Original Article

Effect of non-steroidal anti-inflammatory drugs on the increasing the incidence of colonic anastomosis in rats

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Abstract: Background: Anastomotic leakage is one of serious complications of colorectal surgery. Research is inconsistent about whether non-steroidal anti-inflammatory drugs influence the healing of colorectal anastomoses and increase the incidence of anastomotic leakage. Objective: To study the influence of NSAIDs on the healing of rat colonic anastomoses. Design: This was an animal randomized-control trial. This study was approved by the ethical committee of Yangpu Hospital, Tongji University. Intervention: 90 healthy Sprague-Dawley rats were randomly divided into 6 groups of 15 rats/group. Trail was performed in C (control group) with no drugs, group M with morphine for analgesia, group F with flurbiprofen axeil, group L with lornoxicam, and group P with parecoxib sodium. Main outcome measures: The main outcomes measures were serological indexes including vascular endothelial growth factor, prostaglandin E2, hydroxyproline, and C reactive protein; histological specimens from the anastomotic stoma tissue including the collagen proportion, and hydroxyproline, cycloxygenase-2, and vascular endothelial growth factor content; physical indicators, including stoma fracture pressure, fracture strength and anastomotic leakage. Results: No significant difference was observed among the indices of each group (P > 0.05). A significant difference occurred after operation (P < 0.05), with the data for groups K and M being dramatically higher than those for group F. Limitation: The study was nonblinded. Conclusion: The postoperative usages of non-steroidal anti-inflammatory drugs can decrease the strength of anastomotic tissue, and increase the incidence of anastomotic leakage.

Keywords: Non-steroidal anti-inflammatory drugs (NSAIDs), colonic anastomotic stoma, anastomotic leakage

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) have analgesic, anti-inflammatory and antipyretic effects. They perform these functions by acting on arachidonic acid, and prostaglandins synthesis, via epoxide hydrolase inhibition. Recently, using NSAIDs with anesthetics has significantly improved perioperative analgesia with good clinical result, leading to increase use for post-operative analgesia [1, 2].

Anastomotic leakage is one of serious complications of colorectal surgery [3-7]. However, research is inconsistent about whether NSAIDs influence the healing of colorectal anastomoses and increase the incidence of anastomotic leakage. Moreover new type NSAIDs, flurbiprofen axeil, lornoxicam, and parecoxib sodium are widely applied for perioperative analgesia.

In this paper, the effect of NSAIDs on the healing of colorectal anastomoses in rats was assessed. The review provides a reference for the reasonable and safe usage of NSAIDs, and discusses the possible mechanism through which they affect the healing of colorectal anastomoses.

Materials and methods

Rat selection and feeding

We selected 90 male Sprague-Dawly rats aged 6-8 weeks, and fed with common fodder. The operation was started after the rats were fed with adaptive feeds and unlimited water to drink for 1 week. The rats were fastened for 12 hours before their operation, but allowed water. The rats were fed on postoperative day. Feeding was common at all other time.
Experimental groups

Experimental rats were randomly divided into 6 groups, each containing 15 rats: Group C as a control group without any perioperative drugs, group M with morphine for analgesia, group F with flurbiprofen axetil, group L with lornoxicam, group P with parecoxib sodium, N group as a control group undergoing sham operation.

Operative methods

Rats were anesthetized via tail vein injections with midazolam (5 mg/kg) and fentanyl (0.05 mg/kg). Each anesthetized rat was fastened at supine position, and its abdomen was shaved and disinfected with ethanol and chlorhexidine. The rat was then covered with an aseptic towel. Then, a 2-cm central abdominal incision was made and the colon was transected. Next, we sewed up colorectal end-to-end anastomoses with 6-0 non-absorbable sutures, and sutured peritoneum, muscle, and skin using 1-0 non-absorbable sutures. All operations were completed under aseptic conditions.

