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PROTECTIVE EFFECTS OF QUERCETIN ON THE HEALING PROCESS OF EXPERIMENTAL COLONIC ANASTOMOSIS IN RATS

S. E. Duzcu[1,*], H. Ozturk[2], A. Cetinkaya[3], O. M. Yis[4] and H. Ozturk[5]

[1]Bolu Abant Izzet Baysal University, Medical School, Pathology, Bolu, Turkey
[2]Bezmi Alem Vakıf University, Medical School, Pediatric Surgery, İstanbul, Turkey
[3]Bolu Abant Izzet Baysal University, Medical School, Physiology, Bolu, Turkey
[4]Bolu Abant Izzet Baysal University, Medical School, Biochemistry, Bolu, Turkey
[5]Department of Pediatric Surgery, Istanbul Private Memorial Hizmet Hospital, Istanbul, Türkiye
*Correspondence E mail: serdoganduzcu@hotmail.com

ABSTRACT

The protective effects of quercetin on colonic anastomosis in rats were investigated by mechanical, biochemical and histopathological parameters. Twenty-one male, Sprague–Dawley rats (240–250 g) were used in this study. Group 1, (Sham-control): The abdominal cavity was entered and after the cecum and colon were exposed, they were reinserted into the abdomen without any procedure. Group 2 (Colon anastomosis+untreated): The abdominal cavity was entered and, 2 cm colon was resected from the distal cecum and the colon was anastomosised end-to-end. Group 3 (Colon anastomosis + Quercetin treatment): In addition to the procedure applied in group 2 rats, after colon anastomosis, quercetin was administered at a dose of 50mg/kg by oral gavage for 7 days. The results were evaluated with mechanical, biochemical and histopathological parameters. In the group 2, anastomotic burst pressures on the eighth postoperative day were decreased compared to the group 1. The burst pressure measurements were significantly higher in the group 3 compared to the group 2. MPO and MDA values in the group 2 showed a significant increase when compared to the group 1. However, these values were significantly decreased in group 3 rats compared to group 2 rats, and SOD values were increased. When the histopathological parameters in the group 2 were compared with the groups 1 and 3, significant changes were found on Colonic anastomosis, anastomotic healing, breaking strength, reactive oxygen species, quercetin, rat the negative side. On the other hand, when quercetin treatment group was compared with group 2, a statistically decrease in inflammatory parameters and mucosal and muscular damage and increased angiogenesis were detected. The results of our study showed that quercetin treatment has positive effects on the healing of colon anastomosis and these effects are based on its antioxidant and anti-inflammatory properties.

Keywords: Colon, anastomosis, surgery, wound healing, quercetin, antioxidant, rat

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INTRODUCTION

Especially colorectal cancers, ischemic and ulcerative colitis, Crohn's disease, mechanical bowel obstructions, trauma, recurrent diverticulitis are the diseases in which colorectal surgical operations are frequently applied (Vasiliu *et al*., 2015). Anastomotic leakage, with an incidence of 1-19%, continues to be a fatal complication of colorectal surgery (Vasiliu *et al*., 2015). In addition, anastomotic leakage also causes prolonged hospital stay and high healthcare costs (Snijders *et al*., 2012, Bakket *et al*., 2014, Nachiappan *et al*. 2014). Therefore, there are many studies aimed at preventing and improving anastomotic leakage (Morks *et al*., 2011, Vasiliu *et al*., 2015). Colonic anastomosis leaks may develop due to various causes such as ischemia, inappropriate surgical technique, tension in the anastomotic line, composition and function of the gut microbiome, local infection, and obstruction at the distal anastomosis (Bielecki and Gajda, 1999, Gaines *et al*., 2018).

One of the important reasons that play a role in the pathogenesis of ischemia-reperfusion injury is the formation of increased reactive oxygen species (ROS) (McCue and Phillips, 1991). ROS can react with cellular membrane components such as phospholipids leading to lipid peroxidation (Bonventre, 1993). On the other hand, glutathione peroxidase (GPX), catalase (CAT) and superoxide dismutase (SOD) enzymes form some cellular defense systems against ROS damage (Greene and Paller, 1992, Bhattacharya, 2015). An increase in ROS production causes an overproduction of malondialdehyde (MDA), one of the end products of polyunsaturated fatty acids peroxidation in cells. Therefore, MDA is known as

a marker of oxidative stress and antioxidant status (McCue and Phillips, 1991, Greene and Paller, 1992, Bonventre, 1993, Bhattacharya, 2015). In addition, one of the important markers of oxidative stress and inflammation is myeloperoxidase (MPO), which is characterized by strong pro-oxidative and pro-inflammatory properties, mainly released by activated neutrophils (McCue and Phillips, 1991, Greene and Paller, 1992, Bonventre, 1993, Bhattacharya, 2015).

