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The significance of removing oxygen-derived free radicals in the treatment of acute and chronic duodenal ulceration in the rat

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Abstract—Rats infused for 24 h with pentagastrin (4 $\mu g\ kg^{-1}\ min^{-1}$) and carbachol (0·8 $\mu g\ kg^{-1}\ min^{-1}$) developed acute duodenal ulceration (100%) and hyperchlorhydria ($69 \pm 5{\cdot}3$ $\mu mol\ h^{-1}$ vs $14 \pm 0{\cdot}9$ $\mu mol\ h^{-1}$, $P < 0{\cdot}001$, $n = 10$). The animals were then given daily by gavage, saline, allopurinol with dimethyl sulphoxide (DMSO) or cysteine with methyl methionine sulphonium bromide (MMSB). Two days after the infusion, 10 rats (100%) given saline and 7 rats (70%) given allopurinol and DMSO, or cysteine and MMSB, showed duodenal ulceration. Five days after the infusion, 8 rats (80%) given saline, 3 rats (30%) given allopurinol and DMSO, and 2 rats (20%) given cysteine and MMSB had duodenal ulceration. Seven days after the infusion, only 5 rats (50%) given saline still had duodenal ulceration. Daily intramuscular injection of reserpine (0·1 mg kg^{-1}) for 6 weeks produced chronic duodenal ulceration (90%) and hyperchlorhydria ($47 \pm 3{\cdot}1$ $\mu mol\ h^{-1}$ vs $12 \pm 0{\cdot}9$ $\mu mol\ h^{-1}$, $P < 0{\cdot}001$, $n = 10$). Animals were then given daily by gavage, saline, allopurinol and DMSO, or cysteine and MMSB. Five days after reserpine, 10 rats (100%) given saline, 8 rats (80%) given allopurinol and DMSO, and 7 rats (70%) given cysteine and MMSB showed duodenal ulceration. Ten days after reserpine, 9 rats (90%) given saline, 3 rats (30%) given allopurinol and DMSO, and 4 rats (40%) given cysteine and MMSB had duodenal ulceration. Fifteen days after reserpine, 8 rats (80%) receiving saline and only one rat (10%) receiving allopurinol and DMSO or cysteine and MMSB had duodenal ulceration. Twenty days after the reserpine treatment only 6 rats (60%) given saline still had duodenal ulceration. The results show that removing oxygen-derived free radicals stimulates the healing of acute and chronic duodenal ulceration in the rat.

Lam & Sircus (1975) and Sircus (1979) reported that 60% of duodenal ulcer patients have acid secretion within normal limits and that acid and pepsin secretion by the stomach does not change with ulcer relapse or remission; the latter, therefore, suggests a change in duodenal resistance. Furthermore, duodenal ulceration may heal despite acid hypersecretion and the latter may exist without duodenal ulceration. It, thus, appears that factors detrimental to the integrity of the duodenal mucosa may be responsible for the development and persistence of its ulceration.

Oxygen-derived free radicals such as superoxide anion and hydroxyl are cytotoxic and promote tissue damage (McCord 1974; Fridovich 1978; Butterfield & McGraw 1978; Del Maestro et al 1980). It is not known whether such radicals impair the healing of duodenal ulceration.

This study was, therefore, designed to investigate the influence of removing oxygen-derived free radicals on the rate of healing of duodenal ulceration in the rat.

Materials and methods

Animals. Sprague-Dawley rats of either sex, 200–300 g, were housed in cages with wide mesh wire bottoms to prevent coprophagy. Food was withheld for 24 h before H^+ output studies or infusion of saline or pentagastrin with carbachol. After pyloric ligation, rats were fasted until killed.

