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Protective Effect of Nigella Sativa in an Animal Model of Colon Anastomosis With Ischemia/ Reperfusion Injury

İlhan Bali[1], Fatin Rüştü Polat[1], Mehmet Aziret[2], Selim Sözen[1], Cem Oruç[3], Ufuk Coşkunkan[1], Seyfi Emir[1], Bülent Bilir[4], Ahmet Koç[5]

[1]*Namık Kemal University Faculty of Medicine, Department of General Surgery, Tekirdağ, Turkey*

[2]*Sakarya University Faculty of Medicine, Department of General Surgery, Sakarya, Turkey*

[3]*Mustafa Kemal University Faculty of Medicine, Department of General Surgery, Hatay, Turkey*

[4]*Namık Kemal University Faculty of Medicine, Department of Internal Medicine, Tekirdağ, Turkey*

[5]*Namık Kemal University Faculty of Medicine, Department of Histology and Embryology, Tekirdağ, Turkey*

Objective: Anastomotic leaks are one of the chief complications after gastrointestinal surgery. The aim of this study was to evaluate whether Nigella sativa administration protects against ischemia/reperfusion injury on healing of colonic anastomosis in rats.

Method: Thirty male Wistar albino rats, weighing between 200 and 240 g, were used in the study. They were randomly divided into three groups (n = 10 for each group): Anastomosis (group 1), anastomosis and ischemia/reperfusion injury (group 2), and treatment group of anastomosis, ischemia/reperfusion injury, and Nigella sativa (group 3). After 7 days, serum, plasma, and colonic tissue were obtained and then all rats were sacrificed. Tissue and serum level of total oxidant status, total antioxidant status, total thiol levels, hydroxyproline, interleukin-6, and TNF-alpha were determined and specimens were histopathologically evaluated.

Results: In the Nigella sativa treated rats, serum hydroxyproline levels were significantly higher, while tissue levels were significantly lower than those seen in group 1 and group 2 ($P = 0.007$, $P = 0.01$, respectively). In the Nigella sativa group, the serum levels of TNF-α were significantly lower than those seen in group 1 and 2 ($P = 0.001$). Also, in group 3, the

Corresponding author: İlhan Bali, Namık Kemal University Faculty of Medicine Department of General Surgery, 59100 Tekirdağ, Turkey.
Tel.: +90 5066600999; Fax: +90 2822509928; E-mail: ilhanbali@yahoo.com

tissue IL-6 level was significantly higher than that seen in group 1 and group 2 ($P = 0.009$). The histopathologic analysis showed less edema and inflammatory cell infiltration in the Nigella sativa treated group, as well as a statistically significant difference according to the Chiu classification ($P < 0.05$).

Conclusion: The results of this study indicate that Nigella sativa has a protective and therapeutic effect against ischemia/reperfusion injury on the healing of colonic anastomosis in rats.

Key words: Anastomosis – Anastomotic leaks – *Nigella sativa* – Ischemia/reperfusion injury

Anastomotic leaks are one of the chief complications, in terms of mortality and morbidity, following gastrointestinal surgery.[1] Many factors, including infections, blood supply, suture materials and surgical techniques, age, sepsis and use of steroids, affect, either positively or negatively, the postoperative wound healing process.[1,2] Ischemia and sepsis are the main causes of anastomotic leaks.[3]

Ischemia-reperfusion (I/R) injury triggers a sequence of cellular events due to inadequate oxygen (O2) delivery to the tissues, a problem that impacts all organs and systems.[4] Oxidative stress (OS) results from the failure of a sufficient amount of O2 reaching the tissue and can lead to metabolic-related conditions,[5] as well as other serious issues, such as multiple organ failure. Reperfusion, a critically important procedure for restoring the supply of blood to tissue that has been damaged as a result of ischemia, can cause reperfusion injury, which is the onset of inflammation or oxidative stress due to the return of the supply of blood to the tissues.[6] OS mediators, such as activated polymorphonuclear leukocytes (PMNs), cytokines, platelet-activating factor (PAF), and reactive oxygen species (ROS), which cause lipid peroxidation and protein oxidation, have been reported to play an important role in I/R injury.[1,2,7] The ROS that form after circulation is provided in ischemic tissue are responsible for I/R injury. Several successful experimental studies have been performed showing that I/R injury can be reduced or prevented with various pharmacologic agents.[1,2,7–10] I/R can frequently occur in low flow mesenteric ischemia secondary to various injuries and the performance of critical surgical procedures, such as trauma, vascular surgery, intestinal and colon surgery, and organ transplantation, and results in high morbidity and mortality.[1–3,8,12]

