Effect of Short Term Oral Administration of Silymarin on Healing of Colonic Anastomosis in Rats

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Abstract

Objective- Leakage after colon anastomosis is the most common complication with the highest mortality rate. Silymarin possesses potent anti-inflammatory, antioxidant, reparative and antibacterial activities and therefore has been used to accelerate different experimental wound healing models. This study was aimed to investigate effects of orally administered silymarin following colonic resection and anastomosis in terms of histopathological and mechanical parameters.

Design- An experimental study.

Animals- Twenty four male mature Wistar rats

Procedures- The rats were divided randomly into two groups. After aseptic preparation, descending colon was exteriorized thorough laparotomy. Then, colon resection and end to end anastomosis was performed in all rats. The control rats were gavaged with 1 ml normal saline post operatively as placebo while the rats in the treatment group received 50 mg/kg silymarin suspended in 1 ml normal saline for five consecutive days. At the 7th day, all rats were euthanized.

Results- Necropsy finding showed that leakage, adhesion formation and peritonitis scores in treatment group were significantly decreased. On histopathology, decreased inflammatory cell infiltration was observed in the treatment group. While, the scores of angiogenesis, cell proliferation, and collagen deposition were significantly increased in the treatment group. No significant difference was observed in bursting pressure when control samples were compared to the silymarin treated ones. Furthermore, mechanical properties (including: maximum load, yield load, and absorbed energy) in treatment group were significantly increased compared to control group.

Conclusion and Clinical Relevance- The results of the present study showed that oral administration of silymarin following colonic anastomosis in rats improves the structural indices of wound healing and its mechanical characteristics with lower rates of anastomotic complications.

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1. Introduction

Healing of colon wall injuries is slower than other parts of the gastrointestinal tract and is associated with more complications such as leakage, rupture, and infection, which can lead to prolonged hospitalization, increased treatment costs, and even mortality. Mechanical stress from the contraction of the muscular layer in order to empty the feces and the high intraluminal microbial load are the main etiology of these complications. Therefore, its optimal and prompt healing after surgeries is a challenge for colorectal surgeons. A variety of techniques in terms of suture placement have been recommended to avoid the complications of colon anastomosis. Recently, studies have been focused on the use of adjuvant methods such as tissue engineering methods and the administration of various medications with reparative properties. The time-consuming and cost-intensive products of tissue-engineering have effectively limited their application despite the potential benefits to the patient. It is important that the method of therapy to be efficient, cost effective and readily available to support the patient with minimal side effects. Synthetic medications (such as antibiotics and anti-inflammatory drugs) although can promote the repair process, their use might be accompanied by numerous adverse effects including alteration of the normal intestinal microbial flora, diarrhea, bone marrow depression, and gastrointestinal mucosal damage, which might not be well tolerated in patients who have recently underwent a major surgery. Silymarin is a promising phytotherapeutic agent extracted from Silybum marianum possesses reparative, antimicrobial, anti-inflammatory, and antioxidant properties. Recent studies in the field of wound healing advocated its use for treatment of experimental dermal wound healing. Increased proliferation of fibroblasts, collagen synthesis, and vascularization have been reported followed by silymarin administration. In the field of ulcerative colitis, oral administration of silymarin has previously led to a reduction in ulcerative lesions associated with lower degree of neutrophil infiltration in the colon mucosa in mouse and rat models. A randomized and placebo-controlled clinical trial showed that orally administered silymarin helped patients to remain in remission state of ulcerative lesions due to its anti-oxidative capacities. Since silymarin has been accepted as a safe herbal product with low incidence of adverse effects in therapeutic doses, it may have merit in the management of colon anastomosis.

The present study was aimed to investigate effects of orally administered silymarin following colonic resection and anastomosis in rats.

