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Effect of Postoperative Diclofenac on Anastomotic Strength and Histologic Healing in Rabbit Small Intestine

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In this experimental study, we investigated the effects of a 5-day postoperative treatment with the nonsteroidal anti-inflammatory drug (NSAID) diclofenac on anastomotic healing in rabbits. NSAIDs are widely used analgesics in today's "fast-track surgery," raising concerns about their potential negative effects on healing in humans. A total of 33 New Zealand White female rabbits underwent laparotomy and 2 separate end-to-end anastomoses of the ileum. The animals were randomized to receive subcutaneous diclofenac 4 mg/kg/d (17 experimental rabbits) or subcutaneous isotonic saline 0.1 mL/kg/d (16 control rabbits) postoperatively. On the fifth postoperative day, the animals were humanely killed, and anastomotic leakage, anastomotic breaking strength, and histopathologic changes were evaluated. Breaking strength in the diclofenac group was 21% lower than in the placebo group ($P=0.027$). Anastomotic leakage was found in 4 rabbits in the diclofenac group (26.7%). The rabbits treated with diclofenac demonstrated a 16% lower collagen deposition compared with the placebo group ($P=0.008$). In our study, postoperative treatment with diclofenac had a negative effect on the anastomotic healing and strength in the ileum of rabbits. Caution should be taken in the use of diclofenac after gastrointestinal surgery.

Key words: Diclofenac – Intestinal anastomoses – Anastomotic leakage – Tensile strength – Wound healing – NSAID

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Anastomotic leakage is a severe complication after intestinal surgery, with an occurrence of 1% to 3% after small intestine resection, 3% to 4% after colonic resection, and 10% after rectal resection with an associated mortality of up to 40%.¹⁻⁴ It also leads to increased morbidity caused by abscess formation, peritonitis, ileus, and sepsis, which can result in prolonged hospitalization and increased cost.⁵ Common risk factors include smoking, alcohol abuse,⁶ American Society of Anesthesiologists score over 3, malnutrition,⁷ cardiovascular disease,⁸ male sex,⁹ and technical errors.

Healing of the intestinal wall is an organized and complex process that initially involves inflammatory mediated enzymes such as cyclooxygenases (COX-1 and COX-2). Inhibition of COX-2 is suspected to interfere with normal wound healing in the epithelial tissue of the gastrointestinal tract.¹⁰ The collagen concentration fluctuates during the healing process, showing an initial decrease owing to collagenase activity during the inflammatory phase, and then an increase during the proliferation phase.^{11,12}

Nonsteroidal anti-inflammatory drugs (NSAIDs) are an important component in today's fast-track treatment of postoperative pain and can reduce the need for opioids by approximately 30%. In the present experiment, we chose to study diclofenac, an NSAID with a fourfold higher selectivity for COX-2 compared with COX-1 (IC₈₀: 0.23 mM versus 1.0 mM). However, at the relevant therapeutic level, 70% of COX-1 would also be inhibited.¹³ Retrospective studies focusing on NSAIDs with a predominant inhibition of COX-2 indicate a clear trend toward increased anastomotic leakage after postoperative pain treatment.¹⁴⁻¹⁶ Animal studies show more inconclusive results, perhaps because most were performed on rats, which have a significantly different immune response from humans.¹⁷⁻¹⁹ Previous studies show that diclofenac decreases collagen concentration, but there are conflicting results regarding the correlation between anastomotic breaking strength and peri-anastomotic collagen concentration.^{17,20-22}

The aim of this experimental study was to investigate whether diclofenac inhibits the anastomotic healing process as assessed by increased leakage, reduced tensile strength, and histopathologic changes.

Methods

Animals

A total of 33 New Zealand White female rabbits with a mean weight of 2.85 kg (range, 2.25–3.58 kg)

were included. They were kept in wire cages at room temperature (18°C to 20°C), with 40% to 60% humidity, and a 12-hour light/dark cycle. The cages included wood shavings and hay. The animals had access to water and were fed a standard laboratory diet, Altromin 2120 (Brogaarden, Gentofte, Denmark), once daily. The rabbits were acclimatized for 1 week before the procedure.

