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Original Research

Effect of hesperetin on inflammatory and oxidative status in trinitrobenzene sulfonic acid-induced experimental colitis model

Fatin Rustu Polat[1*], Ihsan Karaboga[2], Muhammed Semih Polat[3], Zeynep Erboga[4], Ahsen Yilmaz[4], Savas Güzel[4]

[1] Tekirdag Namik Kemal University Medical Faculty, Department of Surgery, Tekirdag, Turkey
[2] Tekirdag Namik Kemal University, School of Health, Tekirdag, Turkey
[3] Istanbul University, Engineering Faculty, Istanbul, Turkey
[4] Tekirdag Namik Kemal University, Medical Faculty, Department of Histology and Embryology, Tekirdag, Turkey
[5] Tekirdag Namik Kemal University, Medical Faculty, Department of Medical Biochemistry, Tekirdag, Turkey

Correspondence to: polat22@hotmail.com

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Abstract: In our study, the effect of hesperetin on inflammatory and oxidative status in trinitrobenzene sulfonic acid (TNBS)-induced experimental colitis model was investigated through different methods. Eighteen Wistar albino male rats were divided in to three groups: Group I (Control, n = 8; 1 ml physiological saline), Group II (Colitis, n = 8; 1 ml TNBS), Group III (Hesperetin, n = 8; 1 ml TNBS and 100 mg/kg hesperetin). Macroscopic and microscopic scores were calculated to determine the damage to the colon at the end of the experiment. Serum tumor necrosis factor-α (TNF-α) and tissue interleukin-6 (IL-6) levels were determined using the ELISA method. Myeloperoxidase (MPO), superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) levels were investigated spectrophotometrically. The TUNEL method was used for the detection of apoptotic cells in the colon tissue. Inducible nitric oxide synthase (iNOS) and nuclear factor-kappa-B (NF-κβ) expression in the colon were determined immunohistochemically. Hesperetin administration has shown to significantly reduce levels of MPO, MDA, and proinflammatory agents (TNF-α, IL-6, and NF-κβ). It has also been proven to inhibit mucosal apoptosis. This study indicates that hesperetin is protective against TNBS-induced colitis model via antiinflammatory, antioxidant and antiapoptotic effects.

Key words: Hesperetin; TBNS-induced colitis; NF-κβ; iNOS; IL-6; MPO.

Introduction

The pathogenesis of ulcerative colitis is multifactorial and has not yet been fully explained. Genetic predisposition, mucosal barrier damage, intestinal flora changes, immune response impairment, and interactions among these factors are known to play important roles in the pathogenesis of this disease (1, 2). Inflammation is usually responsible for the tissue damage. Immune response produced as a result of inflammation causes lymphocyte proliferation, neutrophil accumulation, and increased cytokine production (3, 4).

Cytokines, such as interleukin-1α (IL-1α), interleukin-1 β (IL-1β), IL-6, interleukin-8 (IL-8), interleukin-23 (IL-23), TNF-α play important roles in the onset and development of ulcerative colitis (5). In addition, the expressions of inflammatory proteins cyclooxigenase-2 (COX-2) and iNOS are thought to play an important role in the regulation of inflammation (6, 7). Excessive expression of NF-κβ has been observed in colon biopsy samples obtained from patients with ulcerative colitis. The production of NF-κβ is upregulated by TNF-α, interleukins, chemokines, and DNA-damaging agents during inflammation (8).

Oxidative stress may be a potential etiologic or triggering factor for ulcerative colitis (9). During an inflammatory response, changes in oxidative parameters, such as MDA, MPO, and glutathione, occur due to oxidative stress (10). During acute inflammation, a severe increase is observed in the MPO activity of neutrophils as a result of polymorphonuclear leukocyte infiltration. MPO is one of the most important components of the innate immune system, wherein the inflammatory response is at the forefront (11, 12). MPO level, which indicates neutrophil infiltration and is a strong parameter in the determination of oxidative stress, is frequently used for colitis experimental procedures (13, 14).

Hesperetin (5,7,3′-trihydroxy-4′-metoxyflavanone) is a natural compound belonging to the flavanone class of flavonoids (15). It is found in abundance in citrus fruits, such as lemon, lime, tangerine, and orange. Studies have shown that hesperetin has antioxidant, anti-inflammatory, anticarcinogenic, and antihypertensive effects (16-19).

