number of neurons and on the immunoreactivity of eight putative neurotransmitters in the myenteric plexus of the colon have been assessed. No differences were found between the treated animals and their controls, indicating that the drug does not kill myenteric neurons. These results agree with recent observations on the effects of senna in rats and mice, and do not support earlier claims that myenteric neurons are killed by anthraquinone purgatives.

In the human 'cathartic colon' due to excessive intake of anthraquinone purgatives such as senna, the large intestine becomes atonic and dilated. This condition was attributed by Smith (1972a, 1973) to loss of neurons from the myenteric plexus. She described similar changes in mice (Smith 1968, 1972b), and it has since been widely believed that prolonged administration of anthraquinone purgatives kills the intrinsic neurons of the colon (Sladen 1972; Smith 1972a; Cummings 1974; Oster et al 1980). However, we could not confirm the alleged neuronotoxicity of senna (Kiernan & Heinicke 1989), so we examined danthron (1, 8-dihydroxyanthraquinone), which is the simplest purgative anthraquinone (Oster et al 1980).

Materials and methods

Male Long-Evans rats (94–114 g) were given 0·04% (w/v) danthron (1,8-dihydroxyanthraquinone, Sigma Chemical Co., St. Louis, MO) in 0·25% Na_2CO_3 and 3% sucrose, instead of drinking water. The Na_2CO_3 was needed to dissolve the danthron, and the sucrose was necessary to induce the rats to drink adequate amounts of the solution. Control rats were given 0·25% Na_2CO_3 and 3% sucrose. A second control group consisted of rats receiving plain tap water. The mean daily intake of water was 210 mL kg^{-1} in both groups of rats, and the treated animals rats consumed, on average, 79 mg kg^{-1} of danthron per day.

After four months, the rats were exsanguinated under ether anaesthesia, and the intestines were removed and weighed. The colons were flushed with saline and incubated in a modified Krebs solution (composition in mM, after Furness 1969: NaCl, 132·2; KCl, 4·7; $CaCl_2$, 2·8; $MgCl_2$, 0·6; NaH_2PO_4, 1·3; $NaHCO_3$, 16·3; Na_2SO_4, 0·6; dextrose, 7·7) containing colchicine (10^{-5} gl L^{-1}) and bubbled with 5% CO_2–95% O_2, for 4 h at 37°C. This procedure enhances immunohistochemical staining of neuronal somata (Costa et al 1980b) by suppressing rapid anterograde axonal transport. Each colon was then measured and cut into 3 cm lengths, which were threaded onto glass rods and immersed in appropriate fixatives: 60 min in modified Carnoy (Mendelson et al 1983) for neuron counting, or 18 h in buffered picrate-formaldehyde (Costa et al 1980a) for immunohistochemistry. Whole-mount preparations were made, consisting of the external muscular layers and the included myenteric plexus.

Correspondence to: J. A. Kiernan, Department of Anatomy, The University of Western Ontario, London, Ontario, N6A 5C1, Canada.

Neuronal somata were selectively stained with cuprolinic blue as previously described (Heinicke et al 1987). The length of each stained, mounted specimen was measured, so that overall shrinkage could be determined. The neurons were counted, and (with allowance for shrinkage) the total number of neurons in a 1 cm length of fresh colon was calculated (Heinicke et al 1987).

Preparations for immunohistochemical staining were defatted and treated with octylphenoxypolyethoxyethanol (Triton X-100; Sigma, St. Louis, MO) to facilitate penetration of antibodies (Costa et al 1980a). The specimens were incubated (65 h, 4°C) in primary antisera diluted according to the suppliers' recommendations in 0·1 M sodium phosphate buffer, pH 7·4, containing 1% goat serum and 0·3% Triton X-100. Rabbit antisera to the following were used: substance P, somatostatin, bombesin (ICN Biomedicals Canada Ltd., Montreal), 5-hydroxytryptamine Leu-enkephalin (Immuno Nuclear Corp., Stillwater, MN), cholecystokinin/gastrin, Met-enkephalin (Amersham Canada, Oakville Ont.) and tyrosine hydroxylase (Eugene Tech International, Allendale, NJ). Control specimens were incubated in diluted non-immune rabbit serum. Tissues were processed by the avidin-biotin method using a Vectastain ABC kit (Dimension Laboratories, Mississauga, Ont.). The bound peroxidase was demonstrated by incubation for 15–20 min in 0·025% 3,3′-diaminobenzidine tetrahydrochloride and 0·004% H_2O_2 in 0·1 M Tris-HCl buffer, pH 7·2.

