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Absence of protective role of afferent nerves in early intestinal mucosal alterations induced by abdominal irradiation in rats

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Abstract.

Purpose: To assess the early effects of primary afferent nerve suppression by systemic treatment with the neurotoxin capsaicin in an acute model of abdominal irradiation in rats (10 Gy, gamma).

Materials and methods: Changes in myeloperoxidase (MPO) activity, calcitonin gene-related peptide (CGRP) tissue content, number of mast cells and apoptotic cells were determined in jejunum and ileum in four groups of rat male Wistar (vehicle sham-irradiated, vehicle irradiated, capsaicin sham-irradiated and capsaicin irradiated) at 1 and 3 days post-irradiation.

Results: In vehicle irradiated rats, CGRP was significantly increased from the first day after irradiation in jejunal mucosa; MPO activity increased in both segments at day 3 but not at day 1 after irradiation; the number of detectable mucosal mast cells dropped to nearly zero on days 1 and 3, while the apoptotic cells in the intestinal mucosa were significantly increased at day 1. Similar results were obtained for mast cells and apoptosis in capsaicin irradiated rats as compared to capsaicin sham-irradiated rats, while MPO activity was significantly increased and CGRP concentration in jejunal mucosa significantly decreased from the first day in these rats in comparison with capsaicin sham-irradiated rats.

Conclusions: Intestinal sensory innervation seems not to have a major protective role against a radiation-induced intestinal inflammatory reaction.

1. Introduction

Application of local doses of ionizing radiation for radiotherapy of abdominal and pelvic malignancies leads to acute gastrointestinal symptoms seen within hours to days, as well as to chronic radiation enteropathy within months to years (Yeoh *et al.* 1993). Acute radiation enteritis is characterized by diarrhea, nausea and vomiting while chronic enteropathy is dominated by haemorrhage and ulceration. The origin of these symptoms is not clear but they are classically attributed to the breakdown of epithelial integrity associated with a decrease in water absorption combined with excessive secretion and loss of sodium and chloride, leading

to diarrhea and electrolyte imbalance following both whole body (Gunter-Smith 1986) and abdominal irradiation (Empey *et al.* 1992, Chun *et al.* 1997). This mucosal breakdown is preceded by an acute inflammatory response which appears by 4–8 h after abdominal irradiation and has been identified with parameters such as increased microvascular permeability, significant mucosal infiltration of neutrophils or oedema of lamina propria (Buell and Harding 1989). Some studies also reported the effects of both total body or abdominal irradiation on mast cell degranulation (Harari *et al.* 1994, Sedgwick and Ferguson 1994), and which suggested a role for mast cell mediators in the inflammatory response.

It has been reported that abdominal irradiation induces early neurally mediated intestinal responses. For example, abdominal intestinal motor patterns such as giant migrating contractions or retrograde giant contractions occur a few hours after irradiation in dogs (Erickson *et al.* 1994). In the rat colon, neurally evoked electrolyte transport has been found to be dramatically reduced on the first day after exposure to ionizing radiation (François *et al.* 1998). These alterations of intestinal neural functions are supported by increased expression of transmitters of neural afferents, such as substance P (SP), after whole body irradiation (Esposito *et al.* 1996) and vasoactive intestinal peptide (VIP) and substance P after irradiation of upper abdomen in rats (Hockerfelt *et al.* 2000). Furthermore, an increase in CGRP-immunoreactive nerve fibres has been also reported after whole body irradiation in rats (Zhang *et al.* 1998). Other authors have demonstrated modifications in neural functions after different modes of irradiation in human studies, suggesting a significant role for nerves in inflammatory response of radiation-induced enteropathy. Radiotherapy of the abdominal sphere has been shown to modify the innervation of the lamina propria in human bowel (Hirschowitz and Rode 1991) as well as to increase colonic immunohistochemical expression of VIP and SP (Hockerfelt *et al.* 1999, Forsgren *et al.* 2000). It is interesting that little attention has been given to the effects of ionizing

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radiation on the neural control of intestinal function even though this was suggested over 40 years ago (Conard 1956).