Nigella sativa (N. sativa; NS), a herbaceous plant species in the family of Ranunculaceae, is commonly found in Southern Europe and Western Asia, especially in the Mediterranean region.[13] Used in a wide variety of fields, NS seeds and their byproducts have numerous beneficial effects, including antitumor activity,[14] antibacterial activity,[15] anti-inflammatory activity,[16] antioxidant activity,[17] immune system enhancer activity,[18] antidiabetic and antihypertensive activity,[19] and protective action against renal and liver failure, cerebral edemas,[20–22] and I/R, as well as many others.

This study investigated the parameters of oxidant–antioxidant and inflammatory cytokines, such as total oxidant status (TOS), total antioxidant status (TAS), total thiol levels (TTL), hydroxyproline, Interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α), in an I/R injury on the healing of colonic anastomosis in a rat model. Histopathologic changes in the colon were also examined.

Materials And Methods

Ethics

This experimental study was conducted at the Mustafa Kemal University School of Medicine Experimental and Animal Research Center (MKÜ-DAM) between January and May 2015. The study was approved by the Ethics Committee of Faculty of Medicine (dated 02/14/2014 with Ethics Committee approval n°.40595970/27).

Animals

Thirty 28- to 32-week-old male Wistar albino rats, ranging in weight from 200 to 240 g, were evaluated. The rats were maintained under a 12-hour/12-hour light/dark cycle and they were housed three to a stainless steel cage maximum and kept at a room temperature of approximately 22 ± 2°C. Food was withheld 8 hours prior to surgery, but the rats had free access to water. Throughout the study, the rats were provided with rested tap water and standard

pellet feed. The rats were not removed from the experimental animal center.

Experimental design and administration of drugs

The NS seeds were purchased from a local herbalist in Hatay, Turkey, and botanically confirmed by a plant taxonomy specialist in the biology department. The seeds were first broken down into a powder using a mixer and then placed in a steam distillation apparatus to produce volatile oil. This process generated a 0.2% yield, which was collected and administered intraperitoneally to the treatment group at 0.2 mL/kg/day for 5 days. The solution was stored in a domestic refrigerator at +4°C throughout the study. At 7 days post-op, the study rats were sacrificed by cervical dislocation and U-shaped incisions were made to open their abdominal cavities. Biochemical parameters, such as IL-6, TNF-alpha, TAS, TOS, and TTL were determined, and the colonic structures with anastomosis segment were removed for macroscopic and microscopic evaluation. Furthermore, the level of hydroxyproline was measured and the necessary scoring was performed.

The animals were randomized into 3 experimental groups (each with n = 10). Group 1 (Anastomosis) rats were subjected to only left colonic anastomosis and intraperitoneally received 10 mg/kg of 0.9 NaCl. Group 2 (I/R + Anastomosis) rats were subjected to I/R and left colonic anastomosis and intraperitoneally received 10 mg/kg of 0.9 NaCl. Group 3 (I/R + Anastomosis + NS) rats were subjected to surgical induction of I/R and left colonic anastomosis and intraperitoneally received 0.2 mL/kg/day of NS.

The NS solution was given to the treatment group as a single daily dose at the same time every day for 5 days. Sterile insulin injectors were used and the right lower abdomen was selected for intraperitoneal injections.

Surgical procedure

The rats were intraperitoneally anaesthetized with 50 mg/kg of ketamine and 50 mg/kg of xylazine during surgery. Following the administration of anesthesia, the rats were shaved and their skin was cleaned with 10% povidone-iodine. A midline laparotomy (20 mm) was performed and after locating the superior mesenteric artery (SMA), clips were placed on SMA for 30 minutes. The descending colon was detected after inducing ischemia. A single layer and end-to-end anastomosis was performed with individual 6/0 polypropylene sutures (Prolene, Ethicon, Edinburgh, UK) 2 cm above proximal to the peritoneal reflection colon, and then the tissue was reperfused after opening the clips. Following the procedure, 5 cc of NaCl was administered, and the left colon was anatomically placed into the abdomen and then closed with individual 3/0 silk sutures (Silk, Doğsan, Istanbul, Turkey). This procedure was performed for groups 2 and 3. The rats were returned to their cages upon regaining consciousness and were allowed to drink water and feed. At 7 days post-op, the rats were sacrificed by cervical dislocation under anesthesia. Colonic anastomosis was evaluated before colonic tissues were resected and blood was taken for examination. No fistulas or perforations were found during anastomosis.