This project was approved by the Danish National Experimental Animal Inspectorate (J.nr.: 2012-15-2934-00664; influence of drugs on intestinal anastomosis).

Study design

From previous studies in our group, the mean value of the minimal tensile strength (MITS) on small intestinal anastomoses in the rabbit was found to be 1.89 N with an SD of 0.36 N. A difference of 30% between the 2 groups was considered clinically relevant. With a 1:1 randomization, a significance level of 5%, and a power of 80%, the number needed in the study was calculated to be 32 animals (Stata/IC, Version 12.0, StataCorp, College Station, Texas).

The animals were randomized 1:1 (available in the public domain at <https://www.random.org>) into an experimental group of 17 rabbits receiving subcutaneous diclofenac 2 mg/kg (Voltaren, 25 mg/mL, Novartis, Copenhagen, Denmark) twice a day (bid) until the fifth postoperative day (POD5) and a control group of 16 rabbits receiving subcutaneous isotonic saline 0.05 mL/kg bid. The rabbits were humanely killed on POD5.

Surgical procedure

The rabbits were not fasted before the procedure as they are physically almost incapable of regurgitation. The animals were sedated by subcutaneous injection with a combination of fentanyl and fluanisone 0.3 mL/kg (Hypnorm, 10 mg/mL, Vetapharma Ltd, Leeds, UK) and midazolam 2 mg/kg (Dormicum, 5 mg/mL, Roche, Basel, Switzerland). The rabbits were then intubated with an endotracheal tube, size 2.5 to 3.5 mm (Rüsch, Kern, Germany), and if needed, an extra dose of propofol 2 to 5 mg (Propvet, 10 mg/mL, Abbot Laboratories Ltd, Queenborough, UK) was given intravenously. Anaesthesia was maintained with 2% sevoflurane (Sevofluran "Baxter," Baxter A/S, Allerød, Denmark) via an MCM 801 ventilator (Dameca, Rødovre, Denmark). Perioperative analgesia consisted of intravenous fentanyl 1 mL/h (Haldid, 50

µg/mL, Jansen-Cilag, Beerse, Belgium). Hypothermia was prevented by placing the rabbits on a heating pad set to 41°C. To prevent dehydration, 10 mL isotonic saline was administered subcutaneously before surgery. A pulse-oximeter (Nonin 8500V, Nonin Medical, Plymouth, Minnesota) was used to monitor saturation and heart rate during surgery.

A 4-cm midline laparotomy was performed under aseptic conditions after shaving the abdomen. At approximately 20 cm and 40 cm, respectively, proximal to the ileocecal junction, the small intestine was severed and 2 separate end-to-end anastomoses were made. The anastomoses were completed with 14 to 18 interrupted inverted single-layer 5-0 non-resorbable sutures (Ethilon 4-0, Johnson & Johnson Nordic, Birkerød, Denmark). The musculofascial layer was closed with 3-0 resorbable sutures (Vicryl® 3-0, Johnson & Johnson Nordic) and the skin with 4-0 nonresorbable sutures (Ethilon 4-0, Johnson & Johnson Nordic). A combination of sulfadoxine and trimethoprim 0.2 mL/kg (Duoprim Vet, Intervet Danmark A/S, Ballerup, Denmark), a broad spectrum antibiotic registered for treatment of gastrointestinal infections in animals was given intravenously as prophylaxis against infection at the end of the surgery. Postoperative subcutaneous buprenorphine (Temgesic, Schering-Plough Europe, Brussels, Belgium) 0.05 mg/kg was administered every 8 hours for 3 days as pain treatment to the placebo group. The animals' food, water intake, and production of stools were registered daily. Immediate indication to kill the animals was weight loss >20% of preoperative body weight or any clinical sign of acute ileus (*i.e.*, a combination of inactivity, large pupils, and absence of food intake). The animals were humanely killed with an overdose of 2 mL intravenous Pentobarbital (Pentobarbital, 200 mg/mL: KU Life, Copenhagen, Denmark). A repeat laparotomy was performed, and the 2 anastomoses were inspected for macroscopic leakage or infection. The 2 segments were harvested by cutting 5 cm proximally and 5 cm distally to the anastomoses, carefully removed adhesions, and leaving the sutures *in situ*. The 10-cm-long segments were cleansed with saline.

