

Anastomotic healing in a rat model of peritonitis after non-steroidal anti-inflammatory drug administration

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ABSTRACT

The tissue inflammatory response can influence the outcome of anastomotic healing. Anastomotic leakage represents a dreadful complication after gastrointestinal surgery, in particular sepsis and intra-abdominal infections impair the restorative process of colic anastomoses. It has been debated whether the administration of non-steroidal anti-inflammatory drugs (NSAIDs) is a risk factor for dehiscence, since many patients receive NSAIDs in the early postoperative period. Our aim was, for the first time, to analyze the morpho-functional effects of postoperative administration of two commonly used NSAIDs, Diclofenac and Ketorolac, on the healing process of colo-colic anastomoses constructed under condition of fecal peritonitis in a rat model. Sixty adult male rats underwent two surgical procedures: peritonitis induction and colo-colic anastomosis, and were divided into three groups: 20 rats received saline; 20 rats 4 mg/kg Diclofenac and 20 rats 5 mg/kg Ketorolac. We assessed anastomosis strength, morphological features of tissue wound healing, immunohistochemical metalloproteinase 9 (MMP9) expression and collagen deposition and content by Sirius red staining and hydroxyproline level. We found no significant difference in bursting pressure, collagen content and organization and morphological features between the groups, except a significantly reduced presence of inflammatory cells and MMP9 expression in the groups treated with NSAIDs. Our findings showed that Diclofenac and Ketorolac administration did not affect post-surgical healing and did not increase the leakage risk of colo-colic anastomoses during peritonitis.

Key words: Non-steroidal inflammatory drugs; inflammation; colo-colic anastomosis; peritonitis; wound healing; MMP9.

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Contributions: RG, MG, conceived, designed and coordinated the research; GL, performed morphological and IHC studies; FC, performed the surgical procedures; ES, SS, FO, performed some of the experiments; FO, also supervised the procedures applied to the rat model; ES, FC, GL, also contributed to the discussion; RG, GL, MO, ES, MP, MG, were also actively involved in writing and editing the manuscript.

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Introduction

Anastomotic leakage represents a dreadful complication after gastrointestinal surgery. Different risk factors have been evaluated in order to prevent leaks,¹⁻³ and among these, perioperative administration of specific drugs may play a key role. The final stability of the anastomosis is mainly guaranteed by tension-free technique and undisturbed perfusion of the anastomotic tissue; however, the healing process can be influenced by patient specific factors including inflammatory condition of the tissue.⁴

Intra-abdominal adhesions are fibrous adhesions formed between serosal surfaces as result of an inflammatory reaction and occur after abdominal operations and bacterial infections. Non-steroidal anti-inflammatory drugs (NSAIDs) have been used in experimental studies to modulate the initial inflammatory stage and adhesion formation.⁵ They act through the inhibition of the cyclooxygenase enzymes COX-1 and COX-2, which leads to the reduction of the prostaglandin (PG) levels, thus performing their anti-inflammatory, analgesic and anti-hyperalgesic properties. NSAIDs use is, nowadays, a cornerstone in the multimodal management of post-operative pain.⁶ In the last years, however, doubts have been raised regarding their safety in abdominal surgery since they seemed to be associated with a higher risk of serious complications as anastomotic leakage.⁷

The anastomosis strength depends on collagen deposition, which is regulated by COX enzymes through metalloproteinase (MMP) modulation;⁸ in particular, MMP9 is associated with anastomotic leak.⁹ NSAIDs use, reducing COX enzymes, could impair healing by disturbing collagen metabolism.^{10,11}

Emergency surgery for peritonitis is an independent risk factor for anastomotic leak. Sepsis and intra-abdominal infections, in fact, impair the restorative process of colic anastomoses mostly through a reduction of structural collagen synthesis in the submucosa.¹² Therefore, we might suppose that NSAIDs could even more threaten anastomotic healing when used in septic patients with peritonitis and should be used with particular caution in this setting. Nonetheless, experimental data are conflicting and there is still a substantial lack of knowledge on this topic. The purpose of our study was to clarify the morpho-functional effects of postoperative administration of two commonly used NSAIDs, Diclofenac and Ketorolac, on the healing process of colo-colic anastomoses constructed under condition of fecal peritonitis in a rat model. In particular, we assessed anastomosis strength and morphological features of wound healing, as well as immunohistochemical MMP9 expression and collagen deposition and content.