2. Materials and Methods

Study Design

In this experimental animal study, twenty four adult male Wistar rats (weighing 200 ± 20 g) were used. The rats kept in plastic cages on wood chip-type bedding, fed with chow pellet and had free access to water. The animal experiments were performed in accordance with the authors’ Institutional Animal Care Instructions (U1-1395/12/7-3/PD/351). The rats were randomly divided into two equal groups of control and treatment. The rats were anesthetized by an intraperitoneal injection of ketamine (90 mg/kg; Alfasan, Woerden, The Netherlands) and xylazine (5 mg/kg; Alfasan, Woerden, The Netherlands) combination. Following aseptic preparations, the abdomen was approached through a ventral midline incision. Then, the descending colon was carefully exteriorized extra-abdominally and held in situ by two stay sutures using 6-0 Dexon (Covidien, Mansfield, USA). To avoid contamination of abdominal cavity, saline-soaked gauze were used to isolate the colon. Using Westcott ophthalmic scissors, a colonic segment, 1.0 cm of length, was transected and anastomosed end-to-end by simple interrupted sutures using 6-0 Nylon (Ethicon Inc., Somerville, USA). After removal of stay sutures, anastomotic leak testing was performed. In this regard, 0.5
ml normal saline was injected intraluminally while the colon was occluded proximal and distal to the anastomosis and in case of suspected leakage, additional sutures were place. Then, the colon was gently returned into the abdominal cavity and the laparotomy incision was closed in single layer of simple continuous pattern for linea alba and skin, separately. Following recovery from anesthesia, the rats were returned to their cages and in treatment group, 50 mg/kg silymarin (S0292; Sigma-Aldrich Co., St. Louis, USA) suspended in 1 ml normal saline was orally administered using gavage needle for five days after surgery. Control rats received only normal saline for the same period. Seven days post-operation, the rats were sacrificed by anesthesia overdose using intraperitoneal injection of ketamine and xylazine combination. The abdominal cavity was re-opened and the anastomoses were examined macroscopically. Peri-anastomotic abscess, peritonitis, and adhesion formation were recorded. The evaluation was performed as described previously. Adhesion severity was classified as none: no adhesion (score = 0), mild: adhesions mainly between the anastomosis and the omentum (score = 1), moderate: adhesions between the anastomotic site, omentum and a loop of small intestine (score = 2), and severe: extensive adhesions (score = 3). To evaluate the severity of leakage from the anastomosis, the scoring system according to Wu et al. was used in which 0 = no leakage, 1 = mild, abscess formation at the side of anastomosis, 2 = moderate, presence of fecal peritonitis with or without abscess formation, and 3 = severe, leakage-related death.

**Bursting Pressure**

Bursting pressure was measured ex vivo. The anastomoses (n = 6 from each group) were resected en bloc along with a 2.50 cm segment of the colon on either side. After washing out the feces, the proximal end was ligated using a 3/0 silk suture, and a 20-G IV catheter (Unicut, Göppingen, Germany) was secured into the distal end and fixed to the bursting pressure apparatus thorough a T-shaped three way. Bursting pressures were measured with a sphygmomanometer. The colon was placed in a saline-filled container and was inflated with a constant oxygen flow of 1 L per min. The manometer reading was recorded as the bursting pressure when bubbles were observed or a sudden pressure decrease was noted.

**Histopathological Assessment**

Following the measurement of bursting pressure, samples were fixed in a 10% formaldehyde solution and embedded in paraffin. Sections (5 μm) were cut and stained with hematoxylin and eosin (H & E). Sections were studied in terms of infiltration of inflammatory cells, number of mature fibroblasts, and neovascularization were scored according to a previous study. Collagen content was scored according to modified Ehrlich & Hunt as described in a previous study in which score 0 = no evidence, score 1 = occasional collagen fibers, score 2 = light scattering, score 3 = abundant collagen fibers, and score 4 = dense collagen bundles under 100× magnification.

**Mechanical Evaluation**

The remaining sample from each group (n = 6) were subjected to mechanical testing. In this regard, colon including anastomotic site were harvested and mounted on STM-5 tensile device including a 5 kg load cell. The constant velocity of 30 mm/min was used for tensile test until breakage. During each test a load-displacement curve was displayed in real time, and following parameters were recorded: maximum load (N), load at yield point (N), energy absorption (J), and stiffness (MPa).

**Statistical Analysis**

The semi-quantitative histopathological parameters along with scores of adhesion and leakage were analyzed with
Mann Whitney test. The quantitative results of the analysis of the mechanical properties and bursting were compared using paired t-test. All Statistical analyses were conducted using Minitab software (version 16.0, Minitab Inc., Boston, USA) and p values less than 0.05 were considered as significant.

3. Results

Macroscopic Findings

Multiple and strong adhesions were observed in the two rats of control group (median score = 2, range 1-3). The adhesions were formed mostly to the cecum, small intestines, omentum and testes. In the silymarin treated rats, slight adhesions to the omentum were observed (median score = 1, range 1-2), (Figure 1).

Semi-qualitative statistical comparison showed that adhesions was significantly inferior in the group treated with silymarin (Figure 2), (p < 0.05). In control rats, mild to moderate anastomotic leakage into the abdomen were observed (median score = 1, range 0-2). No leakage was found in treatment group (score 0). Statistical analysis showed a significant difference (p < 0.05) between the two groups in terms of leakage. Mild peritonitis and fecal peritonitis (median score = 1, range 0-2) were observed in three and one cases of control group, respectively. While, peritonitis was observed in none of silymarin treated rats (score 0). Statistically, there was a significant difference between the groups for peritonitis (p < 0.05).

