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Gastrointestinal mucosal injury following repeated daily oral administration of conventional formulations of indometacin and other non-steroidal anti-inflammatory drugs to pigs: a model for human gastrointestinal disease

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) vary in their propensity to cause damage in different regions of the gastrointestinal (GI) tract in laboratory animals and humans. This may depend on the type of drug formulation as well as the intrinsic pharmacological properties of the drugs. The purpose of this study was to determine the effects of NSAIDs, with cyclooxygenase 1 and 2 inhibitory activity but with different potency as inhibitors of prostaglandin production, when given orally as tablet/capsule formulations of NSAIDs for 10 days to pigs, a species that has close resemblance in structure and function of the tract to that in humans. Three capsule or tablet formulations of NSAIDs were given orally to pigs for 10 days. GI bleeding was measured by determination of radioactive iron in the faeces from ^{59}Fe-pre-labelled red blood cells. The blood loss was compared with the pathological changes in the GI mucosa observed at autopsy, mucosal myeloperoxidase (MPO) activity as an index of leucocyte infiltration, and plasma and mucosal concentrations of the drugs at termination assayed by high-performance liquid chromatography. Mucosal damage and bleeding varied according to the type of NSAID. Gastroduodenal ulcers and lesions occurred with the cyclooxygenase inhibitors indometacin (indomethacin) (Indocid capsules 10 or 5 mg kg^{-1} day^{-1} b.i.d.), aspirin (USP tablets 150 mg kg^{-1} day^{-1} b.i.d) and naproxen (Apotex tablets 50 or 75 mg kg^{-1} day^{-1} b.i.d.), and there was an increase in the cumulative (i.e. 10-day) blood loss at higher doses of indometacin and naproxen, and with aspirin. There was no statistically significant increase in gastric or intestinal mucosal MPO activity in the non-damaged mucosa with these drugs and this was confirmed by histological observations in non-lesioned areas of the mucosa. Indometacin produced focal ulcers in the caecum but this was not observed with the other drugs. All the NSAIDs produced significant blood loss coincident with gastric ulceration but no increase in gastric or intestinal MPO activity. Plasma concentrations of the non-aspirin NSAIDs were within the range encountered therapeutically in humans. The mucosal concentrations of indometacin in the gastric and intestinal mucosa correlated with mucosal injury. These findings show that: (i) NSAIDs vary in their propensity to produce mucosal injury in different regions of the GI tract according to their pharmacological properties and formulation; (ii) mucosal injury from some NSAIDs may not directly relate to blood loss at low doses of NSAIDs and this may depend on inhibition of platelet aggregation; and (iii) the occurrence of caecal ulcers uniquely observed with indometacin treatment may be relevant to the development of intestinal pathology (e.g. diaphragm-like strictures) seen occasionally in humans. These results suggest that the pig model employed in the present studies may be useful for investigations of GI damage from NSAID tablets/capsules, especially in regions that are generally inaccessible to routine endoscopic investigations in humans (e.g. the proximal regions of the large intestine).

Introduction

Gastrointestinal (GI) permeability changes, inflammation and ulceration are frequently associated with ingestion of non-steroidal anti-inflammatory drugs (NSAIDs) in patients with rheumatic diseases (Bjarnason et al 1987, 1989; Jenkins et al 1987; Roth 1988; Carson & Strom 1992; Rainsford & Quadir 1995). Ulceration