A role for the neurotransmitters of sensory afferents, such as tachykinins or calcitonin gene-related peptide (CGRP), in the control of intestinal inflammation is now accepted (Sharkey 1992, Maggi 1997). Despite some conflicting reports, it is well established that capsaicin-sensitive afferents play a protective role against experimental intestinal inflammation in animals (Reinshagen *et al.* 1996, McCafferty *et al.* 1997, Mazelin *et al.* 1998a, Holzer 1998). In addition, a proinflammatory role of sympathetic nerves has been shown (McCafferty *et al.* 1997). CGRP is released from sensory nerve fibres in the gut wall during the time course of experimental colitis in animals (Eysselein *et al.* 1992, Keates *et al.* 1998) and in inflammatory bowel disease in humans (Eysselein *et al.* 1992). However, in contrast to the effects observed with tachykinin receptor antagonists (Mazelin *et al.* 1998b), CGRP receptor antagonists have been found to aggravate (Reinshagen *et al.* 1998) and CGRP to decrease (Mazelin *et al.* 1999) the severity of experimental colitis, suggesting that CGRP exerts mucosal protection during intestinal inflammation. Since early intestinal alterations induced by abdominal irradiation involve both inflammatory and neuronal pathways, the question arises as to whether sensory afferents play a role in these alterations.

The aim of this study was to determine whether capsaicin-sensitive denervation modifies some parameters which are characteristic of the early mucosal alterations induced by abdominal irradiation in jejunum and ileum in rats. In addition tissue concentrations of CGRP, a mediator characteristic of capsaicin-sensitive afferents (Sternini *et al.* 1987), were measured in tissue extracts of intestinal wall.

2. Materials and methods

2.1. Animals

Experiments were performed in male Wistar rats (Janvier, Le Genest Saint Isle, France) initially weighing 200–250 g. Rats were housed under controlled lighting conditions, with standard diet (Usine d'Alimentation Rationnelle, Epinay-sur-Orge, France) and water provided *ad libitum*. All protocols were conducted according to the French regulations for animal experimentation (Ministry of Agriculture, Act 87-848, 19 October 1987).

2.2. Capsaicin treatment

Sensory denervation was performed according to the protocol described by Holzer (Holzer 1991). Rats

were injected subcutaneously on 4 consecutive days with capsaicin (Sigma Chemical, St Quentin-Fallavier, France; 40 mmol/l in 80% NaCl 0.9%, 10% ethanol and 10% tween 80). A total dose of 125 mg/kg was administered under anaesthesia (ketamine 60 mg/kg, ip; xylazine 5 mg/kg, ip) in doses of 10, 25, 40 and 50 mg/kg on days 1–4, respectively. Control animals received equal volumes of vehicle. The effectiveness of capsaicin treatment was assessed according to Gamse (Gamse 1982) by means of the eye-wiping test, which consists of impaired chemosensitivity of corneal afferents to one drop of 1% NH_4OH instilled into the eye. Animals treated with capsaicin that showed any wiping movement were excluded from the study. Capsaicin-treated and vehicle-treated animals were irradiated 10 days after treatment.

2.3. Irradiation protocol

Rats were exposed to abdominal gamma (^{60}Co source, 3900 Ci) irradiation with a single dose of 10 Gy (0.96 Gy/min) under anaesthesia induced by pentobarbital (60 mg/kg; i.p.). The irradiation source was placed in the vertical position and collimated to delimit an irradiation field (6 cm) and animals were irradiated in a zone comprised between xyphoid cartilage and iliac crests. Sham-irradiated rats were anaesthetised in the same conditions but not exposed to the radioactive source.

Four groups of rats (six rats in each group) were examined and sacrificed 1 and 3 days post-irradiation: vehicle sham-irradiated, vehicle irradiated, capsaicin sham-irradiated and capsaicin irradiated groups.

2.4. Histological study

Two pieces (5 mm) of both jejunum and ileum were taken from each rat at 10 cm from the ligament of Treitz and 5 cm from the ileo-cecal junction, respectively. Tissues were fixed in 10% neutralized formalin and Carnoy's solution, respectively. The samples were cleared in a vacuum infiltration processor, embedded in paraffin blocks and 5 μm sections were made with a rotating microtome. Formalin fixative was used for routine histological analysis with hematoxylin and eosin staining, whereas Carnoy's fixative was used for identification of intestinal mast cells with Alcian blue-Safranin O staining (Roberts *et al.* 1990). The number of mucosal mast cells was determined under a light microscope ($\times 40$ objective) within 10 randomly selected fields of the mucosa (Wallace *et al.* 1992). The number of apoptotic cells in the crypts of small intestine was determined on hematoxylin and eosin stained sections. The number

of apoptotic cells were counted over complete sections (between 100–200 crypts) and data are expressed as the number of apoptotic cells per 50 whole crypts per animal in order to standardize the procedure (Merritt *et al.* 1996, Potten and Grant 1998). Histological analyses were carried out by a pathologist (Dr J. Pomiès, Histotox, La Rochelle, France) who was unaware of the treatment groups.