The purpose of this study was to examine the effects of hesperetin, whose antioxidative and antiinflammatory properties have been reported, on colon tissue structure, tissue oxidative parameters, and TNF-α, IL-6, iNOS, and NF-κβ expressions, which have important roles in the inflammatory process, in a TNBS-induced experimental colitis model.

Materials and Methods

Animals and experimental design

All experimental procedures were approved by the

Table 1. Scale for macroscopic damage score (22).

Characteristics	Score
Ulceration	
Normal appearance	0
Focal hyperaemia, no ulcers	1
Ulceration without hyperaemia or bowel wall thickening	2
Ulceration with inflammation at one site	3
Two or more sites with ulceration and inflammation	4
Adhesions	
No adhesions	0
Minor adhesions (colon can be easily separated from other tissue)	1
Major adhesions	2
Diarrhea	
No	0
Yes	1
Thickness	
Maximal bowel wall thickness (x), in millimeters	X
	Total score

local animal ethics committee of Namık Kemal University (Protocol no: NKUHADYEK-2017/03/04). A total of 24 adult male Wistar albino rats were used. The animals were fed ad libitum in pathogen-free cages and under optimal laboratory conditions (temperature: 20±2 °C, humidity: 50%, and light: 12-h dark/12-h light).

The rats were randomly categorized into three groups: Group I (Control, n = 8), Group II (Colitis, n = 8), Group III (Hesperetin + Colitis, n = 8). Group III rats were pretreated with hesperetin 3 days prior to colitis induction. Hesperetin treatment, which was continued for 7 days after colitis induction, was administered via an oral gavage needle at a dose of 100 mg/kg/day by dissolving it in 1 ml of physiological saline solution (20).

The animals were not fed for 24 h but given free access to water before colitis induction. The rats in the experimental groups were administered intracolonic TNBS (Sigma, MA, USA [25 mg/rat; TNBS, 50% (v/v), dissolved in a physiological saline–ethanol mixture; total, 1 ml] in the Trendelenburg position, with a 6-Fr catheter placed 8 cm proximal to the anus after anesthetization with ketamine (21). After 45 s of TNBS application, the colons were washed with physiological saline solution and TNBS was removed from the colon lumen. Additionally, Group I was administered 1 ml of physiological saline solution intracolonically using the same technique. Group III was administered hesperetin for 7 days using the oral gavage method after colitis induction. Groups I and II were not subjected to any treatment. The body weights of the rats were measured and recorded before colitis induction (at the beginning of the experiment) and at the end of the experiment.

Collection of tissue samples

At the end of the experiment, intracardiac blood was collected from the rats under xylazine–ketamine (Rompun; Bayer, Istanbul; Ketalar; Pfizer, Istanbul, 10/90 mg/kg, intraperitoneal) anesthesia, and serum samples were kept under −80° C until ELISA was performed. Moreover, the intra-abdominal regions were opened at the end of the experiment, and the presence of any perforation/adhesion was examined. Biopsy samples (8 cm in length) were collected from the colons. Colon and body weights were measured with an accuracy of up to 0.01 g using an electronic balance.

Macroscopic and microscopic evaluations

The rat colon samples were macroscopically evaluated according to the method described by McCafferty et al. (Table 1) (22). A portion of the colon tissue was separated for biochemical analyses and stored at −80° C until further use. Colon samples were fixed with formaldehyde solution with 10% buffer before performing light microscopy; following paraffin inclusion, paraffin blocks were acquired. Slices (5 μm in thickness) were taken from paraffin blocks and stained with hematoxylin–eosin (H&E); histological changes in the colon were examined; and microscopic scoring was performed. The H&E-stained slices were microscopically evaluated according to the method described by Obermeier et al. (Table 2) (23).

Immunohistochemistry and TUNEL assay

The slices obtained from the paraffin blocks were marked by the streptavidin–peroxidase method using iNOS (Abcam, ab15323) and NF-κβ (Abcam, ab7970) antibodies. Dilution and incubation times were optimized according to the manufacturer's instructions. TUNEL assay using MerckMilipore (S7101 ApopTag® Plus Peroxidase In Situ) apoptosis Kit was performed to observe apoptotic cells. Mayer's hematoxylin solution was used for counterstaining in immunohistochemistry and staining in TUNEL assay. The number of cells showing positive staining for NF-κβ, iNOS, and in the TUNEL assay on the colonic wall were counted to determine the positively stained cell count for each group in a unit area (mm^2). Microscopic evaluations and cell counts were performed using a light microscope (Olympus CX-40, Olympus, Japan) and a Kameram image analysis program (Kameram II, Argenit, Istanbul, Turkey).