Quercetin (3,3',4',5,7-pentahydroxyflavone), a flavonol, is widely found in a variety of fruits and vegetables (Lee at al., 2003). It is characterized by two benzene rings linked through a heterocyclic pyrone ring in chemical structure (Suganthy *et al*., 2016). Quercetin has been suggested to have protective effects in various pathological conditions such as cardiovascular diseases, metabolic and neurodegenerative disorders, diabetes, cancer and obesity due to its antioxidant and anti-inflammatory properties (Dok-Go *et al*., 2003, Cho *et al*., 2006, Comalada *et al*., 2005). However, to our knowledge, there is no published report on the protective effect of quercetin on the healing process of colonic anastomosis. Therefore, in this study, we aimed, for the first time, to investigate the protective effects of quercetin on colon anastomosis by mechanical, biochemical and histopatological parameters in an experimental model.

MATERIALS AND METHODS

Animals: Twenty-one male, Sprague–Dawley rats (240–250 g) were used in this study, which were obtained from the Bolu Abant Izzet Baysal University (BAIBU) (Bolu, Turkey) Animal Care and Research Laboratories. The Institutional Animal Care and Use Committee of BAIBU approved all procedures to be performed on the experimental animals (Number/ID of the approval(s): 2019/36). Routine animal care guidelines and the Guide to the Care and Use of Laboratory Animals (1996) were essential in the practice of this study's procedures. Free access to water and food for the rats along with a 12-h dark/light cycle in a temperature-controlled room were also provided. All of the rats were first placed on a homeothermic table to maintain a 37 °C body temperature. Anesthesia was administered with an intraperitoneal injection of xylazine (10 mg/kg) and ketamine hydrochloride (100 mg/kg of Ketalar). Following a right femoral venous catheterization, fluid replacement was performed during the process with 3 mL kg 1 h 1 of Lactated Ringer solution by using an infusion pump.

Surgical procedure and study design: Briefly, the rats were anesthetized and then fixed in a supine position. After disinfection of the abdomen area with iodine cotton ball, to acquire access to the abdominal cavity, a 5-cm craniocaudal midline incision of the skin and abdominal musculature was made in all experiments. The cecum was then identified and moved outside of the peritoneal cavity and onto sterile gauzes that were hydrated with sterile saline solution to prevent dehydration. For proximal anastomosis, the colon was transected two centimetres distal from the cecum and an end-to-end anastomosis was created using 6/0 vicryl sutures (Vicryl 6/0, Ethicon, Inc). After performing the anastomosis, the intestines were repositioned and the abdomen was closed in two layers, a running suture for the muscle layer (Vicryl 4/0, Ethicon, Inc) and interrupted sutures for the skin (Monocryl 4-0, Ethicon, Inc).

Group 1, **(Sham-control) (n=7):** The abdominal cavity was entered with a midline incision, and after the cecum and colon were exposed, they were reinserted into the abdomen without any procedure. The abdominal wall was closed with 4/0 vicryl.

Group 2 (Colon anastomosis+untreated) (n=7): The abdominal cavity was entered as described in the surgical procedure above. After the cecum was found, 2 cm colon was resected from the distal cecum and the colon was anastomosised end-to-end with 6/0 vicryl. After the anastomosis, the intestines were placed in the abdomen. The abdominal wall was closed with 4/0 vicryl.

Group 3 (Colon anastomosis + Quercetin treatment) (n=7): In addition to the procedure applied in group 2 rats, after colon anastomosis, quercetin (Sigma-Aldrich) was administered at a dose of 50mg/kg of quercetin dissolved in 1 ml of normal saline by gavage for 7 days. Quercetin dissolving and doses were selected according to previous studies (Naghizadeh *et al*., 2021, Dong *et al*., 2018, Uylaş *et al*. 2018, Tóth *et al*, 2017).