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and haemorrhage from NSAIDs occurs mainly in the gastroduodenal region of the GI tract (Rampton 1987; Carson & Strom 1992; Huang & Hunt 1996; Roth 1988). There is now considerable evidence that the incidence of upper GI ulceration and haemorrhage in humans varies considerably with different NSAIDs (Carson & Strom 1992; Rainsford & Quadir 1995) and is probably lower in rheumatic patients receiving the newer cyclooxygenase 2 selective drugs (Bombardier et al 2000; Hawkey et al 2000; Silverstein et al 2000). Many conventional NSAIDs have been reported to increase permeability and cause inflammation in the intestinal tract distal to the duodenum (Bjarnason et al 1987, 1989; Jenkins et al 1987; Rampton 1987; Jenkins et al 1991; Huang & Hunt 1996), which may lead to ulceration, perforation and the formation of diaphragm-like structures in the lower bowel (Langman et al 1985; Lang et al 1988; Morris et al 1991; Halter et al 1996; Zalev et al 1998). This has given rise to the suggestion that NSAIDs may also be particularly deleterious in this region (Bjarnason et al 1986, 1987; Jenkins et al 1987; Rampton 1987; Jenkins et al 1991). This view is supported by observations that some, but not all, NSAIDs cause mucosal lesions and ulcers in the intestinal tract (principally the ileo-jejunal region) of rats and dogs (Duggan et al 1975; Billingham & Tucker 1979; Rainsford 1982, 1983, 1988a, b; Brune et al 1987; Anthony et al 1994; Nygard et al 1994). This may be related to the enterohepatic circulation of these drugs (Duggan et al 1975; Rainsford 1983, 1988b; Brune et al 1987).

The variability in GI ulcerogenicity among different NSAIDs may be related to: (i) their intrinsic pharmacokinetic and pharmacological properties (e.g. degree of cyclooxygenase 1/cyclooxygenase 2 selectivity; Vane and Botting 1995); and (ii) the influence of drug formulations (e.g. capsules, tablets) or excipients.

Among the non-primate species, the pig closely resembles humans in respect of anatomy and physiological functions in the GI tract (Bustad & McClelland 1966). Recent studies have shown the utility of this species for studying the influence of drug formulations on their GI transit (Davis et al 2001). Moreover, pigs also resemble humans in respect of the histological and pathophysiological changes in the development of gastric ulcers, including those attributed to stress (Bustad & McClelland 1966; Huber & Wallin 1967; Muggenburg et al 1967; Norton et al 1972), and those induced by NSAIDs (Rainsford 1978, 1988b; Rainsford & Willis 1982; Rainsford et al 1995). We have previously studied the gastric ulcerogenic effects of various formulations of aspirin, benoxaprofen and diclofenac (Voltaren) alone or with misoprostol (Cytotec) in this species (Rainsford 1978, 1982; Rainsford & Willis 1982; Rainsford et al 1995). The studies with aspirin formulations suggest that the degree of ulceration varied according to the type of formulation (Rainsford 1978). In this study, we examined the effects of three NSAIDs having differing pharmacological properties given as their conventional oral formulations on the GI tract of pigs and compared GI injury with the faecal blood loss, mucosal leucocyte accumulation and plasma and mucosal concentrations of these drugs.

Materials and Methods

Healthy male Landrace pigs (previously assessed and determined to be parasite-free), 13–20 kg, were obtained from Reinins Farm (St Agatha, Ontario, Canada) and maintained at the Central Animal Facilities of McMaster University. They were initially acclimatized for 5–7 days, housed individually in dog metabolism cages and fed Purina no. 177 pig chow. Each pig received an intravenous injection into an ear vein of 1 mL 100 μCi $^{59}FeCl_3$ in sterile saline 5–7 days before initiation of the experiment using methods described previously (Rainsford et al 1995). Blood samples (approx. 1–2 mL) were collected in heparinized vials from an ear vein in the opposite ear to that where the injection was given from tranquillized (3 mL of 100 mg mL^{-1} ketamine) animals on Days 2, 5 and 7 thereafter for determination of the total blood radioactivity by gamma counting. Faeces were initially collected from animals for the baseline measure of GI blood loss, then collected twice daily (1000–1100 h and 1600–1630 h) thereafter.