Microscopic Findings

The number of inflammatory cells (including neutrophils, lymphocytes, and macrophages) in silymarin treated group was significantly decreased compared to the control group, (p < 0.05) for all comparisons (Figures 3 and 4). According to histopathology, angiogenesis was elevated in silymarin treated samples compared to the controls, (p < 0.05). Number of mature fibroblasts in silymarin treated group was significantly higher than the control group, (p < 0.05). Scattered thin collagen fibers with random orientation in the anastomotic site were observed in control samples. In silymarin treated group, deposition of new collagen bundles were well organized (Figures 4 and 5). The statistical analysis revealed significant increase in collagen content in treatment group compared to the control group (p < 0.05).

Bursting Pressure

The mean bursting pressure for control and treatment groups were recorded as 151.33 ± 13.82 and 159.00 ± 23.07, respectively. There was no significant difference in colon bursting pressures between the groups (p > 0.05).
Figure 3. Infiltration of inflammatory cell was significantly higher in control group (A) compared to treatment group (B). White arrows indicate the new capillaries in control (C) and treatment groups (D). Ovoid shaped immature fibroblast (blue arrows) possess visible cytoplasm including a pale nuclei and were dominantly observed in control group (E). Mature fibroblasts exhibit dense and fusiform nuclei were mostly seen in treatment group (F). In control group (G) haphazardly oriented collagen fibers were observed. In treatment group (H), thick collagen bundles were observed (H & E, 400×).

Figure 4. Boxplot for infiltration of inflammatory cells. Asterisks indicate statistically significant difference between the groups.

Figure 5. Boxplot for histopathological evaluations of repairs. Asterisks indicate statistically significant difference between the groups.

Mechanical Properties

The failure mode was breakage of the specimens at the anastomotic site. Statistical analysis of the data extracted from the load-displacement curves revealed significant increase in mechanical properties including the maximum load, yield load, and energy absorption in the treatment group compared to the control group ($p < 0.05$), (Table 1). However, the slight increased level of stiffness observed in the treatment group was not significant when compared to that of control group ($p > 0.05$).

4. Discussion

Resection and anastomosis are standard treatments for colorectal neoplasia and severe chronic ulcerative colitis. In spite of recent advances in surgical techniques and perioperative care, the anastomosis leak occurs in up to 30% of patients. Considering the negative consequences of leakage (such as increased morbidity, mortality, prolonged hospitalization, increased medical expenses and resource use) development of techniques and pharmacological agents that help increase the anastomotic
Table 1. Mechanical properties of rat colons seven days after anastomosis (n = 6). Data are presented as mean ± SD.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Maximum load (N)</th>
<th>Yield load (N)</th>
<th>Energy absorption (J)</th>
<th>Stiffness (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.70 ± 0.10</td>
<td>0.28 ± 0.09</td>
<td>7.79 ± 0.95</td>
<td>0.29 ±0.12</td>
</tr>
<tr>
<td>Treatment</td>
<td>1.60 ± 0.08*</td>
<td>0.65 ± 0.13*</td>
<td>17.78 ± 1.03*</td>
<td>0.32 ± 0.21</td>
</tr>
</tbody>
</table>

Asterisks indicate statistically significant difference between the groups ($p < 0.05$).

repair and decrease the rate of leakage has been focused in recent years. With regards to reparative, antimicrobial, anti-inflammatory, and antioxidant properties, silymarin has been successfully used in experimentally induced ulcerative colitis.\textsuperscript{18} The present study revealed beneficial effects of oral administration of silymarin on the healing of rat colonic anastomosis. Anastomotic leak is known as the most common cause of death after colorectal surgery. Although no death was recorded in this study, according to the results of macroscopic evaluations, the rate of leakage and resultant peritonitis was significantly higher in control group compared to the silymarin treated rats. Peritonitis is one of the main consequences of anastomotic leakage.\textsuperscript{19} Thus, any treatment could decrease the risk of leakage could also prevent this life threatening complication. Contamination of the abdominal cavity with intestinal contents due to the anastomotic leakage results in peritonitis, sepsis, even mortality.