Tissue preparation: In anesthetized rats on the eighth post-anastomosis day, the anastomotic site was dissected with a 2-cm margin at each site of the anastomosis. After the *ex vivo* measurement of bursting pressure (Bosmans *et al*., 2017), tissue samples were divided in two equal pieces: they were either submersed in 4% paraformaldehyde or measurements of the tissue antioxidant enzyme activities and lipid peroxidation or stored **at −80 °C until needed (3 months).**

Bursting pressure: Burst pressure was measured by a method previously described (Bosmans *et al*., 2017). First, a 5 cm intestinal segment covering the anastomosis line was resected and the distal part of the anastomosis was clamped. A catheter was placed at the proximal end and circumferentially ligated with a single polyglactin 4/0 suture (Vicryl, Ethicon). The intestine was dipped in phosphate buffered saline. Air was infused with a manometer (Medex Inc.) and pressure was manually increased by inflating the colon. Burst pressure was defined as the intraluminal pressure (mBar) at which air leakage was first observed from the anastomosis.

Histological assessment: The tissue samples were placed in 4% formaldehyde solution for histopathological examination and stained with haematoxylin and eosin. The tissue specimens, taken into paraffin blocks, were sectioned at 5 μm and stained with hematoxylene and eosine (H&E). The sections were blindly examined under light microscope (Olympus BH-2, Olympus Corporation, Tokyo, Japan) by two investigators, using a 0-4 Ehrlich and Hunt numerical scale as modified by Phillips *et al* (Phillips *et al*., 1992), The evaluated parameters were inflammatory cell infiltration, fibroblast activity, fibrosis, neoangiogenesis, necrosis, mucosal and muscular damage (Shomaf, 2003)). Each studied parameter was evaluated individually using a numerical scale from 0 to 4 as follows: 0 (-) = no evidence; 1 (+) = occasional evidence; 2 (++) = light scattering; 3 (+++) = abundant evidence; and 4 (++++) = confluent fibres or cells.

Measurements of myeloperoxidase, malondialdehyde and superoxide dismutase activities: The colon tissue samples were washed with phosphate buffered saline and were stored at -80°C until the day of biochemical analysis. When analysis began, the homogenate was centrifuged at 4°C and 1,600 g for 10 m, and the supernatant was removed. Myeloperoxidase (MPO) and superoxide dismutase (SOD) were measured using commercially available, enzyme-linked, immunosorbent assay kits, according to the manufacturer's instructions (ELISA Kit for MPO: Wuhan USCN Business CO., Ltd., Hubei, PRC, and ELISA Kit for SOD: Cayman Chemical Co., Ann Arbor, MI.USA). Malondialdehyde (MDA) was measured using commercially available, colorimetric assay kits, according to the manufacturer's instructions (Thiobarbituric Acid Reactive Substances (TBARS, TCA Method) (Cayman Chemical Co., Ann Arbor, MI.USA). Bicinchoninic acid (BCA) protein assay was used for the quantitation of tissue total protein (Thermo Fisher Scientific Inc., Rockford, IL, USA).

Statistical analyses: All values were expressed as mean ± standard deviation. The significance of the data obtained from oxidative stress-associated parameters was evaluated using analysis of variance (ANOVA). Differences between means were analyzed via post-analysis test after ANOVA (Tukey's b test). The Mann-Whitney U test and Kruskal-Wallis test were also used to compare statistical analysis of the histologic data. P values of < .05 were considered significant.

RESULTS

One rat each in the control and treatment groups died on the fourth day and were excluded from the study. New rats were added to replace the dead rats. In the remaining subjects of the total study group, there was no evidence of anastomotic leak or wound infection and no further deaths.

Bodyweight change: The changes in body weight in grams in the groups 1, 2 and 3 were 9, 15, and 12, respectively. In all the experimental groups, body weight decreased from the day of the experiment to the day of sacrifice, but there were no other differences between the group 2 and 3.

Anastomosis bursting pressure: Burst pressure values measured on the eighth postoperative day were significantly different between the three groups *($p < 0.001$)*. In the group 2, anastomotic burst pressures were decreased compared to the sham-control group *($p < 0.001$)*. On the other hand, burst pressure measurements on the eighth postoperative day were significantly higher in the group 3 compared to the group 2 *($p < 0.001$)* (Figure 1).