Used drugs and time

Drugs were delivered via tail vein injections as follows. Group C (medication control): 1 ml intravenous 0.9% NaCl, 2 times/day for 3 days. Group M: 10 mg/kg of intravenous morphine, 2 times/day for 3 days. Group F: 10 mg/kg of flurbiprofen axetil (50 mg/5 ml Kaifen, Beijing Tide Pharmaceutical Co. Ltd.; national drug approval number: H20041508), 2 times/day for 3 days. Group L: 2 mg/kg of intravenous lornoxicam (8 mg of specification, Beijing Tide Pharmaceutical Co. Ltd.; national drug approval number: H20041508), 2 times/day for 3 days. Group P: 8 mg/kg of intravenous parecoxib sodium (Dynastat, 40 mg of specification, Pharmacia and Upjohn Company; national drug approval number: J20080045), 2 times/day for 3 days.

Collecting and saving experimental specimens

First, blood samples were taken from the orbital veins of each rat on the day before surgery and used for detecting the serological indicator; in the 7 postoperative days, serum from the abdominal inferior vena cava was used. Second, the rats were anesthetized, the sutures removed, and the abdomen opened to observe and determine the level of abdominal adhesion using Nagler’s classification method [8]. Third, we resected the colon from 3 cm proximal and 3 cm distal to the stoma. The colon was then flushed with phosphate-buffered saline and placed in isotonic saline solution to determine the burst pressure and facture strength of the stoma within 10 min. Finally, the stoma was divided into 2 equal parts. One part was kept at -80°C for biological experimentation. The other part was fixed by 10% formalin for immunohistochemistry.

Experimental specimen detection

Serological indicators: The concentrations of vascular endothelial growth factor (VEGF), prostaglandin E2 (PGE2), hydroxyproline (HYP), and C-reactive protein (CRP) in preoperative and postoperative serum from rats were measured by enzyme-linked immunosorbent assay (ELISA).

Histological specimens: Determined specimens were the collagen area, HYP, COX-2, and VEGF. COX-2 and VEGF in the stoma tissue were evaluated via immunohistochemistry and Western blotting, respectively. At the same time, we determined the collagen proportion with Masson’s stain and IPP6.0 determination software.

Observed abdominal indicators and the physical indicators of fracture strength: (A) Observed abdominal indicators. We observed whether anastomotic leakage, infection, or abscesses occurred. We recorded abdominal adhesions using Nagler’s classification method. (B) Physical indicators of stoma strength. The stoma burst pressure was measured using pressure test device for hollow abdominal viscera tissue strength and pressure in experimental animal models [9].

Experimental data processing

The normal distribution for different groups was analyzed using variance, and the data amount was tested using the chi-square test. A $P$ value of less than 0.05 implied a significant difference. The measurement data were expressed as mean ± standard deviation. We analyzed data using SPSS software version 13.0.

Results

Effect of NSAIDs on serum VEGF, PGE2, HYP, and CRP

Differences in VEGF, PGE-2, HYP, and CRP among the groups were not significant ($P >$
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0.05) in preoperative serum (Figure 1). Statistically significant differences in VEGF were observed between the group C and the groups L, P, and F at 7 days postoperatively (all \( P = 0.000 \)).

Statistically significant differences in PGE2 was observed between group C and group M (\( P = 0.000 \)). Significant differences were observed between groups C, L and F (\( P = 0.048, 0.003 \), respectively), except between group C and P. PGE2 increased in group M compared with the group L, P, and F, and the differences were significant (\( P = 0.019, 0.028, 0.001 \), respectively).

The data of HYP for group C significantly increased compared with those for group L, P, and F (\( P = 0.000, 0.000, 0.000 \), respectively); significant differences were observed between groups M, L, P, and F (\( P = 0.005, 0.009, 0.003 \), respectively).

Differences in CRP between group C, L, P, and F were significant (\( P = 0.000, 0.000, 0.000 \), respectively). Significant differences also existed between groups M, L, P, and F (\( P = 0.000, 0.000, 0.000 \), respectively).

Effect of NSAIDs on HYP, COX-2, VEGF, and collagen production in the stoma tissue

Effect of NSAIDs on HYP in histological specimens: A significant difference was observed between group C and group F (\( P = 0.009 \)), but the differences were not significant between group C, L and P (\( P = 0.065, 0.086 \), respectively). No significant differences were observed between the M group and the L, P, and F groups (\( P = 0.302, 0.358, 0.062 \), respectively) (Figure 2).