ELISA

Serum IL-6, MPO, and TNF-α levels were measured

Table 2. Scale for microscopic damage score (23).

Characteristics	Score
Epithelium (E)	
Normal morphology	0
Loss of goblet cells	1
Loss of goblet cells in large area	2
Loss of crypts	3
Loss of crypts in large areas	4
Infiltration (I)	
No infiltrate	0
Infiltrate around crypt basis	1
Infiltrate reaching to L. muscularis mucosa	2
Extensive infiltration reaching to L. muscularis mucosa and thickening of the mucosa with abundant Edema	3
Infiltration of the L. submucosa	4
Total microscopic score represents the sum of the E + I score	Total score

using the ELISA method. IL-6 (Novex, Invitrogen, KMC0061), MPO (Novex, Invitrogen, EMMPO), and TNF-α (Novex, Invitrogen, KRC3011) kits were used in the ELISA studies.

Lipid peroxidation, oxidative enzyme, and MPO analysis

MDA, SOD, and CAT enzyme levels were measured spectrophotometrically for the evaluation of oxidative parameters in the colon tissue. MPO assay was performed according to the method described by Wei et al. using 4-aminoantipyrine/phenol solution as a substrate for MPO-mediated H_2O_2 oxidation (24). MDA, a lipid peroxidation product, was measured using the double-boiled thiobarbituric acid reactivity method described by Draper and Hadley (25). SOD activity was measured according to the method described by Durak et al. (26). CAT activity, which signifies the removal of toxic H_2O_2 from the cells, was determined according to the method described by Aebi and Bergmeyer (27).

Statistical analysis

Data were evaluated using the PASW (PASW Statistics 18.0.0, SPSS Inc, Chicago, IL, USA) statistics program. The numerical parameters of the groups were evaluated using a non-parametric test (Kruskal–Wallis), and the significance of the values obtained in the two-way comparison was measured using the Mann–Whitney U-test. P values of <0.05 were considered statistically significant.

Results

Macroscopic results

The macroscopic images of the colon, damage scoring, and colon weight findings are presented in Figure 1. In the colon macroscopic examination performed at the end of the experiment, the mucosal structure of Group I was observed as normal. In Group II, edema, ulceration, and hyperemic regions were observed in the mucosa (Figure 1b). Fewer ulcers and hyperemic areas were observed in Group III, which were treated with hesperetin (Figure 1c). When the colon macroscopic scores were examined, a significant decrease was observed in Groups II and III compared with that in Group I and Group II, respectively ($p < 0.05$, Figure 1d). Regarding the colon weight findings, there was a significant increase in Group II compared with that in Group I and a significant decrease in Group III compared with that in Group II ($p < 0.05$, Figure 1e).

Microscopic results

General tissue structures and microscopic damage

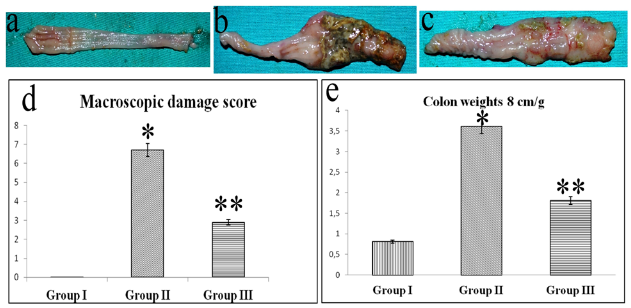

Figure 1. Macroscopic damage score and colonic weight findings of groups (a: Group I; b: Group II; c: Group III; d: Macroscopic injury score; d: colonic weights; *= $p < 0.05$ compared with Group I; **= $p < 0.05$ compared with Group II).