Groups of 3–6 pigs each were randomly allocated to received: (i) 5.0 or 10 mg kg^{-1} day^{-1} indometacin (indomethacin) (Indocid; Merck Sharpe & Dohme, Montreal, QE, Canada) capsules or 10 mg kg^{-1} day^{-1} indometacin as an aqueous suspension in Pluronic 10 mg kg^{-1} day^{-1} b.i.d.; (ii) 150 mg kg^{-1} day^{-1} aspirin tablets (USP) b.i.d.; (iii) 75 mg kg^{-1} day^{-1} naproxen (Apotex; Apotex, Mississauga, ON, Canada), tablets b.i.d.; and (iv) placebo (1 tablet a.m. and 2 tablets p.m.). The drugs were given for 10 days using procedures as described previously (Rainsford & Willis 1982; Rainsford 1988b; Rainsford et al 1995). The animals were fasted from 1600 h on Day 9, allowed free access to water and were killed by an overdose of 10–15 mL of 50 mg mL^{-1} pentobarbitone sodium 2 h after the final dose of drug on Day 10.

After collection of the blood samples for the drug assays, a full autopsy was performed. The washed gastric and intestinal mucosae were separately placed on a tray and colour photographed. The mucosal damage was determined by counting of a number of mucosal lesions and ulcers and the area of mucosal injury with a millimetre rule. Sections of fundic and jejunal mucosa (approx. 1 cm diam.) were randomly selected and frozen (−20 °C) for later myeloperoxidase (MPO) assay. Randomly selected sections of mucosae as well as those with ulcers or lesions were fixed in 4% w/v formaldehyde in 0.01 M sodium phosphate-buffered saline (to pH 7.0) for subsequent paraffin-embedding and histological examination. These sections were stained with haematoxylin and eosin, or periodic acid Schiff's reagent with Alcian blue.

Plasma from blood samples as well as gastric and jejunal mucosa obtained at autopsy were assayed for indometacin according to previous methods (Rainsford et al 1992). Naproxen was assayed by an adaptation of the high-performance liquid chromatography (HPLC) method for indometacin (Rainsford et al 1992), in which the plasma samples were extracted in 4:1 diethyl ether/dichloromethane in 0.1 M citrate-phosphate buffer (pH 3.0) and 2 μg mL^{-1} ketoprofen added as an internal standard.

Gastric and intestinal mucosae (50 mg frozen weight) were homogenized using a silanized glass mortar and Teflon pestle in 3.0 mL ice-cold 0.2 M citric acid with 20 μL of 100 μg mL^{-1} ketoprofen as internal standard. The homogenates were then centrifuged at 2000 *g* for 10 min and the pellets re-homogenized in 3 mL of 0.2 M citric acid. The homogenizer tube was rinsed with 1 mL of 2 M citric acid after each homogenization and the washings were combined with the homogenates before centrifugation. The supernatants were combined and stored at −70 °C. The thawed fractions were subsequently extracted with 15 mL dichloromethane in salinized glass-stoppered tubes, the organic layer evaporated to dryness over nitrogen gas and the dried extract reconstituted in 200 μL acetonitrile/H_2O (50:50) and centrifuged at 2000 *g* for 10 min. The supernatants were then transferred to WISP vials (Waters, Milford, MA, USA) and placed in the Waters Model 717 HPLC autosampler. HPLC was performed using a Waters 510 pump (flow rate 1 mL min^{-1}) using isocratic conditions with a mobile phase of acetonitrile/0.05 mM sodium acetate pH 4.5 (45:55 v/v) gassed with helium and a Vydac C18 5 μm column 25 cm × 4.6 mm i.d. The injection volume was 50 μL. Monitoring was performed using a Waters-Millipore UV detector at 255 nm, and data processed from a Waters system Interface Module using Waters Millennium Chromatography Manager version 2.10 software. Linear responses for indometacin, naproxen and the internal standard, ketoprofen, were obtained over the range of 0.5–100 μg mL^{-1}, with the limit of detection being 0.5 μg mL^{-1} for all drugs. Recoveries of the drugs averaged 86% and 88% for indometacin and naproxen, respectively.