Assay of TAS, TOS

Venous blood was drawn into blood tubes and serum was separated from the cells by centrifugation at 1500 g for 10 minutes. The serum samples were then stored at -80 °C until the analyses.

Assay of total antioxidant status (TAS)

TAS was measured in the serum using the Erel method.[24] In this measurement method, the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid radical (ABTS radical) was used. The ABTS molecule oxidizes the $ABTS^{+}$ molecule in the presence of hydrogen peroxide. The ABTS radical, depending on the amount of antioxidants and antioxidant capacity, loses the blue and green colors. This color change is evaluated by measuring the absorption values at 660 nm. There is an inverse relationship between the amount of antioxidant in the sample and the color change. TAS levels were measured using commercially available kits (Rel Assay, Gaziantep, Turkey) that enabled assays to be conducted with excellent precision values, lower than 3%. The reaction rate can be adjusted by using the Trolox equivalent antioxidant capacity (TEAC), with the results expressed as Trolox equivalent / L.

Assay of total oxidant status (TOS)

The TOS measurement was performed with the fully automated colorimetric method developed by Erel.[25] With this new method, oxidants present in the sample oxidized the ferrous ion-o-dianisidine

complex to ferric ion. TOS levels were measured using commercial available kits (Rel Assay, Turkey) and the results were expressed in terms of micromolar hydrogen peroxide equivalent per liter (μmol H2O2Eqv/L).

Assay of total thiol levels (TTL)

A spectrophotometric assay based on 2,2-dithiobis nitrobenzoic acid (DTNB) was used for total thiol assay.[26] An aliquot of serum was mixed with Tris-EDTA buffer, then DTNB was added. After 15-minute incubation at room temperature, the absorbance was measured at 405 nm. A reagent blank without sample and a sample blank with methanol instead of DTNB were prepared in a similar manner. GSH (50-100 μmol/L) solution was used as calibrator. TTL levels were expressed in terms of μmol/L.

Homogenate preparation and tissue protein determination

After sacrificing the animals, the colon tissue was quickly removed and cleaned, using an ice-cold solution of isotonic NaCl for the removal of blood-spots, and then dried with blotting paper. Weighed samples of colon tissue was homogenized in ice-cold PBS (0,01 M, pH = 7.4). The homogenate was centrifuged at 5,000g for 5 minutes at 4 °C. The resulting supernatants that accumulated were used to assay IL-6, TNF-α, and hydroxyproline levels. Tissue protein contents were determined using the Biuret method for tissue measurement of IL-6, TNF-α, and hydroxyproline.[27]

Assay of hydroxyproline

Hydroxyproline concentration in serum and the supernatant of colon tissue was determined. Hydroxyproline levels were measured using rat ELISA kits (Eastbiopharm, Hangzhou, China, Cat. Nr. CK-E300191) in accordance with the provided protocol. Absorbance was observed to be at 450 nm on microplate reader. Finally, colon tissue supernatant and serum hydroxyproline concentrations were expressed as ng/mg protein and ng/mL, respectively.

Assay of IL-6

Biochemical measurements were performed at Mustafa Kemal University School of Medicine Biochemistry Laboratory. Levels of IL-6 protein were measured in colon tissue supernatants and serum. The rat IL-6 ELISA kit was used to determine the levels of IL-6 protein (Elabscience, Hamburg, Germany, Cat. Nr. E-EL-R0015). The colon tissue supernatants and serum were transferred to a microtiter plate and processed according to the manufacturer's instructions. Absorbance was observed to be at 450 nm on microplate reader. Finally, colon tissue supernatant and serum IL-6 concentrations were expressed as pg/mg protein and pg/mL, respectively.

Assay of TNF-α

The Elabscience rat TNF-α ELISA kit (Cat. Nr. E-EL-R0019) was used to measure colon tissue supernatants and serum TNF-α concentrations. The colon tissue supernatants and serum were transferred to a microtiter plate. The concentration of TNF-α was determined according to the manufacturer's instructions. Absorbance was observed to be at 450 nm on the microplate reader. Finally, colon tissue supernatant and serum TNF-α concentrations were expressed as pg/mg protein and pg/mL, respectively.