2.5. MPO activity measurement

Intestinal segments (8–10 mm in length) adjacent to those used for microscopic evaluation were rinsed and stored at -80°C for no more than one week. MPO activity was determined using the method of Bradley *et al.* (1982). Briefly, samples were homogenized with a T25 Ultraturrax in phosphate buffer (50 mM, pH 6.0), snap-frozen and thawed three times, and centrifuged for 15 min at 8000 *g* at 4°C . The pellets were sonicated with 500 μl of hexadecyl trimethylammonium bromide (HTAB 0.5%, w/v, in 50 mM phosphate buffer). After centrifugation for 15 min at 8000 *g* at 4°C , 100 μl of supernatant was mixed with 3 ml of buffer containing phosphate buffer 50 mM, 0.167 mg/ml of *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. Absorbance at 450 nm was determined with a spectrophotometer (DU-640, Beckman, Gagny, France) for 2 min. One unit of MPO was defined as the quantity catalysing the decomposition of 1 μmol of hydrogen peroxide to water per minute at 25°C . MPO from human leukocytes was used as standard. Protein content was measured by absorbance at 750 nm with a Bio-Rad protein assay kit (Bio-Rad, Ivry sur Seine, France). MPO activity was expressed in units per gram of protein.

2.6. Radioimmunoassay for CGRP

CGRP concentrations were determined in control and irradiated rats using jejunal and ileal segments (5 cm in length) adjacent to those collected for MPO activity and histology determinations. Tissues were dissected on ice and separated into muscular and mucosal layers, weighed and boiled in 20 vols (w/v) of 0.1 N HCl for 10 min. They were then homogenized using a T25 Ultraturrax and centrifuged at 1700 *g* for 10 min. The supernatants were stored at -20°C for later neutralization and radioimmunoassay (RIA). Neutralization of tissue extracts was performed by addition of NaOH (1 M) to a final pH of 7. CGRP immunoreactivity was directly determined on the neutralized tissue homogenate supernatant according to a RIA protocol (Peninsula Laboratories, St Helens, UK) using rabbit antiserum.

The RIA for CGRP was performed using rabbit antibody RAS 6006 raised against rat CGRP. The sensitivity of this assay is 3 pg/tube. In brief, aliquots of 100 μl of rehydrated antiserum were incubated for 16–24 h at 4°C with standards or the unknown samples in a final volume of 200 μl ; 15 000 cpm/100 μl of ^{125}I -CGRP (specific activity: 1717 Ci/mmole) was added and incubated once again for 16–24 h at 4°C . To perform the second antibody separation, 100 μl of dilute goat anti-rabbit IgG serum (GARGG 500) and 100 μl of dilute normal rabbit serum (NRS 500) were added. Precipitates were allowed to form for 2 h at room temperature, then 500 μl of RIA buffer was added. RIA tubes were centrifuged at 1700 *g* for 20 min at 4°C . Then the supernatants were discarded by aspiration and the ^{125}I radioactivity in the pellet was determined in a gamma counter (Cobra II auto-gamma, Packard, Rungis, France).

2.7. Statistical analysis

Results are expressed as means $\pm$ SEM. Multiple comparisons were performed with non-parametric analysis of variance and followed by Dunn's multiple comparison post-test. Statistical significance was accepted if $p < 0.05$.

3. Results

3.1. Effects of irradiation

Following 10 Gy abdominal irradiation, food intake was markedly decreased by $54.8 \pm 6.2\%$ and $88.2 \pm 4.0\%$ on days 1 and 3 respectively ($n=4$). This attenuated food intake was associated with a reduction in body weight ($7.7 \pm 0.6\%$ and $9.3 \pm 0.8\%$ decrease on days 1 and 3, respectively; $n=6$).