Source and preparation of drugs. Drugs, except allopurinol, were supplied by Sigma (St. Louis, MO, USA). A 0·1 mg mL^{-1} solution of reserpine was prepared by dissolving 8 mg powder in 0·1 mL glacial acetic acid and the volume made up to 80 mL with double distilled water. A 5% solution of each of DL-cysteine, methylmethionine sulphonium bromide (MMSB), and dimethyl sulphoxide (DMSO) was prepared by dissolving 1 g of the powder in double distilled water. A 5% solution of allopurinol (Burroughs Wellcome Co., Research Triangle Park, NC, USA) was prepared by dissolving 1 g of the powder in 20 mL double distilled water containing the molar equivalent of 1 M NaOH. Saline was given to control animals. Solutions were freshly prepared each day. Gavage was under light ether anaesthesia (diethyl ether BP) using a 6 FG Infant's Feeding Tube 400/420 (Portex Ltd, Hythe, UK).

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Surgery. Animals were anaesthetized by inhalation of diethyl ether or by intraperitoneal injection of 25 mg kg^{-1} pentobarbitone (Sagatal, May and Baker, Dagenham, UK) into the left iliac fossa. When indicated, supplementary doses of Sagatal were given to maintain narcosis. Pyloric ligation and tracheostomy were as described by Salim (1988a, b).

Preliminary studies. Preliminary studies were done in groups of ten male or female rats, 210–240 g, to determine the effect of the test radical removing agents on H^+ output of the pylorus-ligated rat.

Pentagastrin with carbachol was used to produce acute, and reserpine to produce chronic, duodenal ulceration as described by Gaskin et al (1975) and Salim (1987) and the H^+ output measured (Table 1).

Pentagastrin and carbachol groups. Male and female fasted rats 240–290 g, were fitted with cannulae inserted into the dorsal subcutaneous tissue under ether anaesthesia and placed in individual cages for 24 h, during which time pentagastrin (4 $\mu g\ kg^{-1}\ min^{-1}$) and carbachol (0·8 $\mu g\ kg^{-1}\ min^{-1}$) in 0·9% saline or saline alone were infused at 0·75 mL h^{-1}. The cannulae were removed and animals anaesthetized with pentobarbitone and submitted to tracheostomy (to overcome respiratory distress from intubation) and orogastric intubation with a 6 FG tube. The gastric fasting secretion was recovered by slowly instilling 1 mL of double distilled water and collecting all gastric contents. These were then collected every 15 min for 1 h. The animals were then killed with ether and their duodena were independently examined macroscopically and histologically for the presence of ulceration (a sharply demarcated and clearly punched out oval or round breach in the mucosa). The H^+ output (μmol) was determined by titration to pH 7·0 with 0·1 M NaOH and expressed as the mean for each study group.

Other animals subjected to the infusion were allowed access to food and water and were then given daily, by gavage, either 1 mL cysteine with 1 mL MMSB or 1 mL allopurinol with 1 mL DMSO. These animals were killed 2, 5, 7 or 10 days later. Before each killing they were fasted for 24 h and their gastric fasting secretion was collected and their duodena were examined as above.

Reserpine groups. Male and female rats, 260–300 g, were treated daily with intramuscular injections of reserpine 0·1 mg kg^{-1} ($n = 120$) or saline 1 mL kg^{-1} ($n = 120$) for six weeks. Animals were then given daily, by gavage, under light ether anaesthesia 2 mL saline, 1 mL cysteine with 1 mL MMSB, or 1 mL allopurinol with 1 mL DMSO, and killed after 5, 10, 15 or

Table 1. Production of acute and chronic duodenal ulceration in the rat.

Experimental group	n	Duration of treatment	% Incidence showing duodenal ulceration	μmol H^+ output h^{-1} (mean ± s.e.m.)
Saline 0·75 mL h^{-1} s.c.	10	24 h	0	14 ± 0·9
Pentagastrin 4 μg kg^{-1} min^{-1} s.c. carbachol 0·8 μg kg^{-1} min^{-1} s.c.	10	24 h	100	69 ± 5·3*
Saline 1 mL kg^{-1} d^{-1} i.m.	10	6 weeks	0	12 ± 0·9
Reserpine 0·1 mg kg^{-1} d^{-1} i.m.	10	6 weeks	90	47 ± 3·1*

* $P < 0·001$ Mann-Whitney U test comparing control group with treatment group.