Histopathologic analysis

A histopathologic examination was performed at the Mustafa Kemal University School of Medicine, Department of Pathology. Tissue samples from the left colon, fixed in a 10% formaldehyde solution, were divided into 5 μm pieces and separated for histopathologic examination. Tissues were examined by a pathologist who was blind to which group the samples belonged. Histopathologic analysis and measurement of the severity of ischemic damage were performed using the following ischemic damage classification developed by Chiu[28]: 0: Normal mucosal villus; 1: Subepithelial Gruenhagen's area congestion; 2: Epithelial layer moderately separated from lamina propria, 3: Massive epithelial separation of villus slices; peeling in a few places, 4: Villus separated from lamina propria and stretched, dilated capillary; 5: Digested and diffuse lamina propria; bleeding and ulceration. A semiquantitative analysis was conducted on the histological parameters used in the assessment of wound healing, which was developed by Verhofstad.[29]

Statistical analysis

The data obtained from this study were analyzed with the help of PASW Statistics 18 for Windows (Statistical Package for Social Sciences Inc., Chicago,

Table 1 Comparison of serum cytokines, oxidative and antioxidative parameters in the group (n=10, mean ± SD)

	Group 1	Group 2	Group 3	*P*
Hydroxyproline (ng/lt)	457 ± 22	505 ± 95	562 ± 100	**0.007**
IL-6 (pg/mL)	111 ± 106	168 ± 110	136 ± 60	0.18
TNF-α (pg/mL)	77 ± 23	136 ± 81	26 ± 8	**0.001**
TAS (mmol/L)	1.32 ± 0.07	1.36 ± 0.2	1.29 ± 0.1	>0.05
TOS (μmol/L)	6.8 ± 0.4	6.2 ± 1.4	7.5 ± 2.8	>0.05
TTL (μmol/L)	221 ± 32	199 ± 35	206 ± 57	>0.05

IL-6, Interleukin-6;, TAS, total antioxidant status; TNF-α, tumor necrosis factor alpha;
TOS, total oxidant status; TTL, total thiol level.

Illinois). Normal distribution of groups was calculated by using the Shapiro-Wilk test, and the Bonferroni-corrected Kruskal-Wallis H test was employed for comparisons involving 3 or more groups. Results were expressed as mean ± SD (min-max). The correlation between the variables was examined using Fisher's exact test. One-way analysis of variance test was used for comparisons between groups with normal and homogeneous distribution The significance level was set at 0.05 and if $P < 0.05$, there was significant difference, whereas if $P > 0.05$, there was no significant difference.

Results

There was no mortality during the study.

Measurements of TAS, TOS, TTL, IL-6, TNF-α and hydroxyproline

The mean serum hydroxyproline values were 457 ± 22, 505 ± 95, and 562 ± 100 ng/lt in groups 1, 2, and 3, respectively, and in group 1, the level of hydroxyproline was higher than the level found in groups 2 and 3. A significant difference was found in the mean hydroxyproline values ($P = 0.01$) of groups 2 and 3. Mean tissue levels of IL-6 were 111 ± 106, 168 ± 110, and 136 ± 60 pg/mL in groups 1, 2, and 3, respectively, and when groups were compared to each other, no significant differences in mean serum IL-6 values were observed ($P = 0.18$). The mean serums TNF-α values were 77 ± 2, 136 ± 81 and 26 ± 8 pg/mL in groups 1, 2, and 3, respectively. A significant difference was detected in the mean serum TNF-α values ($P = 0.001$) of groups 2 and 3. However, TAS, TOS and thiol activities were similar to each other but no statistically significant difference was found between groups 2 and 3 ($P > 0.05$) (Table 1).

The tissue hydroxyproline value was significantly higher in group 1 in comparison with the values seen in groups 2 and 3 ($P = 0.01$ in all groups). Additionally, the mean tissue IL-6 levels were 39 ± 3, 45 ± 5, and 43 ± 2 pg/mL in groups 1, 2, and 3, respectively, and when groups were compared to each other, significant differences in mean serum IL-6 values were observed ($P = 0.009$). In group 1, the level of TNF-α was the highest among all groups, but there was no statistically significant difference among groups (Table 2).