Materials and Methods

Animal models and experimental design

Sixty adult male Wistar rats (weight 550-600 g) were provided by the Italian National Institute on Aging (INRCA) IRCCS (Ancona, Italy) and were accustomed to laboratory conditions for one week. The animals were housed two per cage at 22°C with a 12-h light/dark cycle and were allowed free access to standard rodent chow (2518CR, Charles River Laboratories, Calco, LC, Italy) and water. All animals underwent two surgical procedures: peritonitis induction and colo-colic anastomosis. Through a mid-line laparotomy, the caecum was back-filled with feces and ligated just below the ileocecal valve with a 2-0 silk tie. The anti-mesenteric side of the caecum was punctured once with a 19-gauge needle below the ligature, according to the model proposed by Wichterman.^{13,14} The animals were then placed in single cages in cabinets with constant temperature and ventilation, and they had

free access to water and chow. After 24 h (day 0), the abdomen was reopened and the ligated caecum was resected. The left colon was transected approximately 3 cm above the peritoneal reflection, and an end-to-end anastomosis was constructed under an operating microscope using a single layer of eight interrupted inverting sutures (Prolene 6-0, Ethicon, Pomezia, RM, Italy). All animals were given Imipenem cilastatin (MSD Italy, Rome, Italy) 40 mg/kg intraperitoneally, starting after the anastomosis construction and continuing until day 3.

Immediately after anastomosis construction, rats were randomly assigned to one of the three groups. Twenty rats received saline (2 mL, subcutaneous; Control group, CG), 20 were administered 4 mg/kg Diclofenac (Sandoz GMBH, Kundl, Austria) (subcutaneous; Diclofenac group, DG) and 20 were administered 5 mg/kg Ketorolac (Recordati, Milan, Italy) (subcutaneous; Ketorolac group, KG) until post-operative day (POD) 3.

During follow-up, rats were checked twice daily by the designated veterinary doctor for signs of reduced wellbeing, including apathetic behavior, anorexia, piloerection and passage of stool; any suffering or symptoms of illness were recorded and any animal that was excessively pained, indicating septic shock, was sacrificed in a CO₂ chamber according to guidelines. Animals were euthanized in a CO₂ chamber at POD 5 for anastomosis evaluation.

The experimental protocol was conducted according to the Directive 2010/63/EU on the protection of animals used for scientific purposes and was approved by the Animal Research Ethics Committee of the Italian National Institute on Aging (INRCA) IRCCS (Ancona, Italy) and by the Italian Ministry of Health (license n. 874/2016 – PR).

Macroscopic evaluation

On POD 5, re-laparotomy was performed. The abdomen was examined to detect signs of anastomotic leakage according to a score proposed by Yauw *et al.*,¹⁵ where “0” indicates no signs of leakage, “1” anastomotic abscesses, “2” free pus or large abscesses, and “3” fecal peritonitis or visible dehiscence.

Bursting pressure

A 4-cm segment of the descending colon with the suture line in the middle was carefully resected including adhesions and surrounding tissues. Intraluminal faeces were removed. The segments were connected to an infusion pump on the proximal extremity and to a pressure transducer on the distal extremity. They were filled with saline containing methylene blue (rate 2 mL/min) and the intraluminal pressure was continuously monitored. The maximum pressure documented before rupture was recorded as anastomotic bursting pressure. The site of rupture was also noted. Dehiscent anastomoses were recorded as 0 mmHg.

Histological examination

After the measurement of bursting pressure, anastomotic segments of 1 cm in length were carefully cleaned from adhering tissues and opened at the mesenteric side. The specimens were longitudinally transected to obtain two samples containing the anastomosis in the middle: one was fixed for 24 h in 10% neutral buffered formalin solution and embedded in paraffin for histopathological analysis, and the other one was stored at -80°C for tissue hydroxyproline content evaluation.