Effect of NSAIDs on COX-2, VEGF and collagen in histological specimens: Immunostaining results for COX-2, VEGF and collagen were showed in Figure 3. Results of semi-quantitative integrals on effect of NSAIDs on COX-2 expression in histological specimens were showed in Table 1. No significant differences in COX-2 were observed in group C, M, and N (\( P > 0.05 \)), but COX-2 was significantly higher in group C than in groups L, P, and F \( [\chi^2 = 7.002 (P = 0.03), 9.479 (P = 0.024), 13.000 (P = 0.005) \), respectively]. Significant differences in COX-2 were observed between groups M, L, P, and F \( [\chi^2 = 6.613 (P = 0.037), 9.571 (P = 0.023), 13.114 (P = 0.004) \), respectively].

Results of semi-quantitative integrals on effect of NSAIDs on VEGF expression in histological specimens were listed in Table 1. VEGF expression in group L was lower than that in group C, M, and N, with significant differences \( [\chi^2 = 9.078 (P = 0.011), 14.330 (P = 0.001), 11.830 (P = 0.003) \), respectively]. VEGF expression did not show significant difference among groups C, M, and N or among groups L, P, and F (\( P > 0.05 \)).
Figure 3. Micrographs showing COX-2 (C1, M1, L1, P1, F1) and VEGF (C2, M2, L2, P2, F2) detected by immunohistochemistry, and Masson's staining for collagen fibers (C3, M3, L3, P3, F3) in the stoma tissue. C (1~3): control group; M (1~3): morphine group; L (1~3): lornoxicam group; P (1~3): parecoxib sodium group; F (1~3): flurbiprofen axetil group. Magnification 40×.
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The collagen area was significantly greater in group C than in groups L and P (P = 0.014, 0.026, respectively). The group M was also had significantly greater collagen area than group L and P (P = 0.010, 0.019, respectively), but no significant differences were observed between groups C and M or groups L, P, and F (P > 0.05) (Figure 4).

Gene expression of COX-2 and VEGF in the stoma tissue: The real time PCR analysis of both COX-2 and VEGF gene expression showed in Figure 5.

Compared with groups C and M, VEGF expression in group L decreased, and significant differences were observed (P = 0.002, 0.001, 0.002); group F showed lower expression than groups C and M with significant differences (P = 0.007, 0.003). VEGF expression in group P was lower than that in group M with a significant difference (P = 0.041); the differences in VEGF expression were not significant among groups C and M, as well as among groups L, P, and F (P > 0.05).

Protein expression of COX-2 and VEGF in the stoma tissue: Protein expression of COX-2 and VEGF in the stoma tissue was detected by the Western blot assay (Figure 6).

Differences in COX-2 expression were not significant among groups C and M or groups L, P and F (P > 0.05), but COX-2 expression increased in group C compared with groups L, P and F (P = 0.00) and was higher in group M than in groups L, P, and F (P = 0.00 for each).

VEGF expression was significantly lower in group L than in group C and M (P = 0.011, 0.018, respectively), but no significant differ-

### Table 1. Effect of NSAIDs on COX-2 and VEGF expression in histological specimens (Immunohistochemistry)

<table>
<thead>
<tr>
<th>Group</th>
<th>COX-2</th>
<th>VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>M</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>L</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>P</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>F</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>++</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>+++</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

Note: using semi-quantitative integrals. 1) positive cells: ≤ 5% is 0 point, 6%-25% is 1 point, 26%-50% is 2 points, 51%-75% is 3 points, > 75% is 4 points; 2) negative strength: colorless is 0 point, light yellow is 1 point, yellow is 2 points, tan is 3 points. Then the total positive points multiplied by total negative points: the result is negative with 0 point; and 1-4 points is weakly positive (+); 5-8 points is positive (++); 9-12 points means intense positive (+++).
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Table 2. Effect of NSAIDs on Intra-abdominal Adhesions in Rats

<table>
<thead>
<tr>
<th>Degree</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group C</td>
<td>0</td>
<td>4</td>
<td>10</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Group M</td>
<td>0</td>
<td>3</td>
<td>11</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Group N</td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group L</td>
<td>0</td>
<td>2</td>
<td>9</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Group P</td>
<td>0</td>
<td>4</td>
<td>6</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Group F</td>
<td>0</td>
<td>6</td>
<td>4</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>

There was a single case of anastomotic leakage and 3 deaths in group F. There were fourth degree adhesions in one of the dead rats. Another died from its organs being eaten by other rats. The third death had evidence of anastomotic obstruction, proximal intestinal dilatation, an empty distal intestine, a thin gastrointestinal mucosa, erosion, intra-abdominal infection, pus exudates, and fourth degree adhesions.