Table 2. Effect of saline, allopurinol, dimethyl sulphoxide (DMSO), DL-cysteine, and methyl methionine sulphonium bromide (MMSB) on the rate of healing of acute duodenal ulceration in the rat.

Experimental group	% Incidence of animals showing ulceration and their μmol H^+ h^{-1} (mean ± s.e.m.) after the infusion							
	2 d		5 d		7 d		10 d	
	%	H^+ output	%	H^+ output	%	H^+ output	%	H^+ output
Saline								
Saline 2 mL i.g.	0	13·6 ± 0·5	0	11·9 ± 0·6	0	12·5 ± 0·3	0	13·4 ± 0·2
5% allopurinol 1 mL i.g.								
5% DMSO 1 mL i.g.	0	12·8 ± 0·7	0	12·5 ± 0·5	0	13·2 ± 0·6	0	12·8 ± 0·6
5% DL-cysteine 1 mL i.g.								
5% MMSB 1 mL i.g.	0	11·9 ± 0·6	0	12·7 ± 0·3	0	12·9 ± 0·7	0	11·9 ± 0·7
Pentagastrin and carbachol								
Saline 2 mL i.g.	100	12·4 ± 0·5	80	13·1 ± 0·6	50	11·7 ± 0·3	30	12·6 ± 0·5
5% allopurinol 1 mL i.g.								
5% DMSO 1 mL i.g.	70	12·8 ± 0·6	30	12·7 ± 0·5	0	12·5 ± 0·7	0	13·3 ± 0·6
5% DL-cysteine 1mL i.g.								
5% MMSB 1 mL i.g.	70	12·9 ± 0·3	20	12·9 ± 0·7	0	13·2 ± 0·5	0	11·8 ± 0·7

Pentagastrin (4 μg kg^{-1} min^{-1}) and carbachol (0·8 μg kg^{-1} min^{-1}) or saline (0·75 mL h^{-1}) were infused subcutaneously for 24 h.
i.g.: instilled into the stomach by orogastric intubation every day.

Table 3. Effect of saline, allopurinol, dimethyl sulphoxide (DMSO), DL-cysteine, and methyl methionine sulphonium bromide (MMSB) on the rate of healing of chronic duodenal ulceration in the rat.

Experimental group	% Incidence of animals showing ulceration and their μmol H^+ output h^{-1} (mean ± s.e.m.) after the injections							
	5 d		10 d		15 d		20 d	
	%	H^+ output	%	H^+ output	%	H^+ output	%	H^+ output
Saline 1 mL kg^{-1} d^{-1} i.m. for 6 weeks								
Saline 2 mL i.g.	0	11·6 ± 0·6	0	12·4 ± 0·2	0	12·6 ± 0·5	0	13·2 ± 0·6
5% allopurinol 1 mL i.g.								
5% DMSO 1 mL i.g.	0	12·4 ± 0·3	0	13·1 ± 0·7	0	13·4 ± 0·7	0	12·8 ± 0·8
5% DL-cysteine 1 mL i.g.								
5% MMSB 1 mL i.g.	0	12·1 ± 0·7	0	11·4 ± 0·3	0	11·9 ± 0·3	0	13·1 ± 0·7
Reserpine 0·1 mg kg^{-1} d^{-1} i.m. for 6 weeks								
Saline 2 mL i.g.	100	11·5 ± 0·3	90	12·1 ± 0·5	80	12·5 ± 0·5	60	12·6 ± 0·4
5% allopurinol 1 mL i.g.								
5% DMSO 1 mL i.g.	80	11·8 ± 0·5	30	13·4 ± 0·7	10	13·1 ± 0·7	0	11·8 ± 0·3
5% DL-cysteine 1 mL i.g.								
5% MMSB 1 mL i.g.	70	12·2 ± 0·6	40	11·9 ± 0·3	10	11·7 ± 0·8	0	12·4 ± 0·7

i.g.: instilled into the stomach by orogastric intubation every day.