Anastomotic breaking strength

The breaking strength was tested on the distal segment directly after harvesting using a tensile machine (LF Plus; Lloyds Instruments, Fareham, UK) equipped with XLC 10 N load cell (Lloyds Instruments). We mounted the segment with 10 mm

between the anastomosis and the clamps on each side and measured the minimal force (N) at breaking point with a deformation rate of 10 mm/min. A software program (Nexygen, Lloyds Instruments) was used to calculate a load-strain curve, which showed the MITS that ruptured the anastomosis. The rupture site was classified as being either in the anastomotic line or outside the anastomotic line.

Histopathologic analysis

After removal of the sutures, the proximal anastomotic segment was fixed in 4% formaldehyde. The segment was dehydrated and embedded in paraffin blocks and sliced 3-µm thin. To evaluate inflammatory cell composition, a hematoxylin and eosin (H&E) staining was performed. Masson's trichrome stain was used to assess collagen formation. A conventional binocular Leica DMR light microscope (Leica Microsystems A/S, Herlev, Denmark) with objective 40/0.75 was used.

An experienced pathologist, who was blinded to the randomization, graded the histologic changes of the specimens. All specimens were evaluated twice to ensure the quality of the assessment. The histologic features were inflammatory cell infiltrate, fibroblast deposition, collagen formation, blood vessel growth, foreign body giant cells, and necrosis. Except from foreign body giant cells and necrosis, the features were scored from 1 to 4 (1 = absent or minimally present, 2 = mildly present, 3 = moderately present, 4 = markedly present). Foreign body giant cells and necrosis were scored as present or absent.

Statistical analysis

The potential difference in MITS between the 2 groups and the effect of number of sutures, collagen, and weight loss were assessed by a linear regression analysis using a significance level of 0.05. The model was checked by inspection of the residuals and gave no cause for concerns. To compare the histologic changes, the 2-tailed Fisher exact test was used. The Student *t* test with unequal variances was used to compare the weight loss between the groups. Data were analyzed using Stata/IC (Version 12.0, Stata-Corp). A *P* value < 0.05 was considered statistically significant.

Results

Of the 33 animals, 3 died postoperatively. One rabbit in the diclofenac group was humanely killed at

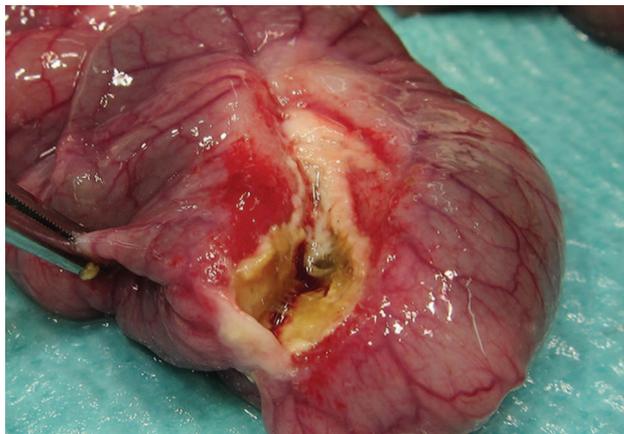


Fig. 1 Encapsulated leakage at POD5 when a part of the adhesion is removed.

POD1 after the discovery of strangulation and necrosis of an intestinal segment due to its entrapment in the abdominal wall, which was caused by the failing of the suture line in the abdominal wall. Two rabbits were found dead in their cages, 1 in the diclofenac group at POD1 and 1 in the placebo group at POD2; autopsy did not reveal cause of death, and there was no sign of anastomotic leakage or infection.

Four rabbits (26.7%) in the diclofenac group had encapsulated feces close to the suture line at POD5 (Fig. 1). The anastomoses were considered as dehiscenced and therefore not amenable to the MITS test. No rabbits in the placebo group had any signs of anastomotic leakage or infection. Both groups had varying amounts of adhesions, and some animals had partial intestinal occlusions without any overt clinical symptoms.