Levels of intra-abdominal adhesion are listed in Table 2. The adhesion degrees among these groups were not significantly different (P > 0.05).

Real-time colonic pressures of each group and differences analysis are showed in Figure 7.

Testing for tissue strength revealed that the burst pressure in group C was higher than that in both groups L and F (P = 0.001, 0.041, respectively), as was that in group M (P = 0.001, 0.049, respectively). However, no significant difference was observed among group L, P, and F.
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Figure 7. Real-time colonic pressures and effect of NSAIDs on burst pressures of stoma specimens.
Discussion

NSAIDs are widely used for postoperative analgesia, with significant effectiveness. In addition, their effects on postoperative anastomotic leakage following proctocolectomy have attracted the attention of researchers. Many factors are able to affect the healing process of colonic anastomotic stomas, including collagen fibers, VEGF, HYP, and COX-2.

Collagen fibers provide the core structure that determines tensile strength; during the anastomotic healing process, the strength of the bonding forces is highly dependent on the submucosal collagen content. HYP is a key component of collagen tissue, occupying some 13% of the total collagen amino acids. Apart from elastin, which contains little HYP (approximately 1%) and collagen, the remaining proteins do not contain HYP.

The healing of anastomotic stomas is dependent on not only the strength and tensile force but also blood flow to the stoma. VEGF has a strong biological effect and is a special mitogen for endothelial cells, which directly stimulate vasculogenesis. At the same time, VEGF can promote the multiplication of vascular smooth muscle cells and the autocrine secretion of the basic fibroblast growth factor. Moreover, it can promote the proliferation of other cells, such as fibroblasts. As an inducible isoenzyme, COX-2 cannot express in most normal tissue but can rapidly synthesize and participate in inflammation under the induction of specific stimulations, such as inflammatory factor and endotoxin. In the inflammatory response, stimulated COX-2 is highly expressed by inflammatory factors and catalyzes PGE2 formed from arachidonic acid. This induces tissue to create VEGF, which in turn leads to a gradual increase in VEGF expression. Therefore, these factors were selected to assess their effects as indicators for anastomotic stomas.

In rats, our results demonstrate that operations could cause increases in serum VEGF, PGE2, HYP, CRP, and WBC, as well as the stoma tissue levels of HYP, COX-2, VEGF, and collagen expression. In addition, applying flurbiprofen axetil postoperatively could decrease the strength of the stoma tissue and increase the incidence of anastomotic leakage. This study has provided important evidence. First, operations without drugs caused dramatic increases in serum VEGF, PGE2, and HYP that were inhibited by NSAIDs. Second, although there were no obvious changes in VEGF, COX-2, HYP, and stoma collagen expression in those operations, postoperative NSAIDs inhibited the expression of those factors. Third, the burst pressure of each stoma decreased postoperatively compared with that in normal rats; this decrease apparently occurred after applying NSAIDs. Thus, we can conclude that NSAIDs affected the healing process of stomas via the regulation of COX-2, VEGF, and collagen synthesis.

Dissection confirmed that most postoperative stomas healed. However, there were significant differences in the measured burst pressures when using the test system for measuring the pressure of abdominal hollow viscera from experimental animals. Thus, NSAIDs may not directly affect the early stage of stoma healing, but could impact later stoma healing because of an inhibition of collagen and HYP synthesis, as well as VEGF creation.

Our experiments revealed that these above research indicators showed reduced trends in groups with NSAIDs. Besides, statistical differences were observed between the NSAID groups and the other groups (the normal group, the operation group without drugs, and the operation group with morphine). However, not all of these differences were significant. The main reasons for this are probably the small sample size (15 rats/group, including deaths), errors with experimental instruments, inadequate skills, and the experimental animals used.

In conclusion, this experimental research reveals the impact of NSAIDs on the healing of colonic anastomoses in rats. It also reveals the effects of NSAIDs on postoperative stomas together with a theoretical basis underpinning our understanding of those effects. Finally, it provides a reference that justifies using NSAIDs.

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Disclosure of conflict of interest

None.

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