3.1.1. Mast cell and structural histology. In the two intestinal segments investigated, the number of histologically detectable mucosal mast cells decreased dramatically (-95%) on the first day after irradiation and mast cells were nearly undetectable on the third day (figure 1). Routine histology revealed neither destruction of the continuity of the epithelium nor any alterations characteristic of inflammation of both mucosa and muscular layer of the small intestine on day 1 and on day 3. In the small intestine there was a three-fold increase in apoptotic cells in the crypts in vehicle irradiated group (18.3 ± 1.7 apoptotic cells/50 crypts; $n=12$, $p < 0.05$) as compared with the vehicle sham-irradiated group (5.9 ± 1.1 apoptotic cells/50 crypts) at 1 day after irradiation. However

Figure 1. Effects of vehicle and capsaisin treatment on mast cell numbers 1 and 3 days after abdominal irradiation. □ vehicle sham-irradiated rats; ■ capsaisin sham-irradiated rats; ▨ vehicle-irradiated rats; ▩ capsaisin-irradiated rats. (means $\pm$ SEM, $n=6$). * $p<0.05$, significantly different from corresponding control values.

at 3 days the number of apoptotic cells was similar to vehicle sham-irradiated group (6.2 ± 0.6 , $n=6$).

3.1.2. MPO activity. On the first day after irradiation, no significant change in MPO activity was observed at the two levels investigated. On the third day, MPO activity dramatically increased at the two levels, the greatest increase (32-fold) being observed in the jejunum (318.5 ± 55.0 versus 10.1 ± 2.3 U/g protein; figure 2).

3.1.3. CGRP content. On day 1, jejunal mucosa CGRP levels were increased ($p<0.05$ versus control; table 1). At this time no changes were seen in ileal CGRP content (table 1). However, on the third day after irradiation, CGRP content significantly increased in small intestine (jejunum and ileum) both in mucosa and muscle layers ($p<0.05$ versus control).

Figure 2. Effects of systemic and capsaisin treatment on myeloperoxidase (MPO) activity 1 day and 3 days after abdominal irradiation. □ vehicle sham-irradiated rats; ■ capsaisin sham-irradiated rats; ▨ vehicle-irradiated rats; ▩ capsaisin-irradiated rats. (means $\pm$ SEM, $n=6$). * $p<0.05$, significantly different from corresponding control values. † $p<0.05$, significantly different from vehicle-irradiated group values.

3.2. Effects of irradiation after sensory denervation

Capsaicin pretreatment did not significantly modify the irradiation-induced decreases in food intake ($6.8 \pm 0.9\%$ and $8.0 \pm 1.1\%$ on days 1 and 3 respectively; $n=4$; means $\pm$ SEM) or body weight ($51.01 \pm 4.6\%$ and $87.6 \pm 2.5\%$ on days 1 and 3, respectively, after irradiation; $n=6$, means $\pm$ SEM).

3.2.1. Mast cells and routine histology. The marked decrease in the number of mast cells observed in the small intestine on the first and third days after irradiation was not significantly modified by capsaicin pretreatment (figure 1). As observed in vehicle-treated rats, neither epithelial disruption nor marked inflammatory processes were detected in either the mucosa or external muscle layers on days 1 and 3 after irradiation in capsaicin-treated rats. Capsaicin pretreatment alone did not cause apoptosis in the small intestine and levels were similar to those described in the previous section (5.9 ± 0.9 , $n=12$, in the capsaicin sham-irradiated group and 5.9 ± 1.1 ,

Table 1. Concentrations of CGRP in mucosa and muscle layers of jejunum and ileum, 1 and 3 days after exposure to 10 Gy abdominal irradiation or sham-irradiation in capsaicin- and vehicle-treated groups.

	Jejunum		Ileum	
	mucosa CGRP	muscle CGRP	mucosa CGRP	muscle CGRP
Sham-irradiated animals				
Control vehicle	6.5 ± 0.5	17.0 ± 1.6	5.8 ± 0.5	16.8 ± 0.9
Control capsaicin	8.8 ± 0.5	9.2 ± 0.3*	6.5 ± 0.5	9.3 ± 0.3*
Irradiated animals				
Day 1 vehicle	20.5 ± 2.2†	15.1 ± 1.7	10.1 ± 1.0	14.5 ± 0.7
Day 1 capsaicin	8.6 ± 1.4*	14.1 ± 0.6†	6.3 ± 0.9	15.8 ± 0.4†
Day 3 vehicle	17.3 ± 2.6†	26.5 ± 0.6†	15.6 ± 1.7†	20.8 ± 1.1†
Day 3 capsaicin	24.0 ± 2.6†	15.9 ± 0.7*†	12.5 ± 1.5†	16.9 ± 0.4*†

Values are means ± SEM, $n=6$ (pmol/g tissue).