20 days of commencing the gavage. Before each killing, rats were denied solid food for 24 h then anaesthetized with pentobarbitone, submitted to tracheostomy and orogastric intubation with a 6 FG tube. The gastric secretion was then collected for 1 h and the H^+ output (μmol) determined as above. Rats were killed by ether overdose and their duodena were examined macroscopically and histologically.

Statistical analysis. Results are presented as mean ± s.e.m. unless stated otherwise. The statistical significance ($P < 0·05$) of observed differences between groups was determined using the Mann-Whitney U test for non-parametric data.

Results

The results are presented in Tables 1, 2 and 3. All rats survived the treatments without any observed distress or changes in activity or weight. During the experiments, the consumption of food and water by the treatment groups was similar to that by control animals.

Preliminary studies. Pylorus-ligation for 2 h was associated with H^+ output of 234 ± 17 μmol (n = 10); 1 mL of 5% solution of cysteine, MMSB, allopurinol, or DMSO had no significant influence on this output.

Pentagastrin with carbachol produced ulceration in the first part of the duodenum in all rats (100%). In addition, some rats (20%) had ulceration in the second part of the duodenum. Ulcers were oval or round and sharply demarcated from the surrounding duodenal mucosa without it heaping at the edges. They ranged in greatest diameter from 3–4 mm. Microscopically, there was extensive necrosis of the mucosa with a variable amount of destruction of the submucosa, sometimes extending into the muscularis propria. The neighbouring tissues showed polymorpho nuclear leukocyte infiltration with oedema and congestion. There was increased mitotic activity of crypt cells and abundant mucous cells both in crypts and surface epithelium. In addition, the mucosa neighbouring the ulcers showed blunting, flattening, and frequently fusion of villi. No pathological changes were detected in the duodenum of animals infused with saline.

Pentagastrin with carbachol significantly stimulated the H^+ output of the rat stomach over 1 h relative to control values ($69 \pm 5{\cdot}3$ μmol vs $14 \pm 0{\cdot}9$ μmol, $P < 0{\cdot}001$, n = 10).

Reserpine produced ulceration in the anterior suface of the first part of the duodenum in animals (90%). Ulcers were round or oval and sharply demarcated with vertical edges and a black colour base. They ranged in greatest diameter from 3–5 mm. The surrounding mucosa was deeply congested forming a 2–3 mm red halo around ulcers without heaping. Microscopically, a narrow-surface layer of fibrinous exudate was observed in all cases. There was extensive necrosis and loss of mucosa and submucosa, sometimes extending into the muscularis propria. Vascular granulation tissue was present in the base of the ulcer and the surrounding tissues were infiltrated with chronic inflammatory cells and fibrous tissue.

No pathological changes were detected in the duodenum of animals injected with saline. Reserpine significantly stimulated the H^+ output of the rat stomach over one hour relative to control values ($47 \pm 3{\cdot}1$ μmol vs $12 \pm 0{\cdot}9$ μmol, $P < 0{\cdot}001$, n = 10).

Healing studies. The stimulation of healing of the secretagogue-induced acute duodenal ulceration and the reserpine-induced chronic duodenal ulceration by the test radical-removing agents is illustrated in Tables 2 and 3.

In all groups, the ulcers observed were similar macroscopically and microscopically to those observed in the preliminary study. They were oval or round, their greatest diameter ranged from 3 to 5 mm, and they extended into the submucosa. Sometimes ulceration extended into the muscularis propria, and this was particularly common with ulceration persisting beyond 5 days. In all cases, the latter ulceration showed chronic inflammatory cell and fibrous tissue infiltration at the base and surrounding tissues. Perforation was not detected.

Healing of duodenal ulceration was confirmed microscopically. The mucosal tissue was intact and defects of the muscularis mucosae and propria were bridged by fibrous tissue without regeneration of muscle. The epithelium of the mucosa grew from the ulcer edges to cover the surface of the ulcer. The new mucosa, initially, was immature and in a regenerating phase but after 20 days it had a mature appearance.