Histopathological evaluation

This study employed the ischemic damage classification system developed by Chiu[25] and the semi-quantitative analysis of histologic parameters developed by Verhofstad[26] to perform the histopathologic evaluation of the resected tissues. The histopathologic scores were higher in group 2 than in groups 1 and 3. According to the Chiu classification, group 3 was the best-preserved among all groups. A significant difference was found between the Chiu scores of group 1 and 2 ($P < 0.05$). Statistically significant differences ($P < 0.05$) were also found when comparing the Chiu scores of

Table 2 Comparison of tissue cytokines, hydroxproline in group (n = 10, mean ± SD)

	Group 1	Group 2	Group 3	*P*
Hydroxproline (ng/lt)	830 ± 199	507 ± 79	508 ± 150	**0.01**
IL-6 (pg/mL)	39 ± 3	45 ± 5	45 ± 5	**0.009**
TNF-α (pg/mL)	129 ± 66	97 ± 63	107 ± 60	0.48

IL-6, Interleukin-6; TNF-α, tumor necrosis factor alpha.

Table 3 Comparisons of Chiu classification and semiquantitative analysis in rats (Mean)

	Group 1 n (%)	Group 2 n (%)	Group 3 n (%)	*P* G1-G2 G1-G3 G2-G3
Chiu clasification[28]				
Normal mucosal villus	8 (80%)	6 (60%)	9 (90%)	**0.009** >0.05 **0.008**
Subepithelial congestion	1 (10%)	2 (20%)	1 (10%)	**0.04** >0.05 **0.047**
EL moderately separated from LP	-	1 (10%)	-	**0.0001** >0.05 **0.0001**
Semiquantitative analysis[29]				
Submucosal/muscular layer	1,7	2	1,5	NS
Mucosal epithelium	3	3	2,5	NS
Edema	0,6	0,5	0,5	>0.05 >0.05 >0.05
Mononuclear cells	2,1	2	1,2	>0.05 **0.008** **0.006**
PMNL	1,9	2,1	1,1	>0.05 **0.009** **0.007**
Necrosis	0	0	0	-
Granulation tissue	2,3	2,5	2	>0.05 >0.05 **0.031**

EL, epithelium; LP, Lamina propria; PMNL, polymorphonuclear leukocyte; NS, nonspecific.

groups 2 and 3. However, there were no statistically significant difference observed between the Chiu scores of group 1 and 3 (Table 3). In the muscular and mucosal layer of anastomosis, a weak integrity was seen in group 2. Re-epithelialization of the wound healing of colonic anastomosis was observed to not have full closure in the left and right of the single-story cubic epithelium of the wound area (Fig. 1). In group 3, minimal mucosal debris was found in the anastomosis area. The wound flaps were microscopically determined to have minimum distance in group 3, a result indicating that group 3 demonstrated the best wound healing (Fig. 3). Infiltration of the lamina propria by polymorphonuclear leukocytes (PMNL), plasma cells, lymphocytes, and eosinophil cells was seen in all groups, while only a minimal degree of cellular infiltration was observed in group 3. Common congestion and hemorrhaging were observed particularly in the anastomosis area in group 2 (Fig. 2). Furthermore, the synthesis of fibroblast and collagen increased in all groups. Statistically significant differences ($P < 0.05$) were found when comparing the alteration of

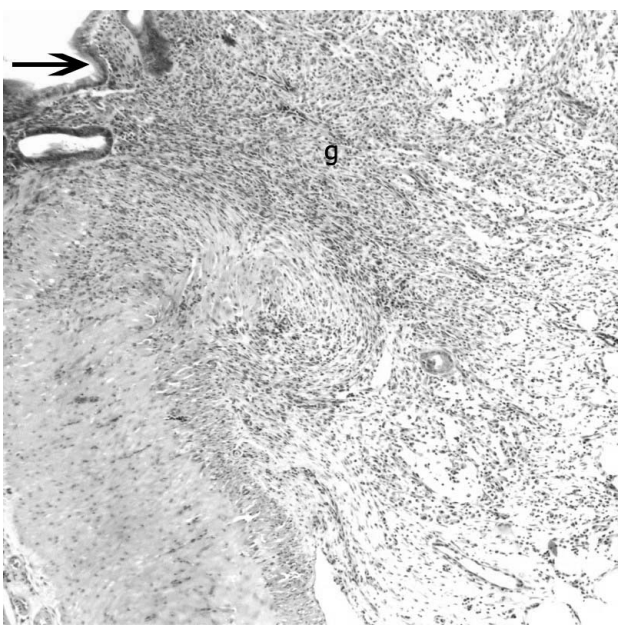

Fig. 1 Group 1: (Anastomosis) Infiltration of granulation tissue, hemorrhage, polymorphonuclear leukocytes, plasma cells, lymphocytes, and eosinophil cells in colonic anastomosis (g = granulation tissue, arrow = epithelium, Hematoxylin–eosin X 200).