Any factor that could lead to tissue hypoxia and ischemia, such as oxygen-free radicals may promote adhesion formation,\textsuperscript{20} which could result in intestinal passage blockage after colorectal anastomosis requiring urgent re-operation. In the present study, oral administration of silymarin resulted in lower rates of adhesion formation in treatment group. These results were comparable with the results of a study by Karimi \textit{et al.} in which intraabdominal administration of silymarin significantly decreased the experimentally induced adhesion formation in rats.\textsuperscript{21} They concluded that the antioxidant activity of silymarin could prevent adhesion formation thorough inhibition of the vascular permeability and exudate formation resulted from toxic influence of free radicals.

Reportedly, silymarin increased fibroblast proliferation and synthesis of collagen bundles,\textsuperscript{7} in a cutaneous wound model in rats. In the present study, elevated collagen deposition and organization of collagen bundles were observed in histopathological evaluations. In this regard, a previous study using silibinin, the main component of silymarin, has been also shown to treat cutaneous wounds in rats by increasing collagen content.\textsuperscript{22} A histopathological study on the patients developing anastomotic leakage revealed reduced deposition of collagen I and III, and a lower quantity of overall collagen after large-bowel surgery.\textsuperscript{23} These data indicate the crucial role of collagen deposition in preventing anastomotic leakage. In other words, the degradation of collagen fibers results in leakage during anastomotic healing. During the inflammatory phase of anastomotic healing, macrophages release collagenase, a high-activity enzyme that results in collagen degradation that causes inferior anastomotic strength.\textsuperscript{24} Preventing collagen degradation during this phase is anticipated to improve the postoperative colonic strength and thus reduce the risk for anastomotic dehiscence and leakage. In this regard, nonsteroidal anti-inflammatory drugs may inhibit postoperative collagen degradation after anastomoses, but they simultaneously may predispose the patient to surgical infections as reported by Mastboom \textit{et al.}\textsuperscript{25} On the other hand, the potential adverse effects of NSAIDs in a patient who recently underwent a major surgery, can be life-threatening and potentially fatal. The reported side effects include large intestinal ulcers, bleeding, and perforation, iron deficiency, anemia, hypoproteinemia, strictures, ulcerations, perforations, diarrhea, and death.\textsuperscript{26}
In the present study, significantly lower infiltration of inflammatory cells was observed in silymarin treated group. It was suggested that due to the lower inflammatory response in treatment group, higher levels of collagen deposition and thus lower rate of anastomotic leakage were observed. It is worthy of note that the collagen content does not reflect the quality of the fibers, cross-linking, and therefore the mechanical stability of the repair. In this respect, the mechanical properties of repairs should be evaluated. The bursting pressure and tensile strength tests are the two most common methods used to evaluate anastomotic strength. The first one represent the resistance of intestinal anastomosis to an elevation in intraluminal pressure, and the latter reflects the anastomotic resistance to longitudinal forces resulted from muscular layer contractions. Reportedly, the measurement of bursting pressure is sensitive to the early phase of anastomotic healing during the first three days after anastomosis. In this study, the difference between two groups in terms of bursting pressure was not significant at day seven after anastomosis. Previously, it has been observed that there is no correlation between the bursting pressure and tensile strength in colonic anastomosis on the 7th postoperative day in rats. Ikeuchi et al. reported that mechanical strength is known as the standard to assess the biological aspects of anastomotic repair. In the present study, the tensile test of anastomoses revealed that silymarin increased the mechanical properties in treatment group. The maximum and yield loads were significantly higher in response to silymarin administration revealing that the anastomoses will tolerate higher distracting forces from peristalsis and therefore will resist dehiscence and consequent leakage. In line with our results, topically administered silymarin improved the tensile strength of experimentally-induced excisional cutaneous wound in rats. The crosslinking and remodeling of collagen naturally occurs after three weeks. Collagen crosslinking occurs to form covalent bonds between collagen fibrils, thus a functional and organized collagen structure is created, which significantly increases the stiffness and maintains the tensile strength of the tissue. In the present study, the stiffness parameter of samples in treatment group were higher when compared to the controls although not significant. To store and release the exerted forces without damage, a high energy-absorbing capacity is required. An insufficient energy-absorption may lead to an increased risk for tissue overload such as dehiscence due to mechanical stress. A tissue containing collagen cross-links is more elastic and durable. Silymarin administration significantly improved the energy absorption of repairs in the treatment group in comparison with the controls. Based on the results of histopathology and mechanical evaluations in the present study, it seem that silymarin could accelerate the healing process of anastomosis in the treatment group. In conclusion, oral administration of silymarin could improve the repair of colon anastomosis in terms of clinical, structural and mechanical properties in rats. Further studies are recommended to determine the hydroxyproline, as a measure for collagen, at the anastomotic site.

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**Conflict of Interests**

The Authors declare there is no conflict of interest.

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