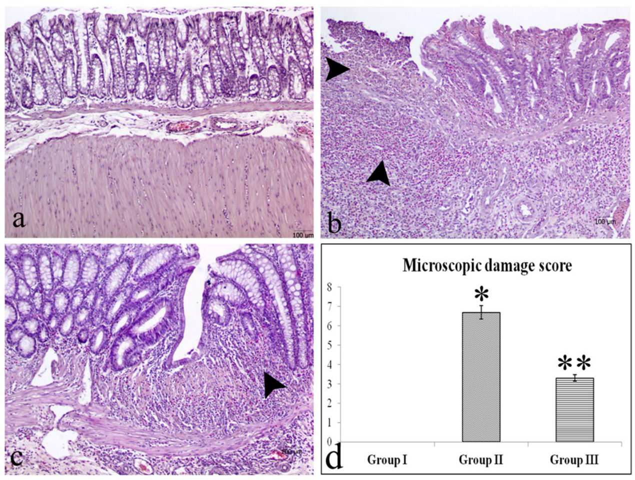

Figure 2. Hematoxylin & eosin staining and microscopic injury scores of groups (a: Group I; b: Group II; c: Group III; d: Macroscopic injury score; d: colonic weights; *= $p < 0.05$ compared with Group I; **= $p < 0.05$ compared with Group II; Magnification: 100X, scale bar: 100 µm, arrowhead: inflammatory cell infiltration in colonic mucosa).

scores are presented in Figure 2. In the examination of the general tissue structure of the H&E-stained colon tissues, the histological structure of Group I rats was normal (Figure 2a). In Group II, histopathological findings such as degeneration in the crypts of Lieberkühn, losses of epithelial and goblet cells, and edema and ulcerations in the mucosa and submucosa were observed (Figure 2b). In Group III showed a slight reduction in histopathological changes compared to Group II. When the colon microscopic damage scores of the groups were examined, there was a significant increase in Group II compared with that in Group I ($p < 0.05$) and a significant decrease in Group III compared with that in Group II ($p < 0.05$, Figure 2c-d).

Immunohistochemical and TUNEL findings

The findings of the immunohistochemical staining performed using anti-NF-κβ antibodies and the average number of positive cells in the groups are presented in Figure 3. The results showed increased staining intensity in Group II. There was a significant increase in the number of NF-κβ-positive cells in Group II compared with that in Group I ($p < 0.05$). The number of NF-κβ-positive cells in Group III was significantly lower than that in Group II ($p < 0.05$).

The findings of the immunohistochemical staining using anti-iNOS are presented in Figure 4. A small number of cells in the colon mucosa showed positive staining in Group I, whereas there was a significant increase in the number of iNOS-positive cells in Group II ($p < 0.05$). In hesperetin-treated Group III, a significant decrease was observed in the iNOS immunoreactivity compared with that in Group II ($p < 0.05$).

The findings of TUNEL staining are presented in Figure 5. TUNEL-positive cells were located in the lamina epithelialis of the mucosa in Group I, inflammation regions in Group II, and glandular epithelium and lamina epithelialis in Group III. When the number of apoptotic cells per field was examined, there was a significant increase in Group II compared with that in Group I ($p < 0.05$), and a significant decrease was recorded in Group III compared with Group II. The distribution of TUNEL-positive cells in the groups is shown in Figure 5d ($p < 0.05$).

ELISA findings

Serum TNF-α levels in Group II were significantly higher than those in Group I ($p < 0.05$). A significant decrease was observed in Group III compared with that in Group II ($p < 0.05$, Table 3). The level of proinflammatory cytokine IL-6 in the serum of the Group II were significantly increased compared to Group I ($p<0.05$), but they were markedly lower in the Group III compared to Group II ($p<0.05$, Table 3).

Table 3. Serum TNF-α and IL-6 levels in group (pg/ml).

	TNF-α	IL-6
Group I	8.9±2.1	16.8±1.6
Group II	23.0±6.2[a]	29.5±5.1[a]
Group III	17.2±2.3[b]	17.2±4.8[b]

a; p<0.05 compared to Group I, b; p<0.05 compared to Group II.