* $p<0.05$, capsaicin treatment compared with vehicle.

† $p<0.05$, irradiated animals compared with sham-irradiated animals.

$n=12$, in the vehicle sham-irradiated group). Capsaicin treatment (22.9 ± 3.0 apoptotic cells/50 crypts; $n=12$) did not modify the increased number of apoptotic cells observed in vehicle irradiated rats (18.3 ± 1.7 apoptotic cells/50 crypts; $n=12$) from the first day after irradiation.

3.2.2. MPO activity. In capsaicin-treated rats on the first day after irradiation, in contrast to that observed in vehicle animals, there was a significant increase (4.5- and 3-fold) in MPO activity (figure 2). In addition, in the jejunum, the increase in MPO activity was different from both capsaicin sham-irradiated and vehicle irradiated rats. However, the increase in MPO activity observed on the third day after irradiation in vehicle rats was not significantly modified by capsaicin pretreatment (figure 2).

3.2.3. CGRP content. Capsaicin pretreatment significantly decreased levels of CGRP in the muscle layers of both jejunum and ileum as compared with control vehicle values ($p<0.05$), but not in the mucosal layer. One day after irradiation, pretreatment with capsaicin significantly ($p<0.05$) decreased CGRP content in jejunum mucosa compared with vehicle pretreatment; in ileum mucosa, CGRP concentrations tended to be lower in the capsaicin rats than in vehicle animals, but this decrease did not reach statistical significance.

Three days after irradiation, there was no difference between the capsaicin group and the vehicle group in the mucosa of both jejunum and ileum; in contrast, CGRP contents in muscle layers were significantly decreased ($p<0.05$) in capsaicin group com-

pared with vehicle group at the two sites investigated (table 1).

4. Discussion

The present study provides evidence that a single dose (10 Gy) of abdominal irradiation increases MPO activity and concentrations of CGRP and decreases mast cell numbers in the small intestine. The routine histology did not detect any epithelial disruption 1 day and 3 days after irradiation, but number of apoptotic cells increased in crypts of the small intestine 1 day after irradiation.

The absence of epithelial damage, which agrees with observations of others (Empey *et al.* 1992, MacNaughton *et al.* 1997) does not exclude functional alterations of the mucosa. The appearance of apoptotic cells is in agreement with other studies (Arai *et al.* 1996, Ruifrok *et al.* 1997) as is the rapid disappearance of mast cells (Cummins *et al.* 1989, Harari *et al.* 1994). However no infiltration of neutrophils was seen histologically at any time, which did not correlate with increased MPO activity at 3 days. Buell and Harding (1989) found an increase in neutrophil infiltration within 12 h after 10 Gy abdominal irradiation in rats. At 24 h, in agreement with the present study, there was no evidence of infiltration. In contrast, in other models of intestinal inflammation, such as that provoked by administration of trinitrobenzenesulphonic acid (TNBS), acute inflammatory responses have been observed with an important increase of neutrophil infiltration at the site of TNBS administration (Miller *et al.* 1993). In the present study, the irradiation was external and was not to a specifically localized site of the gut, like

TNBS-induced administration, but over a large abdominal field. Infiltration of neutrophils was observed histologically only infrequently on 5- μ m sections and so, it is difficult to compare such measurements to those of MPO activity which uses a much larger tissue sample. However, no change in small intestine MPO activity has been observed 24 h and 48 h after whole-body irradiation (MacNaughton *et al.* 1994, MacNaughton *et al.* 1998) and, moreover, a decrease in MPO activity has been reported at the colonic level 48 h after irradiation (MacNaughton *et al.* 1998). On the other hand, an increased jejunal MPO activity has been shown 2 h post-irradiation (MacNaughton and Prud'homme-Lalonde 1995). Thus it seems that data concerning changes in MPO after irradiation are still controversial and the differences between studies seem to depend upon the intestinal segment, the mode of irradiation and the time at which the observations were performed.