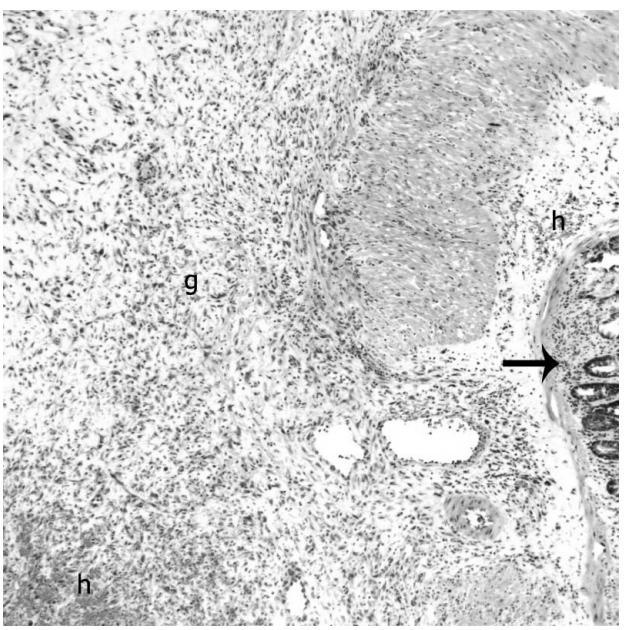

Fig. 2 Group 2: (Anastomosis and I/R) Appearance of mucosal debris, congestion, and hemorrhage in anastomosis area (g = granulation tissue, h = hemorrhage, Hematoxylin–eosin X 200).

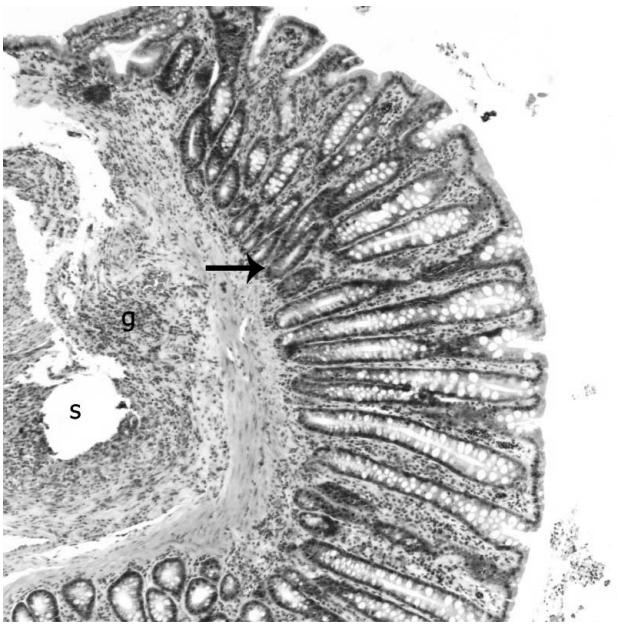

Fig. 3 Group 3: (Anastomosis, I/R, and NS) Minimal congestion and hemorrhaging in anastomosis area (g = granulation tissue, s = anastomosis suture, arrow = epithelium, Hematoxylin–eosin X 200).

mononuclear cells, PMNL, and granulation tissue among all groups (Figs. 1–3). However, there were no statistically significant difference detected between mucosal epithelium and submucosa/muscular layer in groups 1, ,2 and 3 ($P > 0.05$) (Table 3).

Discussion

Anastomotic leaks are one of the major complications associated with colorectal resections, and they have a significant mortality rate (6%–22).[1] The morbidity rate of anastomotic leaks can increase in cases of radiologic intervention, infection, chronic diseases, emergency intervention, and when previous operations have been performed.[2] Despite the reported rate of anastomotic leaks, these rates can vary depending on whether the leak was related to an emergency surgery or an elective surgery, with the former having higher rates. The rate of colonic anastomosis leaks is 1%–2% in elective surgery and 3%–7% in emergency surgery.[3] Ischemia is among the chief causes of anastomotic leaks. The colonic wall or circulatory ischemia can result in leakage disrupting anastomosis integrity.[4] Many studies have shown that there was greater decrease in blood flow of anastomosis than other parts of colon circulation.[1,3,5] Hypovolemia and hypotension due to systemic disease also increases the risk of anastomotic leaks being triggered from the decreased blood supply.[1,5,6]