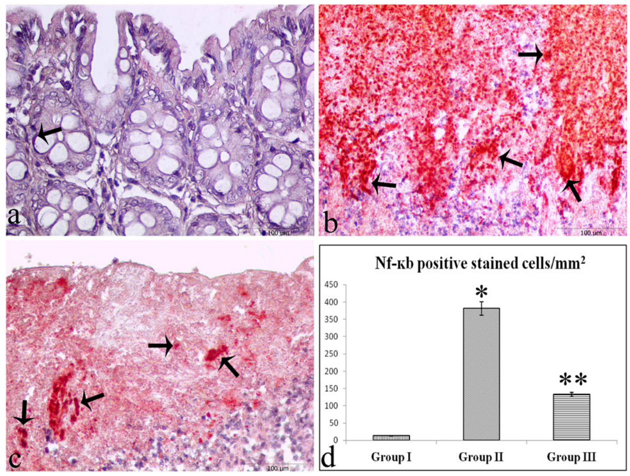

Figure 3. Light microscopy analysis of NF-κβ immunoreactivity of the colonic mucosa (a: Group I; b: Group II; c: Group III; d: NF-κβ-positive stained cell numbers of groups; *= $p < 0.05$ compared with Group I; **= $p < 0.05$ compared with Group II arrow; NF-κB-positive stained cells, magnification: 400X, scale bar: 100 µm, Mayer's hematoxylin counterstain).

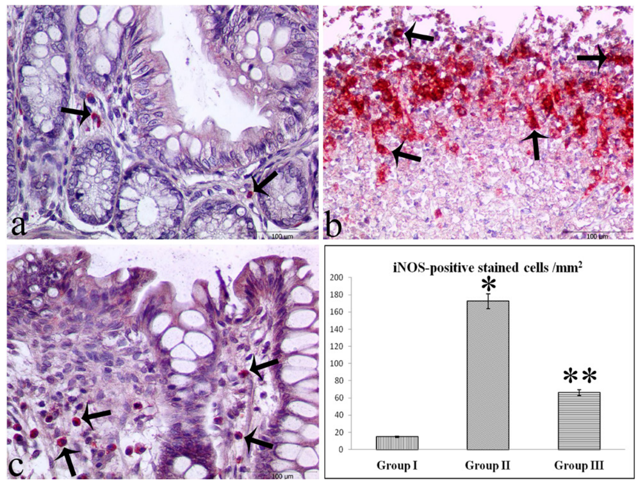

Figure 4. iNOS-positive cells in the colonic mucosa (a: Group I; b: Group II; c: Group III; d: number of iNOS-positive of groups; *= $p < 0.05$ compared with Group I; **= $p < 0.05$ compared with Group II arrow; iNOS-positive cells, magnification: 400X, scale bar: 100 µm, Mayer's hematoxylin counterstain).

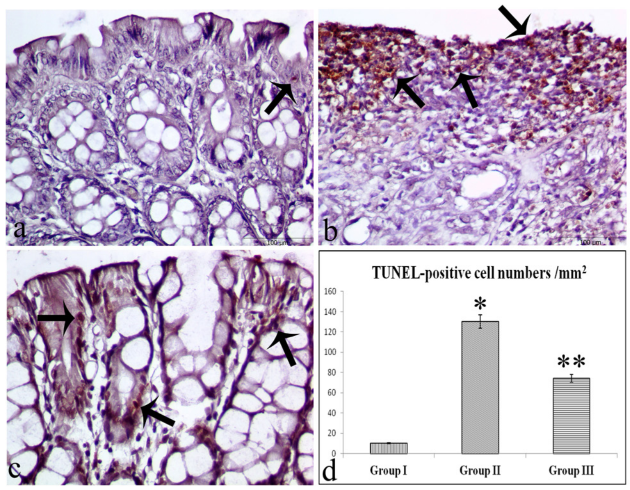

Figure 5. Apoptotic cells in the colonic mucosa (a: Group I; b: Group II; c: Group III; d: number of TUNEL-positive cell of groups; *= $p < 0.05$ compared with Group I; **= $p < 0.05$ compared with Group II arrow; indicate TUNEL-positive cells, magnification: 400X, scale bar: 100 µm, Mayer's hematoxylin counterstain).

Table 4. Effect of Hesperetin on colonic MPO, MDA, SOD, CAT levels.

	MPO (U/mg protein)	MDA (mg/protein)	SOD (U/mg protein)	CAT (K/kg protein)
Group I	0.05±0.02	0.15±0.02	11.3±3.3	0.11±0.04
Group II	0.17±0.4[a]	0.29±0.07[a]	8.0±2.3	0.06±0.01
Group III	0.06±0.03[b]	0.18±0.3[b]	8.9±1.4	0.09±0.01

a; p<0.05 compared to Group I, b; p<0.05 compared to Group II.