Results and discussion

The danthron-treated animals showed no signs of ill health, and the mean weights of the animals in the experimental and control groups did not differ significantly at any time. The faeces of normal rats are hard and dry. After two months of treatment many of the faecal pellets of the danthron-treated rats were large and moist, but the same animals also produced pellets of normal appearance. In rodents, purgation by anthracene derivatives results in the production of soft, wet faecal pellets (Brittain et al 1962). By this standard, doses of danthron comparable to those found effective in mice (Dufour & Gendre 1984) and guinea-pigs (Verhaeren et al 1981) had a somewhat weaker purgative action in our rats.

The small intestines of animals treated with danthron weighed 12·8 ± 0·5 g, which was significantly more than those from control animals (11·5 ± 0·4 g; $P < 0·05$), but the weights of the caecum (8·2 ± 0·8 g) and colon (5·7 ± 0·6 g) were not significantly changed by the treatment. Nor did the treatment significantly change the length of the small intestine (114 ± 0·4 cm) or colon (18·0 ± 1·0 cm). The sennosides act only on the colon, where the active hydroxyanthraquinone is released by bacterial glycosidases (Dreesen et al 1981). However, unconjugated hydroxyanthraquinones like danthron can act also on the small intestine, suppressing the absorption of water and electrolytes by inhibiting an epithelial ATPase (Leng-Peschlow 1980). Thus the greater weights of the small intestines of our treated rats may have been due to increased water content of the digesta.

The neuron counts from control and treated rats did not differ from those of a group of eight normal water-drinking rats when the three groups were compared by one-factor analysis of variance. Thus, the sodium carbonate and sucrose solution (in which the danthron was dissolved) had no effect on the numbers

Table 1. Numbers of neurons in the myenteric plexus of the colon from danthron-treated and control rats.

Portion of colon	Total number of neurons		
	Treated[a] $n^d=6$	Control[b] $n^d=6$	Significance[c]
Proximal half	235 800 ± 58 700	189 400 ± 34 200	NS ($P>0.05$)
Distal half	196 400 ± 24 500	219 600 ± 12 500	NS ($P>0.10$)
Total colon	432 200 ± 61 200	409 600 ± 40 700	NS ($P>0.10$)

[a] Average dose of danthron per kg body weight was 79 mg in 197 mL of 0·025% Na_2CO_3 and 3% sucrose.
[b] Average daily consumption of 0·025% Na_2CO_3 and 3% sucrose was 220 mL per kg body weight.
[c] Refers to the comparison of the values in the preceding two columns by Student's t-test. NS—difference between values is not significant. P—probability of rejecting a true null hypothesis.
[d] n = number of animals in group.

of neurons in the myenteric plexus of the colon. The numbers of neurons in the myenteric plexuses of the colons of the treated and control animals are displayed in Table 1. Treatment with danthron did not significantly change the numbers of neurons.

The immunoreactivity of each antigen was assessed with respect to the following criteria: overall appearance of the plexus; immunoreactive cell bodies and axons within ganglia; unlabelled neuronal somata (outlined by stained neuropil); axons in strands between ganglia; and axons within the smooth muscle. With all the antisera, dark brown staining indicating immunoreactivity was seen in beaded axons in the myenteric ganglia and their interconnecting strands and within the circular muscle of the colon. Immunoreactive perikarya were seen in myenteric ganglia with antisera to all antigens other than tyrosine hydroxylase, an enzyme largely confined to extrinsic (sympathetic) axons (Furness & Costa 1980; Gershon 1981). Specimens incubated in non-immune rabbit serum exhibited a generalized light brown colour in the smooth muscle, but no staining of neuronal somata or axons. A detailed descriptive account of the immunohistochemical appearances in the colon of the normal rat and mouse will be published elsewhere (Heinicke & Kiernan, J. Anat. in press).

No differences were observed between the staining of the myenteric plexuses of treated and control animals with any of the antisera used. There were variations in the distributions of different immunoreactive materials, but each primary antiserum presented a similar appearance in danthron-treated and control animals.

Prolonged administration of danthron did not induce the distended colon that was to be expected if the number of enteric neurons had been diminished (Krishnamurthy et al 1985; Smith 1973, 1982). Although the total number of neurons in the myenteric plexus was not depressed by the treatment, certain subpopulations might have been selectively damaged without significantly affecting the overall count. However, there were no qualitatively discernible changes attributable to danthron in the distributions of several neurotransmission-related antigens known to occur in the enteric nervous system (Furness & Costa 1980; Gershon 1981). The only published report of a neurotoxic effect of danthron is that of Dufour & Gendre (1984), who described vacuoles and lysosomes in axons close to the mucosal surfaces of the small and large intestines of mice. They gave 200 mg kg^{-1} daily by gastric tube, so the concentration in contact with the mucosa was probably much higher than in our rats, which received a mean dose of 79 mg kg^{-1} of danthron in their drinking water.