Anastomotic breaking strength

The diclofenac group had a 21% lower breaking strength ($P = 0.027$) in the MITS test compared with the placebo group (Fig. 2, Table 1). Four rabbits, all belonging to the diclofenac group, were not amena-

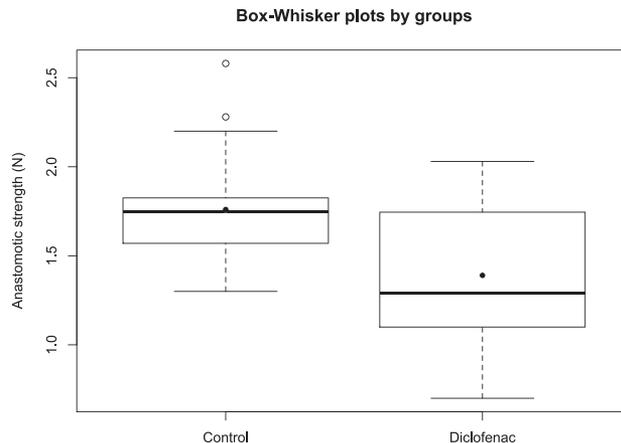


Fig. 2 Box-whisker plots for the MITS values at POD5 by group. Mean and median values are indicated by filled circles and bold lines, respectively. Plot for the diclofenac group is based on the 11 non-dehiscenced rabbits.

ble to the MITS test because of dehiscence. If this condition is related to the diclofenac group, the estimated difference in breaking strength is likely to be conservative. There was no significant correlation between the anastomotic breaking strength and collagen formation ($P = 0.11$). Two ruptures occurred outside the suture line in the diclofenac group and one in the placebo group.

Histopathologic analysis

Histologic investigation of the anastomosis demonstrated a lower collagen deposition in the treatment group by 16.7% ($P = 0.008$) compared with the placebo group (Table 2, Figs. 3 and 4). Infiltration of inflammatory cells was 16.6% ($P = 0.26$) lower in the diclofenac group but the difference was not statistically significant. No significant differences were found in neovascularization, fibroblast deposition, necrosis, or foreign body giant cells.

Both groups had similar postoperative status regarding food and water intake, mobility, and weight, where the mean weight loss was 5.2% for

Table 1 MITS and weight

	Diclofenac (n = 15) ^a	Placebo (n = 15) ^a	P value
MITS (N)	1.39 (0.70–2.03) ^b	1.76 (1.30–2.58)	0.027
Preoperative weight (kg)	2.92 (2.25–3.38)	2.79 (2.25–3.58)	0.39
Weight at POD5 (kg)	2.76 (2.14–3.23)	2.61 (2.15–3.38)	0.28
Weight difference, preoperative–POD5 (kg)	–0.15 (–0.35 to 0.02)	–0.18 (–0.32 to –0.06)	0.44

^aValues are presented as mean (range).

^bBased on the 11 non-dehiscenced rabbits.

Table 2 Distribution of histopathologic rating, mean value, and P value^a

	Inflammatory cell infiltrate				Fibroblast deposition				Collagen formation				Neovascularization				Necrosis		Foreign body giant cells	
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	Yes	No	Yes	No
Diclofenac (n = 15)	5	6	2	2	1	3	5	6	1	6	8	0	8	3	3	1	5	10	6	9
Mean ± SD	2.1 ± 1.0				3.1 ± 1.0				2.5 ± 0.6				1.8 ± 1.0							
Placebo (n = 15)	10	4	1	0	0	1	6	8	0	5	3	7	10	4	1	0	2	13	7	8
Mean ± SD	1.4 ± 0.6				3.5 ± 0.6				3.1 ± 0.9				1.4 ± 0.6							
P value	0.26				0.61				0.008 ^b				0.66				1.00		0.39	

^aHistologic features were rated 1 to 4, apart from necrosis and foreign body giant cells. Fisher exact test was used for testing the statistically significant difference between the 2 groups.

^bStatistically significant.

the diclofenac group and 6.3% for the placebo group (Table 1). There was no significant effect of the number of sutures upon MITS.