MPO activity

MPO activity was determined in randomly selected areas adjacent to lesions, but in non-lesioned mucosal tissues (0.2–0.37 mg wet weight) from the fundus or jejunum. The tissues were homogenized and assayed for MPO as described previously (Krawisz et al 1984; Mullane et al 1985; Rainsford et al 1995), with the enzymic activity being determined using *O*-dianisidine hydrochloride and hydrogen peroxide as substrates (Mullane et al 1985). The initial linear part of the change in absorbance at 460 nm was recorded spectrophotometrically (Mullane et al 1985; Rainsford et al 1995) and used to calculate the initial (linear) rate of change of absorbance for determination of the enzyme activity. Assays were performed on three separate tissue samples from the same animal and the mean values obtained. The protein content of the precipitated mucosal homogenates obtained following centrifugation was assayed using the method of Lowry et al (1951) following solubilization of the pellets in 1.0 M NaOH.

Ethical approval

The study was approved by the Animal Research Experimentation Board of McMaster University and performed under the guidelines of the Canadian Council for Animal Care.

Results

All the pigs dosed for 10 days with the NSAIDs had ulcers and superficial lesions of the gastric mucosa (in both antrum and fundus) of the typical appearance seen in Figure 1A of that from naproxen. However, among the NSAIDs, only 10 mg kg^{-1} indometacin given as the capsule formulation produced ulcers in the caecum (Table 1). These appeared as round, deep craters approximately 2–5 mm in diameter and were visible only on the mucosal surface and not from the serosa (Figure 1B). Histological examination confirmed that these lesions had perforated the muscularis layer and thus could be considered as ulcers. Indometacin given as a suspension in Pluronic also resulted in the development of mucosal lesions in the caecum (Table 1).

The higher dose of indometacin (10 mg kg^{-1} day^{-1}) and aspirin (150 mg kg^{-1} day^{-1}) produced a statistically significant (Student's *t*-test $P < 0.05$ with Bonferroni's

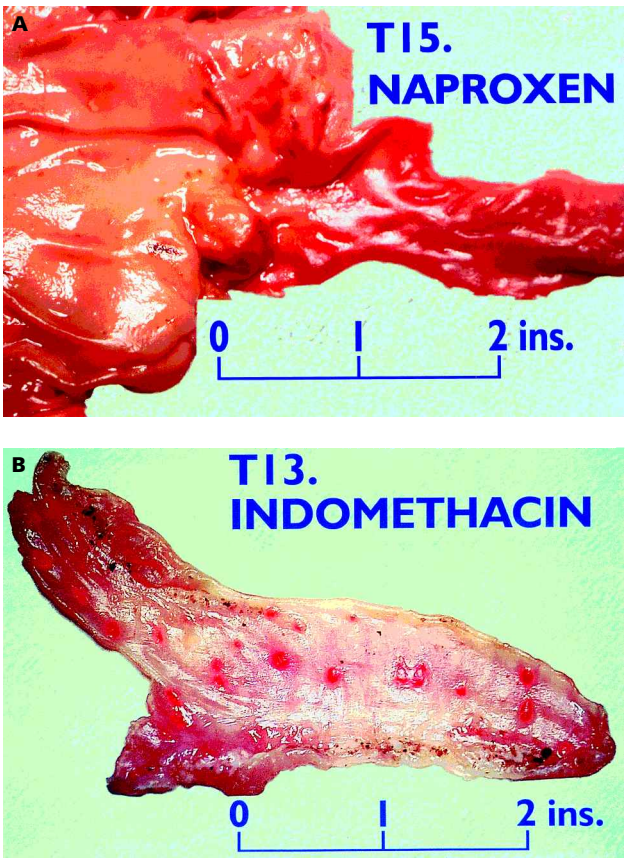

Figure 1 A. Appearance of the gastric mucosa of a pig dosed with naproxen for 10 days showing lesions and ulcers in the fundic and antral mucosa. B. The caecal mucosa of pig no. T13 treated with 10 mg kg^{-1} day^{-1} indometacin (Indocid capsules). Craterous round ulcers are evident from the mucosal surface. The mucosal appearance contrasts with that on the serosal side of the caecum from a pig that had ulcers in the caecum. The ulcers are not visible from the serosal side. The scale is in inches.