We conclude that danthron probably has no direct neurotoxic action on the myenteric plexus, and that the purgative action of the drug is probably due to its action on the absorptive epithelium. Our findings agree with the similarly demonstrated lack of neuronotoxic effects of senna in rats and mice (Kiernan & Heinicke 1989), and do not support the view (Sladen 1972; Smith 1973; Cummings 1974) that hydroxyanthraquinones kill myenteric neurons. Excessive use of laxatives by humans lasts for years, so experiments with small rodents may not mimic the clinical disorder. However, four months is approximately one third of the natural life span of a laboratory mouse, and it may therefore be reasonable to expect signs of chronic toxicity to develop during this time.

This research was partly supported by a grant to J.A.K. from the Medical Research Council of Canada. We thank John Wijsman, Karen Starkey and Gerald Tighe for technical assistance.

References

Brittain, R. T., D'Arcy, P. F., Grimshaw, J. J. (1962) Observations on the use of a mouse bioassay method for investigating purgative activity. J. Pharm. Pharmacol. 14: 715–721

Costa M., Buffa, R., Furness, J. B., Solcia, E. (1980a) Immunohistochemical localization of polypeptides in peripheral autonomic nerves using whole mount preparations. Histochemistry 65: 157–165

Costa, M., Furness, J. B., Buffa, R., Said, S. I. (1980b) Distribution of enteric nerve cell bodies and axons showing immunoreactivity for vasoactive intestinal polypeptide in the guinea-pig intestine. Neuroscience 5: 587–596

Cummings, J. H. (1974) Laxative abuse. Gut 15: 758–766

Dreesen, M., Eyssen, H., Lemli, J. (1981) Metabolism of sennosides A and B by the intestinal microflora: in vitro and in vivo studies on the rat and the mouse. J. Pharm. Pharmacol. 33: 679–681

Dufour, P., Gendre, P. (1984) Ultrastructure of mouse intestinal mucosa and changes observed after long term anthraquinone administration. Gut 25: 1358–1363

Furness, J. B. (1969) An electrophysiological study of the innervation of the smooth muscle of the colon. J. Physiol. (Lond.) 205: 549–562

Furness, J. B., Costa, M. (1980) Types of nerves in the enteric nervous system. Neuroscience 5: 1–20

Gershon, M. D. (1981) The enteric nervous system. Ann. Rev. Neurosci. 4: 227–272

Heinicke, E. A., Kiernan, J. A., Wijsman, J. (1987) Specific, selective, and complete staining of neurons of the myenteric plexus, using cuprolinic blue. J. Neurosci. Methods 21: 45–54

Kiernan, J. A., Heinicke, E. A. (1989) Sennosides do not kill myenteric neurons in the colon of the rat or mouse. Neuroscience 30: 837–842

Krishnamurthy, S., Schuffler, M. D., Rohrmann, C. A., Pope, C. E. (1985) Severe idiopathic constipation is associated with a distinc-

tive abnormality of the colonic myenteric plexus. Gastroenterol. 88: 26–34
Leng-Peschlow, E. (1980) Inhibition of intestinal water and electrolyte absorption by senna derivatives in rats. J. Pharm. Pharmacol. 32: 330–335
Mendelson, D., Tas, J., James, J. (1983) Cuprolinic blue: a specific dye for single-stranded RNA in the presence of magnesium chloride. II. Practical applications for light microscopy. Histochem. J. 15: 1113–1121
Oster, J. R., Materson, B. J., Rogers, A. I. (1980) Laxative abuse syndrome. Am. J. Gastroenterol. 74: 451–458
Sladen, G. E. (1972) Effects of chronic purgative abuse. Proc. Roy. Soc. Med. 65: 288–291
Smith, B. (1968) Effect of irritant purgatives on the myenteric plexus in man and the mouse. Gut 9: 139–143
Smith, B. (1972a) Pathology of cathartic colon. Proc. Roy. Soc. Med. 65: 228
Smith, B. (1972b) The Neuropathology of the Alimentary Tract. Edward Arnold, London. pp 92–98
Smith, B. (1973) Pathologic changes in the colon produced by anthraquinone purgatives. Dis. Colon Rectum 16: 455–458
Smith, B. (1982) The neuropathology of pseudo-obstruction of the intestine. Scand. J. Gastroenterology 71 (17 Suppl.): 103–109
Verhaeren, E. H. C., Verbruggen, A. M., Lemli, J. A. J. M. (1981) The effect of 2,4,6-triaminopyrimidine on increased mucosal permeability of guinea-pig colonic mucosa, following administration of 1,8-dihydroxyanthraquinone, salicylic acid and dinitrophenol. Pharmaceut. Weekbl. Sci. Ed. 3: 815–819

J. Pharm. Pharmacol. 1990, 42: 125–127
Communicated August 10, 1989

Gastric mucosal cytoprotection in the rat by naftidrofuryl oxalate

AWS S. SALIM, *University Department of Surgery, The Royal Infirmary, Glasgow G4 0SF, UK*