Measurements of MPO, MDA and SOD: The mean MPO, MDA and SOD values of the groups are given in figure 2. The MPO and MDA values were significantly different between the groups *(for all $p<0.001$)*. MPO and MDA values in the group 2 show a significant increase when compared to the group 1 *($p < 0.001$, $p < 0.001$ and $p < 0.001$, respectively)*. However, these values were significantly decreased in the group 3 rats when compared to the group 2 rats *($p < 0.045$, $p < 0.001$ and $p < 0.001$, respectively)*. Tissue SOD value in the group 2 show a significant decrease when compared to sham-control group *($p < 0.001$)*. However, these values were significantly increased in the group 3 rats when compared to the group 2 rats ($p < 0.007$)(Figure 2).

Histopathological *assessment:* Histopathological parameters of the all groups are shown in Table 1. Statistical analysis revealed significant changes on the negative side in group 2 compared with groups 1 and 3 in all histopathological parameters: neutrophil ($p < 0.001$), lymphocyte ($p < 0.001$), macrophage ($p < 0.001$), fibroblast ($p < 0.001$), fibrosis ($p < 0.001$), neoangiogenesis ($p < 0.001$), necrosis ($p < 0.001$), mucosal damage ($p < 0.001$) and muscular damage ($p < 0.001$). On the other hand, when quercetin treatment group was compared with group 2, a statistically decrease in inflammatory parameters (neutrophil, lymphocyte, macrophage, and fibroblast) fibrosis and mucosal and muscular damage and increased angiogenesis were detected *(for all $p<0.05$)* (Figure 3A-E).

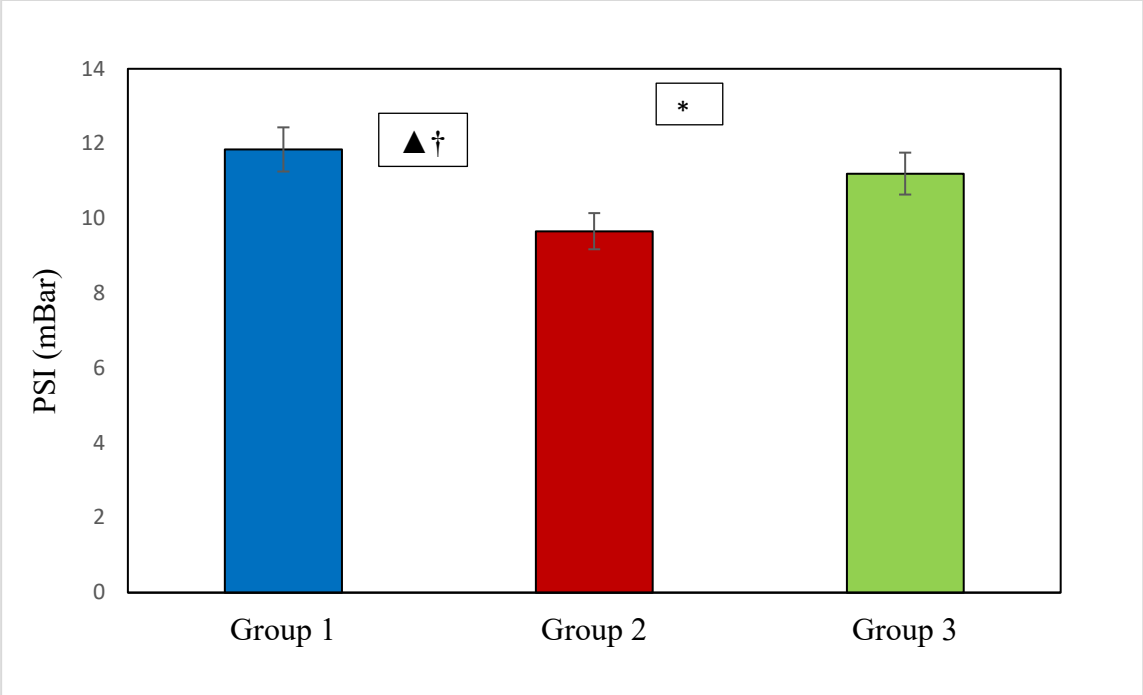

Figure 1. The bursting pressure values of the groups. ▲† In group 2, anastomotic burst pressures were decreased compared to the group 1 and 3. On the other hand, burst pressure measurements on the eighth postoperative day were significantly higher in group 3 compared to group 2. Group 1: Sham-control. Group 2: Colon anastomosis+untreated. Group 3: Colon anastomosis + Quercetin treatment.