Table 1 Summary of the principal gastrointestinal pathological findings in pigs at autopsy, compared with blood loss and plasma drug concentration after 10 days oral dosing with conventional tablet or capsule formulations of non-steroidal anti-inflammatory drugs (NSAIDs).

Treatment	Number animals with damage/total	Gastric mucosal damage (mean ± s.e.)			Cumulative blood loss (mean ± s.e.m.)		Intestinal mucosal damage	Plasma concentrations of NSAIDs ($\mu g\,mL^{-1}$) (mean ± s.e.m.)
		Ulcers	Lesions	Area lesion index	Total (mL)	Specific ($mL\,kg^{-1}$)		
Control/placebo	0/5	0	0	0	29.6 ± 13.1	58.9 ± 29.6	0	0
Indometacin (Indocid)								
$5\,mg\,kg^{-1}\,day^{-1}$	3/3	1.5	7.5	10.5	29.5 ± 3.6	71.5 ± 1.1	Superficial erosions in caecum	0.06 ± 0.03
$10\,mg\,kg^{-1}\,day^{-1}$	6/6	35.7 ± 7.4*	0	214 ± 38	30.7 ± 8.7	115.1 ± 41.9*	32.3 + 18.8 (mean + ulcers in caecum)	0.39 ± 0.10
$10\,mg\,kg^{-1}\,day^{-1}$ + Pluronic	1/2	0	5	16.5	34.6	117.6	Superficial erosions in caecum	0.38
Aspirin (tablets USP)								
$150\,mg\,kg^{-1}\,day^{-1}$	3/3	21	14.0	91	88.5 ± 54.1*	137.8 ± 78.7*	0	ND
Naproxen (Apotex)								
$50\,mg\,kg^{-1}\,day^{-1}$	2/4	3.00 ± 1.35	0	0	41.3 ± 14.6*	94.4 ± 23.3*	1 Duodenal ulcer	48 ± 8.5
$75\,mg\,kg^{-1}\,day^{-1}$	2/2	9.5	0	119	28.4	88.4	0	58

*$P < 0.05$, Student's t-test.

correction) increase in both the total and specific cumulative blood loss over the 10-day period compared with the placebo control (Table 1). Although the lower dose of 5 mg kg^{-1} day^{-1} indometacin did not produce significant loss of blood and no visible injury in the caecum, there were gastric lesions and ulcers present in these animals (Table 1). Naproxen produced gastric ulcers but no haemorrhagic-type lesions and, at higher doses, significantly greater blood loss compared with placebo (Table 1).

Comparisons of the time course of blood loss over the 10 days of treatment with the NSAIDs are shown in Figure 2A–G. The blood loss from aspirin appeared to be uniformly elevated from 2–10 days of oral dosing with this drug (Figure 2J). There appears, qualitatively, to be a trend where the daily blood loss is greater in the first 4–6 days, with a progressive decline thereafter to values that approach those of placebo. This contrasts with the effects of the NSAIDs on the cumulative blood loss where there are clearer differences.

Gastric and intestinal mucosal concentrations of indometacin are shown in Table 2. The drug concentrations in the gastric mucosa of those animals given 10 mg kg^{-1} day^{-1} indometacin capsules appeared about twice those given 5 mg kg^{-1} day^{-1} indometacin. However, the concentrations in the intestinal mucosa of pigs given these two doses of the drug were about the same. Comparison of the gastric mucosal pathology using individual data from the overall scores of mucosal damage with gastric mucosal concentrations of indometacin using the Spearman rank correlation statistical analysis resulted in a Spearman rank statistic, r_s, of 0.905. This shows that there was a high correlation between drug concentrations in the gastric mucosa and mucosal injury. The Spearman correlation obtained in the comparison of intestinal mucosal concentrations with intestinal injury gave an r_s value of 0.45, indicating a poorer correlation between these two parameters than observed in the gastric mucosa.