Abstract—Ischaemic gastric mucosal injury was assessed in the rat by measurement of the area of the injury produced after 6 h by reserpine (5 mg kg^{-1} i.p.) or 5-hydroxytryptamine (5-HT) (50 mg kg^{-1} i.p.). Pretreatment with naftidrofuryl 1 mL, 1% by gavage significantly ($P < 0.001$) protected the rat stomach against the reserpine (24 ± 2.7 mm^2 vs 40 ± 4.7 mm^2, mean ± s.e.m., n = 10) and 5-HT injury (11.4 ± 1.7 mm^2 vs 27 ± 4.1 mm^2, mean ± s.e.m., n = 10). Naftidrofuryl 1 mL 2% by gavage was more effective ($P < 0.001$) in this respect and mucosal injury only developed in 50% of rats injected with reserpine (9.4 ± 1.1 mm^2) and 30% of those injected with 5-HT (3.2 ± 0.4 mm^2). Administration of naftidrofuryl 1 mL 5% by gavage completely protected the rat against both the reserpine- and 5-HT-induced acute gastric mucosal injury. This protection was not associated with any significant influence on the H^+ output.

Clinical and laboratory experience has confirmed the role of ischaemia as a mechanism of acute gastric mucosal injury (Lucas et al 1971; Ritchie 1975). Naftidrofuryl oxalate is a vasodilator (Fontaine et al 1968, 1969) which directly enhances tissue oxidative metabolism by activation of succinic dehydrogenase (Eichhorn 1969; Meynaud et al 1973). This action prompted investigation of the effect of the drug on ischaemic injury of the rat gastric mucosa induced by reserpine (Salim 1987) or 5-hydroxytryptamine (5-HT) (Ferguson et al 1973).

Materials and methods

Animals. Groups of ten Sprague-Dawley rats of either sex, 220–280 g, were fasted for 24 h before experimentation. Animals were housed in cages with wide mesh wire bottoms to prevent coprophagy.

Source and preparation of drugs. Solutions of naftidrofuryl oxalate (Praxilene, Lipha Ltd, West Drayton, Middlesex, England) were prepared by dissolving the powder in physiological saline. All other drugs were supplied by Sigma (St. Louis, MO, USA). A 1 mg mL^{-1} solution of reserpine was prepared by dissolving 80 mg crystalline powder in 0·3 mL glacial acetic acid (BP) and the volume made up to 80 mL with double distilled water. 5-Hydroxytryptamine powder was dissolved in double distilled water to prepare a 10 mg mL^{-1} solution. Injections were administered intraperitoneally into the left iliac fossa using a 25 G needle and gavage was undertaken under light ether anaesthesia using a 6 FG Infant's Feeding Tube 400/420 (Portex Ltd, Hythe, UK). Solutions were freshly prepared each day.

Surgery. Animals were anaesthetized by inhalation of diethyl ether or by intraperitoneal injection of 25 mg kg^{-1} pentobarbitone (Sagatal, May and Baker, Dagenham, England) into the left iliac fossa. When indicated, supplementary doses of pentobarbitone were given to maintain narcosis. The pyloric sphincter was ligated then the abdomen was closed as described by Salim (1988a). Tracheostomy was done as detailed elsewhere (Salim 1988b) to overcome respiratory distress from intubation.

Experimental design. One mL of 1,2 or 5% naftidrofuryl oxalate or 1 mL saline was instilled into the stomach by orogastric intubation. Animals were allowed to recover from anaesthesia then were injected with reserpine (5 mg kg^{-1}), 5-HT (50 mg kg^{-1}), or saline (5 mL kg^{-1}). Five h later, they were anaesthetized with pentobarbitone, submitted to tracheostomy and orogastric intubation with a 6 FG tube. The gastric fasting secretion was recovered by slowly instilling 1 mL of double distilled water and recovering all gastric contents. The gastric secretion was then recovered every 15 min for 1 h and the H^+ output (μmol h^{-1}) determined by titration to pH 7·0 with 0·1 M NaOH using an automatic titrator (Radiometer, Copenhagen). At the end of this hour animals were killed by ether overdose and the stomach removed and opened along the greater curvature. After washing with a direct stream of cold water the stomachs were pinned out and independently examined for the presence of mucosal injury macroscopically, each injury being measured as the maximum length and width, and the surface area (mm^2) calculated. The total injury score was obtained for each animal and the mean injury score calculated for each study group. Sections of injured and apparently uninjured gastric mucosa were examined microscopically.

To minimize day-to-day variation in response to treatment, the study was conducted over several days and animals were allocated to the control and all of the treatment groups on each experimental day.

Correspondence and present address: A. S. Salim, Department of Surgery, Ward 6, Stobhill General Hospital, Glasgow G21 3UW, UK.