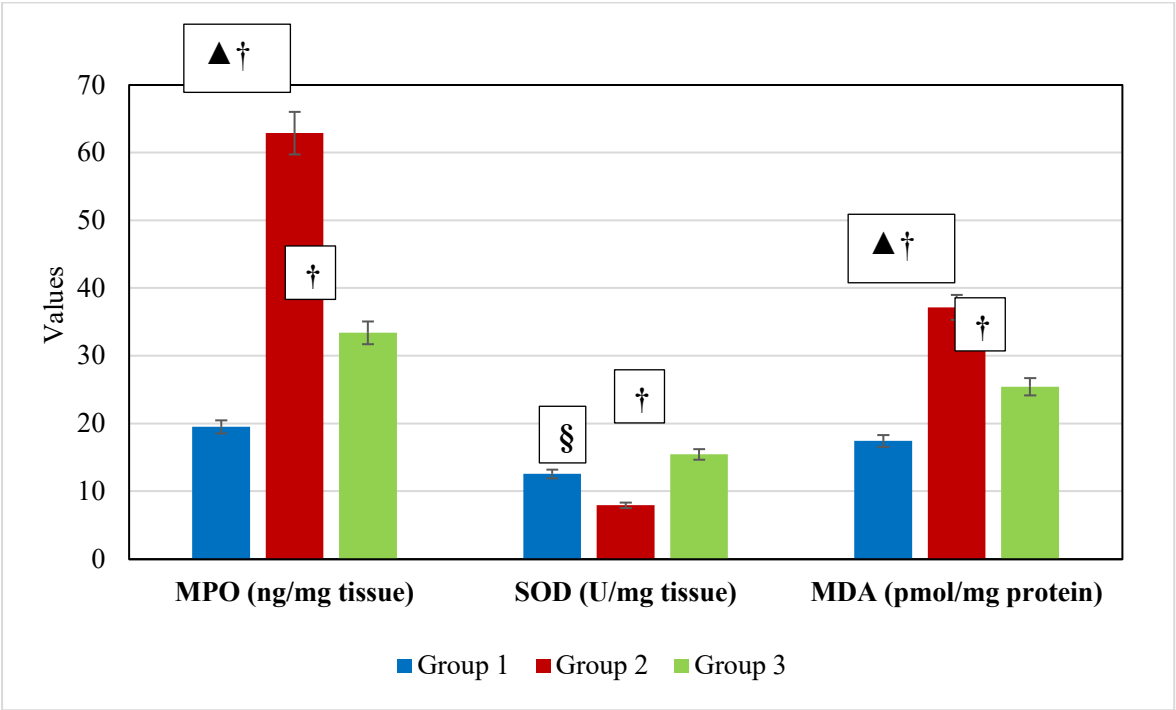

Figure 2. The mean MPO, MDA and SOD values of the groups. ▲† MPO and MDA values in the group 2 show a significant increase when compared to the group 1 and 3. § Tissue SOD value in the group 2 shows a significant decrease when compared to the group 1 and 3. † However, these values were significantly improved in group 3 rats compared to group 2 rats. Group 1: Sham-control. Group 2: Colon anastomosis+untreated. Group 3: Colon anastomosis + Quercetin treatment.