There was no significant increase in mucosal MPO activity in non-damaged sections of the fundus or jejunum after 10 days of NSAID treatment (Table 3). Histological observations confirmed these findings. In contrast, all gastric and intestinal mucosae from lesioned or ulcerated areas examined histologically showed marked infiltration of neutrophils, macrophages, mononuclear cells, lymphocytes and plasma cells, with neutrophils between the lamina propria and muscularis. Areas of fibrosis were also evident in the lamina propria in some sections from gastric ulcers.

The plasma concentrations of the NSAIDs indometacin and naproxen are shown in Table 1. These were within the range encountered during arthritis therapy. Thus, for indometacin, the steady-state plasma concentrations are over the range of 0.3–3.0 μg mL^{-1}, while those of naproxen are 25–75 μg mL^{-1} in humans (Day et al 1982; Lin et al 1987; Rainsford 1996).

Discussion

The main findings from this study in the pig are: (i) the site of mucosal damage induced by formulations of NSAIDs employed clinically and the comparison of this blood loss varies with different drugs; (ii) GI mucosal pathology observed following oral administration of indometacin extends to the development of ulceration in the caecum; (iii) there is a direct correlation between gastric mucosal concentrations of indometacin and mucosal injury in the stomach, but the correlation between these two parameters is less so in the intestinal tract; and (iv) MPO activity (a marker of leucocyte infiltration) is not increased in the undamaged gastric and intestinal mucosa following oral dosage of NSAIDs for 10 days.

Our previous studies in pigs given the conventional formulation of diclofenac sodium (Voltaren) under the same conditions as employed in the present study also showed that this drug produced gastric mucosal ulcers and lesions without significant increase in the loss of blood from the GI tract (Rainsford et al 1995). It was suggested that the lack of effect of diclofenac on blood loss might be related to its weaker effects on platelet aggregation in contrast to the more sustained effect of aspirin, which was found in these studies to increase blood loss coincident with marked development of gastric mucosal ulceration and haemorrhagic lesions; a result confirmed in the present investigations.

Significant blood loss associated with the appearance of gastric ulcers was only evident in pigs given aspirin (Table 1). Ulcers and lesions occurred with indometacin and naproxen without bleeding at doses of the drug that produced plasma concentrations (Table 1) comparable with those achieved during therapy (Day et al 1982; Lin et al 1987; Rainsford et al 1992; Rainsford 1996). These findings with indometacin and naproxen are in agreement with the previous findings with diclofenac (Rainsford et al 1995). Thus, it appears that blood loss determinations may not reveal serious pathology in the GI tract with non-aspirin NSAIDs. The appearance of ulcers and lesions in the caecum of pigs dosed with 10 mg kg^{-1} day^{-1} 1 indometacin capsules or even as the suspension in Pluronic, in contrast to the lack of effects of other commonly used NSAIDs, is also an important finding from these studies.

These observations may have considerable importance for NSAID-associated GI pathology in humans. Thus, the differences observed in GI bleeding by non-aspirin NSAIDs compared with aspirin in humans have been postulated to relate to their relative potential for prolonging bleeding time (Rainsford et al 1995). Aspirin is known to be a more potent inhibitor than other NSAIDs of thromboxane-dependent platelet aggregation because of the irreversible inhibition of cyclooxygenase isoform 1 by aspirin. Since the enzyme is not replaced by de novo synthesis (Vane & Botting 1995), the blockade of platelet aggregation extends for the biological lifetime of this cell (the half time of elimination being approximately 5 days (Hawkey 1992).