Lipid peroxidation and antioxidant enzyme and MPO analysis

Oxidative stress parameters including MPO, MDA, SOD, and CAT levels in the colon tissues were measured by spectrophotometry, and the findings are presented in Table 4. When MPO levels were examined, a significant increase was observed in Group II compared with that in Group I ($p < 0.05$). A significant decrease was observed in Group III compared with that in Group II ($p < 0.05$) When SOD levels of the groups were examined, there was a decrease in Group II compared with that in Group I, but this decrease was not statistically significant ($p = 0.64$). Although there was an increase in SOD levels in Group III compared with that in Group II, this was not statistically significant ($p = 0.81$). When MDA levels of the groups were examined, a significant increase was observed in Group II compared with that in Group I ($p < 0.05$). The MDA level significantly decreased in Group III compared with that in Group II ($p < 0.05$). When CAT enzyme levels were examined, no significant difference was observed between the groups.

Discussion

Anti-inflammatory and antioxidant properties of hesperetin have been previously reported in various experimental studies using different methods (28-30). The present study evaluated the effects of hesperetin on TNF-α, NF-κβ, iNOS, IL-6, and tissue oxidative stress parameters, which have important roles in inflammatory processes.

In various models of colitis created using different methods, several studies have reported an increase in the colon weight associated with inflammatory cell infiltration and edema in the colon mucosa and submucosa, similar to human ulcerative colitis (31, 32). There was a remarkable increase in the colon weight of rats in Group II. Experimental models have reported that many agents possessing anti-inflammatory properties reduce the colon weight and colonic inflammation (33, 34). Hesperetin administration significantly decreased the colon weight and the macroscopic damage score of the mucosa ($p < 0.05$). These macroscopic findings indicate the potential anti-inflammatory activity of hesperetin.

Microscopic evaluations demonstrated severe histopathological changes in the colon mucosa of Group II rats, while these changes were observed to have decreased in Group III rats. Similar findings have been presented in studies that examined the effects of various agents showing protective effects on the colon mucosa (35, 36).

Nitric oxide (NO) is involved in various processes, such as blood flow regulation, vascular permeability, immune regulation, mucosal defense, fluid secretion, and bowel movements in the gastrointestinal tract. NO is produced by the three isoforms of the enzyme NO synthase (NOS): neural NOS, epithelial NOS, and iNOS. iNOS is involved in various processes, including inflammatory processes in the intestines (37). TNF-α expression is not commonly observed in the colon mucosa of healthy people, and its expression increases under inflammatory conditions (38). Experimental models have shown that the onset and development of ulcerative colitis suppresses iNOS gene expression (37, 39). iNOS activity was immunohistochemically determined in our study. In Group II, its expression was increased in the mucosa, especially in the inflamed areas. El Ashmawy et al. reported increased colonic iNOS expressions in a DSS-induced colitis model (39). Studies have reported that agents that are experimentally shown to decrease inflammation also decrease the colonic iNOS level (40, 41). Similarly, hesperetin administration produced a significant decrease in iNOS immunoreactivity ($p < 0.05$). TNF-α levels were determined using ELISA in our study, and a significant increase was observed in Group II, similar to previous studies and human ulcerative colitis ($p < 0.05$) (31, 34). In Group III, there was a significant decrease in serum TNF-α levels ($p < 0.05$). Increased TNF-α levels cause the disruption of the epithelial barrier, secretion of chemokines, and induction of apoptosis (21). TUNEL staining findings were in line with those of previous studies, and a significant increase in the number of TUNEL-positive cells was observed in Group II ($p < 0.05$) (42, 43). Thus, hesperetin administration contributes to the protection of the colon mucosa by suppressing the effects of TNF-α and other apoptotic inducers.