The embedded biopsies were sectioned at 2.5 µm thickness and stained with haematoxylin and eosin (H&E). Light microscopy (Nikon Eclipse E 600, Nikon Instruments, EuropeBV, Kingston, Surrey, England) was used to evaluate the progression of the mucosal anastomotic re-epithelialization, inflammatory cell presence, granulation tissue formation, presence of fibroblasts and collagen distribution according to the parameters (Table 1) modified from Pantelis *et al.*¹⁶

Immunohistochemistry

From the paraffin-embedded tissues, 2.5 µm sections were prepared for MMP9 immunostaining. The sections were deparaffinised, rehydrated and treated with microwave for heat-induced epitope retrieval in 10 mmol/L sodium citrate buffer (pH 6.0) and incubated overnight at 4°C with the monoclonal antibody anti-MMP9 (diluted 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The reaction was revealed using the streptavidin-biotin-peroxidase technique according to the manufacturer's instructions (Dako-Envision Plus/HRP peroxidase kit, Dako SpA, Milan, Italy). Sections were incubated with 3,3-diaminobenzidine (Sigma-Aldrich, Milan, Italy) and counterstained with Mayer's haematoxylin (Bio-Optica SpA, Milan, Italy). As negative controls, the samples were treated as described previously, except that primary antibody was omitted and replaced by non-immune sera (sc-2025, Santa Cruz Biotechnology). We evaluated MMP9 immunostaining in both mucosa and submucosa of the anastomotic area. The number of MMP9 positive stained cells was counted in the selective fields by using a Nikon Eclipse E600 light microscope at x250 magnification and estimated as percentage of the total cell counted.

Collagen distribution and quantification

Sections were stained with Sirius red (365548 Sigma-Aldrich Sirius Red), a widely used histological technique to visualize and analyze the distribution of collagen type I and III and the ratio between young and mature fibers. The quantitative measure of collagen fibers was calculated as the percentage of Sirius red positive collagen content by digital image analysis. This software enables to select the anastomotic area of bowel biopsy by the red, green, and blue (RGB) light channels. The percentage of total anastomotic tissue and collagen area was calculated. After digital image capture, structural collagen in large portal tracts, blood vessel walls, artefacts and lymphoid aggregates were eliminated. Quantification of Sirius red-positive parenchyma was performed at ×100 final magnification; pictures were taken with the Eclipse E800 microscope (Nikon) equipped with a cooled digital camera (Nikon DS-U1, Nikon) and analyzed by Lucia G 5.0 software (Nikon).

Defining the level of hydroxyproline is a good method of evaluating the amount of tissue collagen.¹⁷ Tissue samples were hydrolyzed in 37% HCl at 120 °C for 3 h and allowed to evaporate to dryness. Hydroxyproline levels were analyzed using a commercially available kit (Hydroxyproline Assay Kit, MAK008, Sigma-Aldrich, Milan, Italy) according to the manufacturers' instructions (Multiskan GO, Thermo Fisher Scientific Inc., Waltham, MA, USA). Hydroxyproline content was measured against standard concentrations and expressed as micrograms per milligrams of sample (µm/mg).

Statistical analysis

Statistical analyses were performed using the SPSS 16 package (SPSS Inc., Chicago, IL, USA). All results are shown as mean ± standard deviation (SD). ANOVA and Bonferroni test were used to compare all groups. Pearson's correlation was used to test the relationship between Sirius red staining and hydroxyproline content. Significance was accepted when the P value was <0.05.

Results

Overall observations

Following cecal ligation and puncture the animals exhibited signs of discomfort including dirty nose, ocular exudate, apathetic behavior and diarrhea. No difference was observed among the three groups. Fecal peritonitis was confirmed in all animals at the time of cecal resection. Four rats in CG (20%), 1 rat in DG (5%) and 2 rats in KG (10%) died by POD 2. *Post-mortem* re-laparotomy did not reveal anastomotic dehiscence but colonic ischemia in two cases. Sepsis was considered the cause of death for the remaining rats.