The H^+ output of the experimental groups was similar to that of the groups injected with saline.

Discussion

DL-cysteine and MMSB are sulphydryl-containing agents that bind the electrophilic oxygen-derived free radicals mediating tissue damage (Szabo et al 1981). Allopurinol is a potent scavenger of hydroxyl and an inhibitor of xanthine oxidase, which is responsible for the formation of superoxide radicals, and DMSO is a scavenger of hydroxyl radicals (Del Maestro et al 1980; Flower et al 1980; Moorhouse et al 1987).

The stimulation of gastric acid secretion by pentagastrin and carbachol suggests that the acute ulceration resulted from the erosive action of acid hypersecretion. Two days after treatment with these secretagogues, the H^+ output was similar to that of control values (Table 2), indicating that the acute ulceration was allowed to demonstrate its healing potential in circumstances as near basal as possible.

The stimulation of gastric acid secretion by reserpine is caused by a vagal action on the stomach releasing acid secretagogues (Kim & Shore 1963; Emås & Fyrö 1965) and its persistence for six weeks produces chronic duodenal ulceration in the rat (Salim 1987). The hyperchlorhydria associated with this ulceration (Table 1) suggests that the erosive action of acid hypersecretion was behind its development. Five days after the reserpine treatment, the H^+ output was similar to that of control animals (Table 3) allowing the ulceration to reflect its healing ability in relatively basal circumstances.

The results demonstrate that removing oxygen-derived free radicals by inhibiting their formation and by scavenging stimulates the healing of acute and chronic duodenal ulceration in the rat (Tables 2, 3). Rats treated with radical removing agents after producing acute duodenal ulceration showed complete healing of this ulceration after 7 days of treatment (Table 2). At this time, 50% of the rats without treatment had duodenal ulceration. Treatment with radical removing agents produced complete healing of chronic duodenal ulceration within 20 days of its development; however, without this treatment 60% of rats had the ulceration at this stage (Table 3). The healing of both acute and chronic duodenal ulceration was confirmed microscopically. The mucosa was intact and any defect of the muscularis mucosae was bridged by fibrous tissue without regeneration of muscle. This was similarly the case with any defects of the muscularis propria. The epithelium of the mucosa grew from the ulcer edges to cover its surface. The new mucosa, initially, was immature and in a regenerating phase, but gradually returned towards normal.

The diameter of the acute and chronic duodenal ulcers remaining at the end of the experimental period was not significantly reduced relative to that of ulcers seen in earlier parts of the study. In addition, microscopic examination of these ulcers showed appearances suggesting persistence rather than progress towards healing. It thus appears that removing oxygen-derived free radicals plays a direct role in stimulating healing of acute and chronic duodenal ulceration rather than just enhancing an inevitable healing. Consequently, it follows that oxygen-derived free radicals are detrimental to the integrity of the duodenal mucosa in that they are responsible for maintaining its ulceration.

In man, the current treatment for duodenal ulceration is largely with the H_2-receptor antagonists (e.g. cimetidine, ranitidine) which are widely used to aid healing, and the addition of cytoprotective agents, such as colloidal bismuth or sucralfate, is useful in treating the refractory ulcer. The present observations suggest that removing oxygen-derived free radicals may prove to be an added dimension in the treatment.

The sulphydryl-containing agents cysteine and MMSB also make a contribution to protein synthesis (Turner et al 1977), which is essential for the repair and healing of damaged tissues. This action may enhance the stimulation of healing of acute and chronic duodenal ulceration.

The radical-removing agents used in this study had no

significant influence on the H^+ output of the rat stomach (Tables 2, 3). Their actions, therefore, are independent of inhibition of acid secretion and point to them being cytoprotective. The mechanism(s) of this cytoprotection is unknown; however, the possibility exists that it involves maintaining the integrity of surface mucus and the surface epithelial cells of the mucosa.

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