Acute mesenteric ischemia (AMI) is one of the major diseases of the gastrointestinal tract and has a high mortality rate, despite the advances made in diagnostic and therapeutic modalities.[3,6] The mortality rate of AMI is between 60% and 80% and accounts for 0.1% of all emergency room admissions.[4–6] The overall incidence rate of AMI between 1970 and 1982, diagnosed during either autopsy or operation, in the population of Malmö, Sweden, was estimated to be 12.9/100,000, and the incidence is gradually increasing.[7] The most common causes of AMI are superior mesenteric artery (SMA) embolism (50%), SMA thrombosis (15%–25%), mesenteric venous thrombosis (5%), and nonocclusive ischemia (20%–30%).[5,6,8] The risk factors include blunt abdominal trauma, pancreatitis, abdominal infections, portal hypertension, hypercoagulable states, coronary artery disease, splenectomies, and malignancies.[7,8] The duration of ischemia directly affects the prognosis of the disease, and early diagnosis and treatment is vital. As the resulting damage is biphasic in character, reperfusion also plays as critical a role as ischemia.[5,8] Parks and Granger[9] showed that the majority of intestinal mucosa damage occurred in the reperfusion period of the I/R process. The mucosal damage that occurs in the intestinal I/R model can be reduced by early diagnosis.[1,5,9] However, although foreseen ischemic injury can be reduced by early diagnosis, reperfusion injury and its attendant consequences are still inevitable. After elimination of the causes of ischemia, the passing of the oxygenated blood to ischemic tissue in situ through reperfusion leads to the formation of reactive oxygen species (ROS).[5,10]

ROS, which is formed by a series of reactions and results from molecular oxygen, (superoxide, hydrogen peroxide, hydroxyl) can damage biologic molecules, including nucleic acids, membrane lipids, enzymes, and receptors.[8,10] ROS can lead to impairment of cell function, cell lysis, increased secretion of leukotriene B4 and platelet-activating factor (PAF), neutrophil migration, and eventually even death.[1,5,11] Neutrophils are the primary source of ROS.[1,5,11] Further, inducible nitric oxide synthase (i-NOS), which is activated as an enzyme during I/R injury, synthesizes large amounts of nitric oxide (NO), and since the abundance of NO disrupts the intestinal barrier function, bacterial translocation can occur.[1,5,10–12]

Currently, NS seeds and their byproducts are used in many fields, and they have numerous pharmacologic activities, including protective effect on enterocolitis,[13] antitumor,[14] antibacterial,[15] anti-inflammatory,[16] antioxidant,[17] immunomodulatory,[18] antidiabetic, and antihypertensive,[19,20] and protective action against renal, liver, and cerebral conditions.[20–22] Additionally, NS seeds have protective action against I/R-induced injuries to various organs, such as kidney, liver, and gastric mucosal lesions.[20–23] Thymoquinone, the active constituent of NS seeds, is a pharmacologically active quinone that has several properties, including those mentioned already.[13–19] I/R is related with elevated levels of lipid peroxidase (LPX) and lactate dehydrogenase (LDH) and reduced levels of glutathione (GSH) and superoxide dismutase (SOD). El-Abhar *et al*[23] reported that these biochemical changes were accompanied by an increase in the formation of gastric lesions, which were reduced with NS. NS tended to normalize the level of LDH, GSH, and SOD. The mechanism of action by NS is still unknown, but it seems that these effects may be related to inhibition of eicosanoid generation, namely thromboxane B2, leukotriene B4, and membrane lipid peroxidation.[16] Moreover, Bayir *et al*[30] reported that NS seeds increased the ratio of helper to suppressor T cells and enhanced natural killer cell activity in normal volunteers.[17,30] Haq *et al*[31] reported that the immunomodulatory effect of NS purified proteins was found in mixed lymphocyte cultures and caused changes in the levels of cytokines.

Hydroxyproline is an amino acid essential for collagen synthesis, and collagen synthesis is fundamental for wound healing, as fibroblasts migrate toward the injured area and produce collagen to increase tissue permeability. Hydroxyproline content has been used as an indicator to determine the content of collagen.[32] Ab Rahman *et al*[32] determined that NS seeds possess wound healing activities based on the ability of NS to enhance the proliferation of fibroblasts and promote the level of Basic Fibroblast Growth Factor (bFGF). In our study, we found a higher level of hydroxyproline in the serum and tissue in group 3 than in group 1 and detected a statistically significant difference ($P = 0.007$).