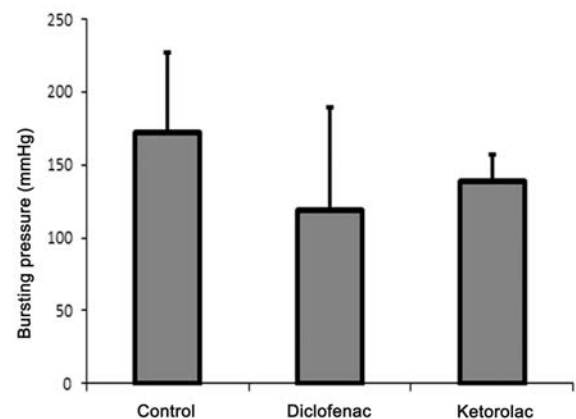


Figure 1. Bursting pressure test according to the group (Control, Diclofenac and Ketorolac) in colo-colic anastomosis of rat peritonitis model. Differences are not significant. Data are mean values ± SD.

Table 1. Scores of morphological features.

	Absence	Incomplete with a single layer of cells	Complete with a single layer of cells	Complete with granular epithelium
Mucosal anastomotic re-epithelialization	1	2	3	4
	Absence	Slight	Mild	Intense
Inflammatory cell presence	1	2	3	4
Fibroblasts	1	2	3	4
Collagen deposition	1	2	3	4
	Complete interruption	Muscle synechia	Complete restitution	-
Muscle layer continuity	1	2	3	-

Macroscopic evaluation

On POD 5, firm adhesions between the anastomotic segment and surrounding intestinal loops were observed in all animals. One rat in CG (6%) and one in KG (5%) showed a perianastomotic abscess (score 1);¹⁵ a visible dehiscence was noted in one rat in DG (5%) (score 3) while the all other animals were scored as 0. There was no difference in the number of leakages among the groups (Table 2).

Bursting pressure

No significant difference in bursting pressure was observed between the groups (CG 172 ± 55 mmHg, DG 119 ± 70 mmHg, KG 139 ± 18 mmHg, $P > 0.05$). Two anastomoses in CG, one in the DG and three in the KG burst outside the anastomotic line (Figure 1).

Histological evaluation

The impact of different treatment regimens on anastomosis wound healing was scored according to Table 1 and the statistical analysis is shown in Table 2.

No gross differences were observed in the architecture of the 5-day-old anastomoses between the control and treated groups (Figure 2). No difference was noticed regarding mucosal re-epithe-

lialization, incomplete with a single layer of cells at the margin level. We found an evident granulation tissue in all groups and similar fibroblast presence, collagen fiber organization and discontinuation of the tunica muscularis. The anastomoses showed a significantly reduced presence of inflammatory cells in the treated groups ($P < 0.05$).

MMP9 immunostaining

MMP9 expression was present in all samples, localized in mucosal and submucosal stromal cells (Figure 2). In the treated groups we found a significant decreased MMP9 expression (DC 18 ± 0.80 and KC 20 ± 1.50) compared to CG (30 ± 2.20 , $P < 0.05$). There was no significant difference between DG and KG.

Collagen quantification

The Sirius red stained fibers were similar in CG (6.27 ± 1.59) and DG (6.99 ± 3.09), although in KG they were lower but not statistically significant (4.29 ± 2.87 ; Figure 3A).

No significant difference for hydroxyproline content was observed (CG 0.212 ± 0.03 , DG 0.172 ± 0.03 , KG 0.198 ± 0.04 ; Figure 3B).