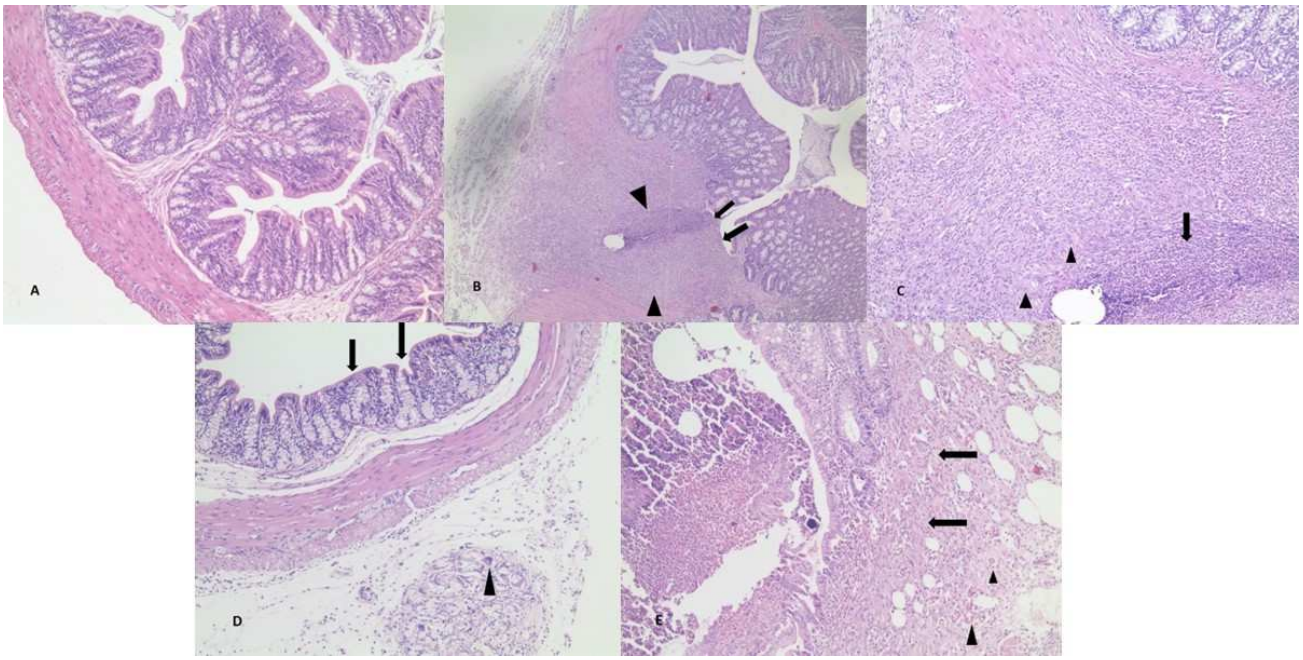

Figure 3. Histopathological representative light microphotographs of the colon belonging to the groups were presented. A: Group 1 (sham-control), regular appearance of tunica mucosa, tunica submucosa, muscularis propria in the colonic mucosa (H&E x100). B: Group 2 (colon anastomosis+untreated), epithelial loss in the colon mucosa (arrow) and intense inflammation in the colon wall (arrowhead) (H&E x40). C: Group 2 (colon anastomosis+untreated), neutrophils (arrow), macrophages (arrowhead) in the colon wall (H&E x100). D: Group 3 (colon anastomosis + quercetin treatment), reepithelialization is completed in the colonic mucosa (arrow), macrophage-like multinuclear giant cells (arrowhead) are present in the adipose tissue (H&E x100). E: Group 3 (colon anastomosis + quercetin treatment), underlying reepithelialization with fibroblast proliferation (arrow) and diffuse neovascularization (arrowhead) consisting of congested vessels (H&E x100).

Table 1. Histopathological parameters of the groups.

Groups	Neutrophil	Lymphocyte	Macrophage	Fibroblast	Fibrosis	Neoangiogenesis	Necrosis	Mucosal damage	Muscular damage
1	0,14±0,37	0,42±0,53	0,14±0,37	0,14±0,37	0,14±0,37	0,14±0,37	0,14±0,37	0,14±0,37	1,0±,14
2	2,85±0,89 ▲	3,71±0,48 ▲	3,14±0,89 ▲	3,28±0,95 ▲	1,85±0,89⁋	1,85±0,89⁋	1,71±0,75 ▲	2,85±0,89 ▲	1,85±1,21⁋
3	1,14±0,3†	1,42±0,53†	1,71±0,48†	1,0±0,81†	1,28±1,11	3,14±0,69§	0,57±0,97	0,42±0,53†	1,0±0,81

▲, ⁋: When the histopathological parameters in the group 2 were compared with the groups 1 and 3, significant changes were found on the negative side. **†, §:** On the other hand, when quercetin treatment group was compared with the group 2, a statistically decrease in inflammatory parameters fibrosis and mucosal and muscular damage and increased angiogenesis were detected.

DISCUSSION

Despite the new tools used for intestinal anastomosis, technological developments in surgery, advances in the treatment of surgical infections, and improvements in pre- and postoperative care conditions, anastomotic leakage that develops after resection and anastomosis is still an important and serious complication of colorectal surgery with high mortality and morbidity rates (Bakker *et al*., 2014, Vasiliu *et al*., 2015, Barlas *et al*., 2018, Sciuto *et al*., 2018). It is known that some factors such as male gender, obesity, preoperative steroid and nonsteroidal anti-inflammatory drug use and radiochemotherapy treatment, long operation time, surgical experience and preoperative blood transfusion are risk factors for anastomotic leakage (Vasiliu *et al*., 2015). We investigated the effect of quercetin on the healing of colon anastomoses in an animal model. Our main findings revealed that quercetin treatment had

positive effects on histopathological values and burst pressure compared to untreated rats.