Cytokines are substances in the peptide or glycopeptide structure that are synthesized, and they secrete stimulated lymphocytes, monocytes, and macrophages cells to increase efficiency of the cells in the immune and inflammatory processes.[32] Cytokines have an important role in normal cell differentiation, mitosis, and cell movement, and as they work as mediator molecules of the immune system, they are known to be important in the pathogenesis of cancer.[33]

There are two subtypes of CD4 + cells, Th1 and Th2, differentiated according to the cytokines associated with their functions. Mossman and Coffman, who were the first to find the expression grouping of Th1 and Th2 25 years ago, helped to clarify the many events in the acquired immunity.[33,34] Th1 cells produce IFN-γ, interleukin-2 (IL-2) and tumor necrosis factor (TNF), activate macrophage, and combat intracellular pathogens, while Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13 secretion.[35] As a result, the Th1 subtype facilitates delayed-type hypersensitivity response, and Th2 cells regulate the response to the antibody.[32,34] TNF-alpha and IL-6 are known as pro-inflammatory cytokines that promote inflammation.[36,37]

According to Haq *et al*,[31] the aqueous extract of NS enhanced TNFα but not IL-4 production in nonactivated, mitogen activated, or allogeneic cells and stimulated IL-3 production by T cells. NS may, therefore, have a stimulatory effect on macrophage directly or indirectly through IL-3. Gholamnezhad *et al*[37] reported in their treadmill exercised rat model that NS may act as a balancing factor in Th1/Th2 lymphocytes in different exercise loads and act as an inhibitory factor on Th2 phenotype in control animals. In our study, we found a lower level of serum TNF-α in group 3 than in groups 1 and 2 ($P = 0.001$). Also, we detected a significant difference in level of tissue IL-6 in groups ($P = 0.009$). These findings suggest that inflammation in the treatment group decreased with NS.

OS causes significant changes in the body as a result of the increased number of lipid and protein oxidation products and the decreased number of antioxidant enzymes.[1,8,9,17,20,37] In the present study, we preferred to measure oxidants and antioxidant capacity simultaneously to assess oxidative stress more exactly. Furthermore, we measured oxidative stress, which was detected using both oxidative and antioxidative parameters, along with the protective effects of NS. We determined TAS and TTL, which reflect the antioxidative status, and TOS to investigate oxidative status using the more recently developed measurement methods by Erel.[24,25] Terzi *et al*[15] reported increased TAS levels and decreased TOS levels in treatment group rats in the study they conducted on the protective effects of NS on intestinal ischemia-reperfusion injury in rats. Yildiz *et al*[21] found that NS relieves the deleterious effects

of ischemia reperfusion injury on the liver via the decrease of oxidative stress biomarkers. Furthermore, Boskabady *et al*[38] demonstrated the preventive effect of the NS extract on the tracheal response and lung inflammation in sensitized guinea pigs, while Havakhah *et al*[39] reported that the NS extract showed protective action against ischemia-reperfusion induced rat kidney damage. In our study, we detected increased serum TOS and TTL levels and decreased TAS levels in I/R and anastomosis groups as compared to the only anastomosis group. However, we observed decreased serum TOS and TTL and increased TAS levels in the NS treated group but they were not statistically significant ($P > 0.05$).

The histopathologic results also suggested the protective effects of NS. In the present study, we detected histopathologic changes in subepithelial congestion, edema, granulation tissue and the infiltration of the majority of polymorphonuclear leucocytes, plasma cells, lymphocytes, and eosinophil cells in lamina propria, especially in group 2. NS group was the best-preserved group among all the groups in terms of Chiu classification ($P < 0.05$) (Table 3) (Figs. 1–3).

The limitations of our study include the absence of an analysis of TOS, TTL, and TAS levels in tissue to detect significant differences with similar studies found in the literature. Furthermore, we did not study the effect of different doses of NS.

Conclusion

Our study results demonstrated that NS is an effective therapeutic option in a rat model of I/R colonic anastomosis. These results can likely be attributed to the positive attenuating effect NS has on advanced inflammation, neutrophil infiltration, and oxidative stress in colonic anastomosis. Further studies are required to clarify the antioxidant and preventive effects of NS in experimental models.

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