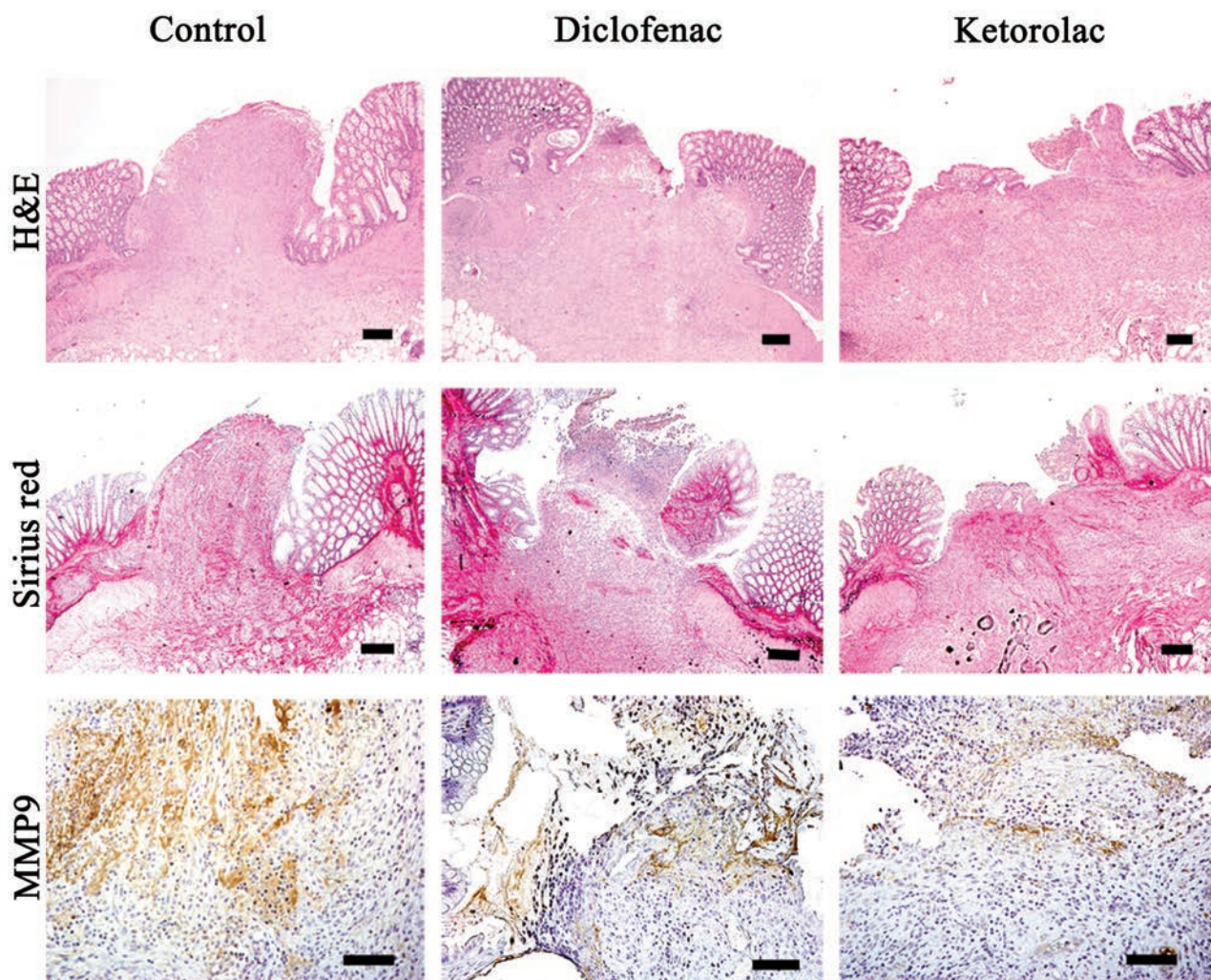


Figure 2. Histological examination of colo-colic anastomoses in control and treated rats. No high differences were observed in all the parameters observed, except for inflammatory cells that were significantly reduced in anastomoses of the treated rats. H&E, 40x magnification; immunoperoxidase, scale bar 100 μ m.

We found a positive correlation between hydroxyproline concentration and Sirius red staining in all groups (CG $P=0.000$, $r=1.00$; DG $P=0.017$, $r=0.983$; KG $P=0.038$; Pearson's correlation, $r=0.997$).