Destruction of sensory afferents by capsaicin did not modify the mast cell depletion observed after irradiation. According to the anatomical relationships between sensory neurons and mast cells, and the well-known ability of substance P, a major tachykinergic mediator of sensory afferents, to degranulate mast cells (Maggi 1997), one can speculate that substance P, which is rapidly released from the intestinal wall after irradiation (Esposito *et al.* 1996) could be responsible in part for the irradiation-induced mast cell degranulation. Such an hypothesis is not supported by our results showing that mast cell depletion occurs independently of the sensory innervation. Similarly, Cummins *et al.* (1994) observed that mast cell degranulation associated with weaning in rats was not modified after destruction of sensory afferents by capsaicin.

In contrast to the marked mast cell depletion no change in MPO activity occurred on the first day post-irradiation. However, a significant increase of MPO activity was found in capsaicin-treated animals, suggesting a role for sensory afferents in the early stage of irradiation-induced inflammation. A protective role of sensory neurons has been observed in different animal models where inflammation was induced by compounds such as TNBS (Reinshagen *et al.* 1996, Mazelin *et al.* 1998a) and sodium dextran sulphate (Domek *et al.* 1997) or ricin (Shea-Donohue *et al.* 1997). The main mediators of sensory afferents are CGRP and tachykinins. Substance P is released from extrinsic and intrinsic neurons during an experimental inflammation in animals (Reinshagen *et al.* 1995) and the number of substance P nerve fibres is increased in ulcerative colitis in humans (Keranen *et al.* 1995). However, following total-body irradiation

of rats tissue levels of substance P were decreased in the ileum (Esposito *et al.* 1996).

In the small intestine CGRP is localized in both extrinsic and intrinsic neurons (Sternini *et al.* 1987) and measurement of CGRP levels in intestinal tissue whilst providing tissue concentration of the peptide does not relate directly to neuronal activity. Nevertheless, the increase of tissue CGRP concentrations 1 day after irradiation may reflect either blocked CGRP release, increased synthesis or decreased catabolism. Consequently, we suggest that, according to the increase of CGRP levels in jejunum 1 day after irradiation in vehicle-treated animals compared to the decrease seen in capsaicin-treated animals 1 day after irradiation, the effect of sensory afferents against irradiation-induced inflammation may depend in part on CGRP at this time. In support of this, a recent study showed an increase in CGRP-immunoreactive nerve fibres in mesenteric arteries in rats, 24 h after a whole-body irradiation; the authors suggested a protective function of CGRP against irradiation (Zhang *et al.* 1998).

In contrast, on the third day after irradiation we did not observe any protective action of sensory afferents on intestinal inflammation as assessed by MPO activity and it may be postulated that the protective action of afferents is overwhelmed by several pro-inflammatory factors. Finally, our study shows that ablation of sensory nerves by capsaicin does not affect the marked enhancement of mucosal apoptosis observed on the first day after irradiation. This suggests that intestinal sensory innervation may not be involved in the protection of the intestinal mucosa against radiation-induced apoptosis. Some data for a role of innervation in apoptosis have been already reported for organs or tissues other than the digestive tract. For example, in newborn rats some muscle fibres in developing striated muscle undergo apoptosis due to lost innervation resulting from the retraction of nerve terminals (Trachtenberg 1998). Moreover, experimental denervation induces a several-fold increase in the magnitude of fibre apoptosis. One important property of CGRP is vasodilatation; thus a reduced release may effectively create tissue anoxia and subsequent apoptosis of intestinal epithelial cells as has been demonstrated in cases of ischaemia-reperfusion in mice (Farber *et al.* 1999). Despite the increase in CGRP levels after irradiation, the irradiation-induced apoptosis does not seem to be associated with CGRP, since capsaicin treatment, which destroys CGRP immunoreactive sensory neurons, did not modify irradiation-induced apoptosis.

In conclusion, sensory afferents do not play a major protective role in the early (1 day) mucosal alterations induced by abdominal irradiation, since

only MPO activity was slightly, yet significantly, increased at this time in capsaicin-treated rats while other parameters, such as apoptosis and mast cell numbers remained unchanged.

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