There would appear to be potential clinical implications from these findings. Thus, the pig could be employed to investigate the clinically important factors underlying the development of indometacin-induced injury in the caecum and nearby regions of the GI tract that are not

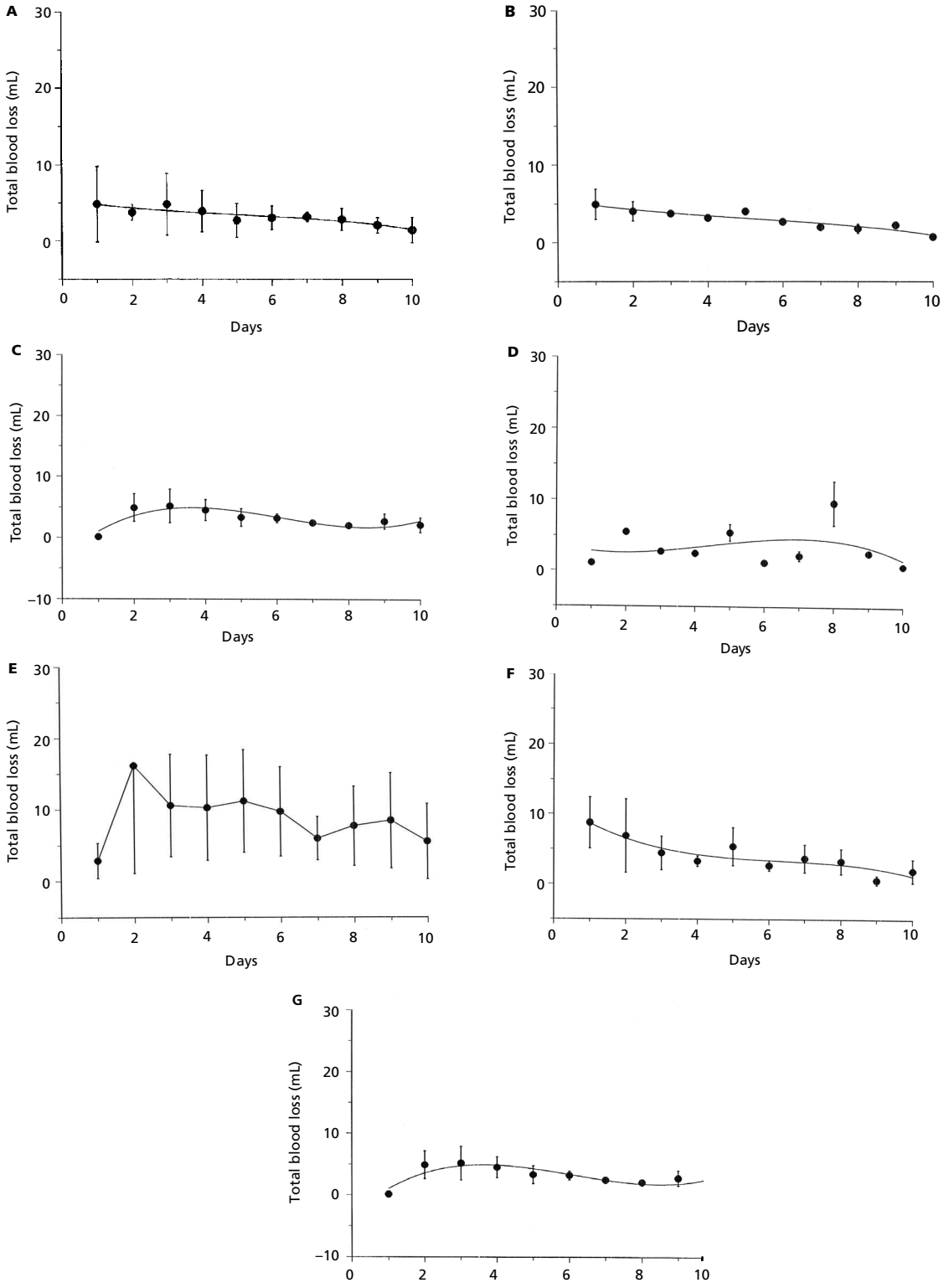

Figure 2 Gastrointestinal blood loss (total mL day^{-1}) from pigs dosed orally each day for 10 days with placebo (A), indometacin 5 mg kg^{-1} day^{-1} (Indocid; B), indometacin 10 mg kg^{-1} day^{-1} (Indocid; C), indometacin 10 mg kg^{-1} day^{-1} (Indocid) + Pluronic (D), aspirin 150 mg kg^{-1} day^{-1} (USP tablets; E), naproxen 50 mg kg^{-1} day^{-1} (Apotex; F), naproxen 75 mg kg^{-1} day^{-1} (Apotex; G). The treatments are identical to those in Table 1. Although no significant changes were observed in individual values of the total daily blood loss, there were significant increases in total cumulative blood loss over the 10-day period of treatment with indometacin 10 mg kg^{-1} day^{-1} (with or without Pluronic), aspirin 150 mg kg^{-1} day^{-1}, naproxen 50 or 75 mg kg^{-1} day^{-1} (compare with Table 1).

Table 2 Gastric and intestinal mucosal concentrations of indometacin following 10 days daily oral dosing of indometacin in pigs.

Dose	Gastric mucosa	Intestinal mucosa
Indometacin		
5.0 mg kg^{-1} day^{-1}	0.21 ± 0.0021	0.047 ± 0.011
10.0 mg kg^{-1} day^{-1}	0.54 ± 0.28	0.051 ± 0.027

Table 3 Effects of non-steroidal anti-inflammatory drugs on gastrointestinal mucosa myeloperoxidase activity.

Treatment	Change in absorbance (μg protein)$^{-1}$	
	Stomach	Intestine
Control	0.55 ± 0.38	0.55 ± 0.38
Aspirin		
150 mg kg^{-1} day^{-1}	0.44 ± 0.19	0.53 ± 0.98
Naproxen		
50 mg kg^{-1} day^{-1}	0.38 ± 0.04	0.40 ± 0.08
75 mg kg^{-1} day^{-1}	0.28 ± 0.15	0.58 ± 0.14
Indometacin		