Discussion

Anastomotic leakage is a harmful complication after colorectal surgery that often necessitates urgent reoperation and increased lengths of hospital stay and in-hospital mortality.³ Many patients receive NSAIDs in the early postoperative period to prevent the formation of prostaglandins that contribute to post-surgical inflammation.¹⁸

It has been debated whether the administration of NSAIDs is a risk factor for dehiscence;^{6,19-23} in fact, they are used to decrease inflammation and prevent adhesion at the anastomosis site but may lead to deterioration or retardation of wound healing, since inflam-

matory reaction constitutes the first, essential phase of wound repair sequence. Daams *et al.*²⁴ showed that anastomosis healing occurred precisely by formation of a fibrotic cap at the serosal side during inflammation stage. Therefore, inflammatory response is either pro-inflammatory, impairing wound healing, or anti-inflammatory, promoting wound healing; a shift in the pro-inflammatory/anti-inflammatory index can influence the outcome of anastomotic repair.²⁵

Some authors reported how NSAIDs can affect the leakage rate and healing²³⁻²⁶ while others showed the opposite.^{27,28} Unfortunately the results are scarcely comparable, because different dosages, ways of administration and length of therapy were used; in addition, various parameters were adopted for anastomosis evaluation. Thus, further investigations about this topic are extremely helpful.

The anastomosis tensile strength is mainly due to the submucosal collagen that is particularly involved in the inflammatory

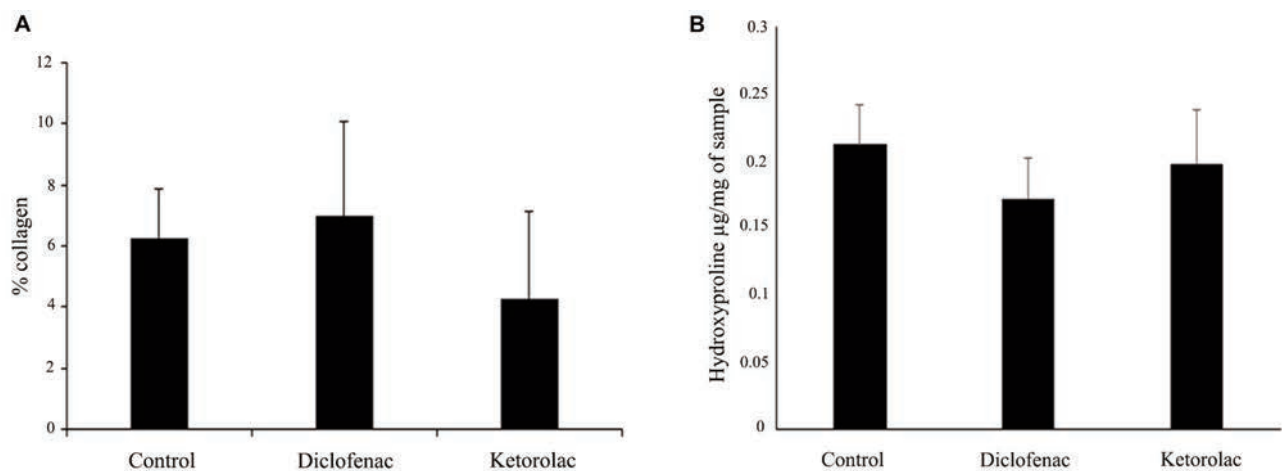


Figure 3. Collagen quantification in colo-colic anastomoses of control and treated rats. A) Sirius red staining showed a similar collagen deposition in the three groups. B) The measurement of hydroxyproline content revealed no statistically significant between control and treated samples. Means \pm SD are shown.

Table 2. Macroscopic assessment of anastomoses.

	CG	DG	KG
Animals, n	20	20	20
Premature death, n (%)	4 (20%)	1 (5%)	2 (10%)
Anastomotic leakage at POD 7, n (%)	1 (5%)	1 (5%)	1 (5%)
Leakage score	1	3	1

CG, untreated anastomosis; DG, anastomosis treated with Diclofenac; KG, anastomosis treated with Ketorolac; POD, post-operative day.

Table 3. Statistical evaluation of wound healing parameters at day 7 post-surgery in rat anastomosis model.