Discussion

Using a rabbit ileum anastomosis model, we have demonstrated that diclofenac has a negative effect on anastomotic healing, resulting in a significant lower breaking strength in the treatment group at POD5 compared with the placebo group. Furthermore, the estimated difference is likely conservative as 4 rabbits in the diclofenac group had dehisced and would probably have shown low breaking strengths if they could have been measured. The reason for testing small intestine anastomosis was the possibility of obtaining a high degree of standardization compared with colonic anastomo-

sis, which is difficult or even impossible to handle in most animals including the rabbit. Furthermore, the healing process should be similar in the 2 organs.

The results from our study are similar to previous studies investigating anastomotic strength in rats and rabbits at POD7.^{17,21} From human studies, early and late leaks may occur. The early leaks are more frequent and occur typically at POD4 or 5 at a time when the healing process shifts from the inflammatory reaction to the proliferative phase,²³ when the strength of the anastomosis is lowest, and it is therefore the most interesting time to investigate. Although leakage often is diagnosed or recognized at POD7 in humans, it is well known that a diagnostic delay of 1 to 4 days is common. At POD5, the fibroblast activity is at its highest level with the collagen-rich granulation tissue leading to increased strength in the anastomosis. During the

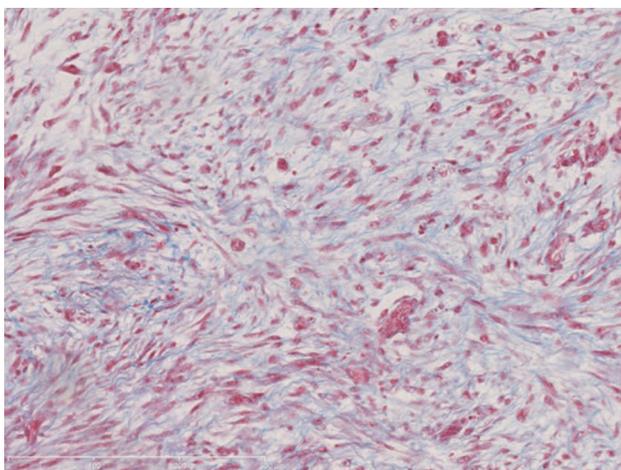


Fig. 3 Masson's trichrome stained section displaying granulation tissue close to the anastomosis for evaluation of collagen formation. Example from the placebo group with a richer collagen formation (blue) and less inflammatory cell infiltrate.

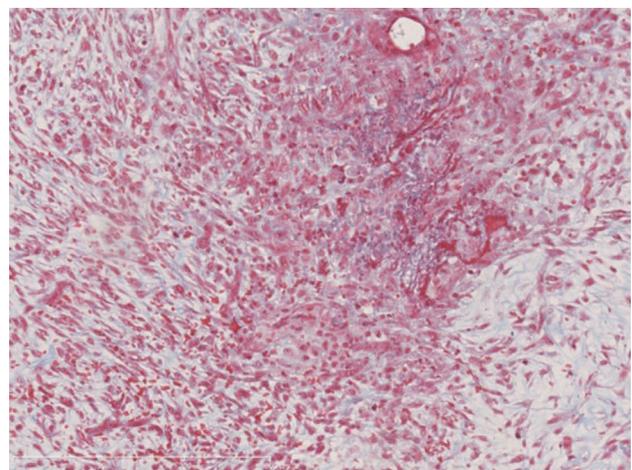


Fig. 4 Masson's trichrome stained section displaying granulation tissue close to the anastomosis for evaluation of collagen formation. Example from the diclofenac group with less collagen formation and more inflammatory cell infiltrate.

proliferation phase, COX-2 expression has been demonstrated to be associated with the mitogenic activity for fibroblast and its production of collagen through basic fibroblast growth factor (bFGF).²⁴

Our results also demonstrate a tendency toward an increased rate of anastomotic leakage in the treatment group. The typical presentation was a small amount feces in direct connection to the suture line and encapsulated by fibrin without signs of peritonitis, pointing toward an early leakage. The subclinical leakage could later have led to complications if the animals had not been humanely killed at POD5. Whether a possible negative effect of NSAIDs is most likely to be manifest during the early acute inflammatory phase or the later proliferative phase is an important question. A recent study concluded that the COX-2 selective NSAID carprofen (no longer on the market for humans) interferes at an early stage in the healing of ileum anastomosis in rats, which showed a marked decline in leakage rate if the treatment was delayed by 48 hours.²⁵ This finding emphasizes the importance of the early effects of NSAIDs in the anastomotic healing process. A previous study also demonstrated a significant reduction of COX-2 enzyme concentration in peri-anastomotic tissue after 3 days of postoperative treatment with diclofenac in rats but did not find a reduction in anastomotic breaking strength.¹⁸