5 mg kg^{-1} day^{-1}	0.42 ± 0.07	0.85 ± 0.25
10 mg kg^{-1} day^{-1}	0.41 ± 0.09	0.67 ± 0.14
10 mg kg^{-1} day^{-1} + Pluronic	0.79 ± 0.06	0.65 ± 0.13

Values are means ± s.d. of assays on individual samples from each animal; see Table 1 for n values.

easily accessible to observation in humans, as well as the development of procedures to obviate this pathology. The reasons for the development of caecal ulcers with indometacin may be related to the enterohepatic circulation of the drug (Duggan et al 1975; Rainsford 1983; Brune et al 1987). The accumulation of indometacin-glucuronide in the caecum of rats has been observed (Ruelius et al 1985) and this may account for the present observations in pigs. Otherwise, the factors accounting for this unique biodisposition from orally administered capsules of indometacin in pigs are not known.

The lack of effects of the drug treatments on mucosal MPO activity in the chronic model of GI damage employed in the present studies contrasts with previous studies in acute models where it has been claimed that gastric mucosal damage is a neutrophil-dependent process (Wallace et al 1990). There may be important adaptive changes that occur in the transition from acute to chronic effects observed with NSAIDs in the genesis of mucosal injury. It is also possible that there may be species differences in response to leucocyte-related injury. It is known, for example, that the NSAID effects on leucocyte accumulation and superoxide production in pig leucocytes resemble those in humans (Mousa et al 1989). These effects may be different to those in rats and rabbits, which were employed in those studies where NSAID-related leucocyte changes during mucosal injury was previously observed (Wallace et al 1990).

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