The balance between proinflammatory and anti-inflammatory cytokines is very important for colon health. Cytokines play an important role in immune regulation and inflammatory response (44). Increased proinflammatory cytokine (TNF-α, IFN-γ, and IL-6) levels were detected in fecal, tissue, and blood samples of patients with ulcerative colitis (45, 46). In our study, the serum IL-6 level was determined using ELISA and was found to be significantly increased in Group II, which was in line with the literature ($p < 0.05$). Khan et al. showed that the administration of caffeic acid phenethyl ester has a mucosa-protective effect as it significantly decreases IL-6 levels in the experimental colitis model (47). In our study, increased IL-6 levels in the Group II significantly decreased with the hesperetin treatment ($p < 0.05$).

Proinflammatory mediators, such as inflammatory cytokines, are controlled with the activation of transcription factors. NF-κβ activation plays an important role in the pathogenesis of inflammatory bowel diseases (48, 49). NF-κβ is considered to be involved in a proinflammatory signal pathway, which activates other cytokines, chemokines and other adhesion molecules that play a role in the expression of TNF-α and IL-1 (37). When NF-κβ is activated in the cell, it moves into the nucleus, binds to DNA, and initiates gene expression (50). In our study, NF-κβ activity in the colon tissue was shown using immunohistochemical methods. NF-κβ expression was upregulated in Group II, whereas it was significantly downregulated in Group III ($p < 0.05$). Both iNOS and TNF-α-suppressive effects of hesperetin can be explained by decreased NF-κβ activation. NF-κβ activation and expression is upregulated in patients with ulcerative colitis. Moreover, the intensity of NF-κβ activation increases with intestinal inflammation (8).

The accumulation of neutrophils in the inflamed intestinal mucosa is a characteristic feature of patients with ulcerative colitis (11). MPO levels increase several-fold in patients with ulcerative colitis compared with healthy people (51). Cytoplasmic granules of neutrophils contain MPO. As a proteolytic enzyme, MPO levels increase directly in proportion with the intensity of activated neutrophils in the colon wall (11). IL-8 released from the activated neutrophils and mesangial cells in ulcerative colitis result in increased neutrophil clustering and MPO expression (37). In our study, a significant increase in MPO level was noted in the colonic mucosa of Group II compared with that in Group I ($p < 0.05$). Hesperetin administration produced a significant decrease in the MPO level ($p < 0.05$). This result shows that hesperetin prevents neutrophilic infiltration by suppressing NF-κβ activation and downregulating the expression of cytokines and chemokines.

Reactive oxygen species (ROS), free radicals, and pro-oxidant molecules released from inflammatory cells during the inflammatory process in ulcerative colitis play a role in the development of colitis (52). SOD has a very strong antioxidant effect and enables the recovery of colonic inflammation in experimental models by decreasing ROS production and oxidative stress (53). In our study, although the SOD enzyme level in Group II was decreased compared with that in Group I, this was not found to be statistically significant. This finding shows similarity to studies by Cetinkaya et al. and Kannan et al. (54, 55). Many experimental studies have reported significantly decreased SOD levels (56, 57). In our study, the SOD level increased in Group III compared with that in Group II, but this was not statistically significant. CAT is an antioxidant enzyme that plays a role in the detoxification of H_2O_2 (58). There was no significant difference in the levels of CAT between the Group II and Group III. On the other hand, some studies have shown that CAT levels significantly decrease in colitis models (57, 59). In contrast to this finding, Colares et al. reported that CAT level significantly increased in the colitis model (60). MDA is a product of lipid peroxidation and an important indicator of oxidative stress in cells and tissues (61). In our study, MDA level significantly increased in Group II, and it was significantly lower in Group III than in Group II ($p < 0.05$). Many experimental studies have reported that MDA significantly increases in the colitis model. Yang-Hong et al. and Xie et al. observed a significant increase in the MDA levels of the Group II in the TNBS-induced colitis models (57, 62). Hesperetin administration produced a significant decrease in the MDA levels of Group III ($p < 0.05$).

These results show that hesperetin decreases inflammation in the TNBS-induced colitis model by decreasing proinflammatory cytokine levels through the inhibition of NF-κβ transcription factor activation. The findings of our study will light the way for future studies.

Conflict of interest

There are no conflicts of interest among the authors.

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Author contributions

Concept -, Design-, Supervision -, Resource -, Materials -, Data Collection and/or Processing - F.R.P., I.K, M.S.P., Z.E.; Analysis and/or Interpretation - F.R.P, I.K, A.Y, SG; LiteratureSearch -, Writing -, Critical Reviews - FRP, I.K.

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