Quercetin is a plant polyphenolic flavonoid, found in many fruits and vegetables such as apples, onions, berries, broccoli, and black and green tea (Pandey and Rizvi, 2009, Singh P *et al*., 2021). It was previously shown that quercetin has a protective effect on ischemia and reperfusion (I/R) injury in various organs (Chen *et al*., 2014, Ali *et al*., 2015, Miltonprabu *et al*., 2017), including liver. Quercetin has been proven to possess anti-inflammatory, (Orsolic *et al*., 2004, Hosseini A *et al*., 2021), antioxidant, (Alrawaiq and Abdullah, 2014, Singh P *et al*., 2021), and oxygen radicalescavenging (Hanasaki *et al*., 1994) activities. Quercetin is an antioxidant, which has been reported to have protective effect on several organs (Renugadevi and Prabu, 2010, Gonza´lez-Esquivel *et al*., 2015, Hosseini A *et al*., 2021). Quercetin can protect cells against apoptosis and necrosis by inhibiting oxidative stress (Liu *et al*., 2010). Quercetin has been shown to have protective effects against myocardial injury through inhibition of the high mobility group protein 1 (HMG-1) pathway in a myocardial ischemia-reperfusion injury (I/R) model (Dong *et al*., 2018)). Jin *et al* suggested that quercetin may alleviate blood-brain barrier dysfunction after global cerebral I/R in rats and that the mechanism may be related to the activation of canonical Wnt/β-catenin signaling pathway (Jin *et al*., 2019). In a hepatic ischemia/reperfusion study in rats, it was suggested that 50 or 100 mg/kg of quercetin significantly decreased serum and tissue MDA levels, and that quercetin was effective in preventing hepatic injury with its antioxidant properties (Uylas *et al*., 2018). In addition, Tóth *et al* concluded that quercetin application attenuated mucosal damage from IR injury by inhibiting neutrophil infiltration which was demonstrated by a lower number of myeloperoxidase positive cells in the lamina propria (Tóth *et al*., 2017). In our experimental study, oxidative stress parameters were evaluated to determine the possible mechanism responsible for the beneficial effects of quercetin on wound healing. MDA and MPO levels were lower and SOD levels were higher in the quercetin administered group compared to the control group. These results showed that quercetin has important antioxidant properties, and this may be one of the possible mechanisms responsible for the beneficial effects.

Quercetin caused the fastest wound closure and markedly improved the oxidative stress. Quercetin treatment increased the expressions of IL-10, VEGF, TGF-β_1, CD31, α-SMA, PCNA, and GAP-43, and decreased the expressions of TNF-α. Early infiltration of inflammatory cells and formation of good quality granulation tissue dominated by fibroblast proliferation, angiogenesis, and collagen deposition in quercetin treated groups was also evident (Kant V *et al*., 2020). Beken *et al* (2020) suggested that pretreatment of the cells with quercetin significantly reduced the expression of AD-induced IL-1β, IL-6, IL-8, and thymic stromal lymphopoietin, while it strongly enhanced the expression of superoxide dismutase-1 (SOD1), SOD2, catalase, glutathione peroxidase, and IL-10. They demonstrated that quercetin promoted wound healing by inducing epithelial-mesenchymal transition, which was supported by the upregulation of Twist and Snail mRNA expression. Burst pressure indicates the mechanical strength of the anastomosis and is a useful parameter to measure the healing process in the first week after anastomosis formation (Kiyama *et al*., 2001, Månsson *et al*., 2002). In our study, burst pressures were found to be significantly higher in the quercetin treatment group on the eighth postoperative day compared to the untreated group. In addition, when compared with the histopathological findings of the untreated group, wound healing was found to be better with decrease in inflammatory cell infiltration, fibroblast activity, fibrosis, necrosis, mucosal and muscular damage, and increase in neoangiogenesis or vascularity in the quercetin treatment group. As a contribution to the literature (Polerà N *et al*., 2019) these findings show that quercetin has important biological activities related to the improvement of the wound healing process.

In conclusion, the results of our study show that quercetin treatment has positive effects on colon anastomosis healing. Thus, in the experimental colorectal anastomosis model we present here, oral treatment of quercetin by gavage improved burst pressures, biochemical and histopathological parameters. It can be argued that the positive effects of quercetin on anastomotic healing are based on its antioxidant and anti-inflammatory properties.

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