	Mucosal anastomotic re-epithelialization score	Inflammatory cell presence score	Fibroblasts score	Collagen deposition score	Muscle layer continuity score
CG	2.4 \pm 0.02	3.0 \pm 0.20	3.0 \pm 0.2	2.3 \pm 0.05	1.2 \pm 0.25
DG	2.4 \pm 0.15	2.3 \pm 0.08*	3.2 \pm 0.8	2.22 \pm 0.08	1.25 \pm 0.20
KG	2.5 \pm 0.10	2.2 \pm 0.05*	3.5 \pm 0.5	2.25 \pm 0.01	1.22 \pm 0.30

CG, untreated anastomosis; DG, anastomosis treated with Diclofenac; KG, anastomosis treated with Ketorolac. *DG and KG vs CG: $P<0.05$. Data were evaluated according to scores showed in Table 1.

phase of wound healing where a balance between collagen lysis and synthesis is essential.²⁹ Therefore, collagen is an interesting healing marker. Its degradation is due to MMP overexpression by cytokine-activated macrophages. Initially MMPs start to increase and play a central role in early organization of the extracellular matrix (ECM), subsequently they decrease and the consequent reconstruction of collagen fibers and remodeling of ECM proteins are reported.³⁰ Prolonged expression and activity of MMPs can cause degradation of collagen and lead to reduction in anastomotic strength resulting in anastomotic leakage that, in turn, can produce continuous inflammatory stimuli with persistent MMP activation.³¹

The intra-abdominal sepsis has been reported to reduce collagen gene expression in the perianastomotic tissue.³² When bowel perforation occurs and a primary anastomosis is then constructed, the presence of enteric pathogens in the peritoneal cavity might induce MMP overexpression, contributing to leak occurrence¹⁰ and giving rise to gut barrier failure and bacterial translocation, mainly through a reduction of epithelium structural integrity.^{10,33}

Since there is a lack of knowledge on the intestinal anastomosis in sepsis condition, we aimed to evaluate the effects that administration of two NSAIDs, Diclofenac and Ketorolac, exert on colocolic anastomosis constructed under condition of fecal peritonitis.

Diclofenac has a selective action on the COX-2 isoform, comparable to Celecoxib and it has been extensively studied in experimental models; as concerns the non-selective COX inhibitor Ketorolac, although it is a very commonly used analgesic drug in intestinal surgery, its effect on intestinal anastomoses has not been largely investigated.

Our results showed that both Diclofenac and Ketorolac did not affect the healing of colocolic anastomoses constructed during peritonitis. Different studies have been carried out, although in experimental models without sepsis. In accordance with our findings, Klein *et al.*³⁴ reported no association between Diclofenac and Ketorolac use and anastomotic leakage; on the contrary, Inan *et al.*³⁵ noticed that Diclofenac administration could reduce the mechanical strength of colocolic anastomoses and hydroxyproline content. Interestingly, Van der Vijver *et al.*²⁰ and Yauw *et al.*¹⁵ showed a negative effect of Diclofenac administration limited to ileal anastomoses, suggesting a specific sensibility to COX-inhibitors of different segments of the gastrointestinal tract.

In our study, MMP9 expression decreased in the animals treated by NSAIDs. MMPs can be released from almost all connective tissue cells present in the bowel in response to inflammatory stimuli, in particular, MMP9 is known to be associated with inflammation in general³⁶ and anastomotic leak.^{8,9,37,38} Excessive MMP9 activation could upset the balance during anastomotic healing such that collagen degradation results in leakage instead of healing. NSAIDs decrease MMP expression by inhibiting COX isoforms,³⁹ in particular MMP9,⁴⁰ and this effect could contribute positively to healing. We also observed a significant reduction of inflammatory cell infiltrate in DG and KG compared to CG. This evidence correlated with the decrease of MMP9 expression, since inflammatory cells are the major source of MMPs.^{27,30}

Successful anastomotic healing is defined by the ability of the previously injured bowel to withstand tensile forces. In our study, bursting pressures of the colocolic anastomoses showed no differences between the groups, indicating a comparable stability of the anastomoses. In terms of the morphological aspects (mucosal re-epithelialization, granulation tissue and fibroblast presence), the treated and untreated anastomoses were similar. Finally, Sirius red staining and hydroxyproline content revealed that Diclofenac and Ketorolac did not alter collagen levels and organization in the perianastomotic tissue.

In conclusion, our findings showed that administration of Diclofenac and Ketorolac did not increase the leakage risk of colo-

colic anastomoses during peritonitis. Further studies are needed in order to elucidate their effect on other biological factors involved in the inflammation and regulation of collagen deposition in wound healing.

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