In the evaluation of anastomotic healing, both bursting pressure and breaking strength are commonly accepted methods. Measuring bursting pressure during the proliferation stage often results in rupture outside the anastomosis.^{20,21} According to Ikeuchi *et al*, who investigated the 2 methods, breaking strength was recommended from POD5 or later because of its greater accuracy to estimate anastomotic healing.²⁶ Although we chose breaking strength as the most suitable test, there were some difficulties in preparation and mounting of the segments as a result of adhesions and torsion in or close to the anastomosis. This may have had impact on our results, but we assume they are equal for both groups. The use of nonresorbable sutures is a major difference from the normal surgical procedure in humans, where resorbable materials are used. However, it is standard to use nonresorbable sutures in experimental studies on tensile strength of intestinal anastomosis to avoid confounding factors such as degradation of suture materials, which may differ among the individual animals.

Hydroxyproline concentration in the peri-anastomotic tissue is often used to measure the amount of collagen. Most studies have not found any correlation between anastomotic strength and hydroxyproline concentration.^{20,21,27} It should be noted that the measure of hydroxyproline concentration in the peri-anastomotic tissue only informs about the total amount of collagen and not necessarily about new collagen production or the quality of cross-linking, organization, and maturity.²⁸ Recently, it was demonstrated that postoperative diclofenac treatment significantly decreased the accumulation of collagen in subcutaneous granulation tissue.²² In the present study, we therefore decided to histologically estimate the new production of collagen in the granulation tissue of the anastomosis and found decreased collagen deposition in the treatment group but no correlation with the anastomotic breaking strength. Further histologic evaluation showed a tendency toward increased infiltration of inflammatory cells in the treatment group, without any difference in fibroblast infiltration, vascular growth, foreign body giant cells, or necrosis compared with the placebo group. As earlier studies have suggested that COX-2-selective NSAIDs inhibit the production of prostaglandin E₂,²⁹ we did expect less infiltration of inflammatory cells in the treatment group. The findings may point toward a delayed rather than a decreased inflammatory response and therefore to a delayed healing process—a possible explanation that correlates with findings on bone fracture healing in rats treated with diclofenac.³⁰

We were unable to find in the literature a recommended dose of diclofenac for rabbits, and there are no studies on the pharmacokinetics of diclofenac in rabbits either in our or other studies to our knowledge. The given dosage of 4 mg/kg/d has been used in previous studies performed on rats^{17,18} and was therefore considered suitable in the present study. This dosage is approximately twice as high as recommended for humans but is still under toxic levels.³¹ A higher dosage than normally administered was used to study the possible existence of adverse effects. The unknown serum concentration of diclofenac in this study could be a confounder, and ideally the serum concentration of diclofenac should have been measured, but we had no access to this.

Our results show that postoperative treatment with diclofenac has a negative effect on anastomotic healing in the ileum of rabbits at POD5,

indicated by reduced breaking strength and a tendency toward increased anastomotic leakage. As in any experimental study on animals, our results cannot be directly extrapolated to humans for various reasons. The study animals may be more sensible to the given surgical intervention with a different healing and recovery process. In the present study model, 2 separate small intestinal anastomoses were performed, which is different from the clinical situation in which only 1 anastomosis is the most common. With these reservations in mind, our group recommends that diclofenac and other NSAIDs with predominant inhibition of COX-2 should be used with caution as analgesic treatment after gastrointestinal surgery. A human randomized controlled trial would be useful to evaluate the risk of postoperative use of NSAIDs after gastrointestinal surgery but could be difficult to implement because of ethical considerations. Future experimental studies should focus on identifying which biological mechanisms NSAIDs interfere with, the differences between the various types of NSAIDs, and the timing of their interaction.

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