Corrective Effect of Verbascoside on Histomorphological Differences and Oxidative Stress in Colon Mucosa of Rats in Which Colon Ischemia–Reperfusion Injury was Induced

Ayşe Nur Değer[1], Filiz Özyiğit[2], Fatma Emel Koçak[3], Zülfü Bayhan[4], Sezgin Zeren[5], Özlem Arık[6], Hakkı Değer[7]

[1]Department of Pathology, Kütahya Health Sciences University School of Medicine, Kütahya, Turkey
[2]Department of Pharmacology, Kütahya Health Sciences University School of Medicine, Kütahya, Turkey
[3]Department of Biochemistry, Kütahya Health Sciences University School of Medicine, Kütahya, Turkey
[4]Department of General Surgery, Kartepe, Kocaeli, Turkey
[5]Department of General Surgery, Kütahya Health Sciences University School of Medicine, Kütahya, Turkey
[6]Department of Biostatistics, Kütahya Health Sciences University School of Medicine, Kütahya, Turkey
[7]Department of Neurosurgery, Kütahya Evliya Çelebi Training and Research Hospital, Kütahya, Turkey

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ABSTRACT

Background: *This study aims to show the corrective effect of verbascoside on histomorphological and biochemical differences in the colon mucosa of rats in which colon ischemia–reperfusion (I/R) injury was induced.*

Methods: *Fifty Sprague Dawley male rats were divided into 5 groups, of control, sham, ischemia (I), I/R, and I/R+verbascoside. Ischemia and reperfusion were applied to the suitable groups for 30 minutes and 120 minutes respectively, and 10 mg/kg verbascoside was administered intraperitoneally. Histomorphological assessment was done in the colon tissues obtained, and the goblet cells were assessed using the Alcian blue method. Proliferating cell nuclear antigen (PCNA), TUNEL, and hypoxia-induced factor 1 (HIF-1α) assays were used to assess oxidative stress with the immunohistochemical method. Malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), total antioxidant status (TAS), total oxidant status (TOS), oxidative stress index (OSI), and total thiol (TT) levels were checked, for a biochemical analysis of oxidative stress.*

Results: *Compared with the I/R group, histomorphological differences were seen to be corrected in colon epithelium in the I/R+verbascoside group. The goblet cell number increased and cell proliferation was increased, as seen with the PCNA assay; and apoptosis was decreased, as seen with the TUNEL assay. HIF-1α expression also decreased in the drug group. In the drug group, SOD, GSH-Px, TAS, and TT levels increased, but TOS, OSI, and MDA levels decreased.*

Conclusion: *It was seen that verbascoside had a corrective effect on histomorphological and biochemical differences caused by I/R injury.*

Keywords: *Biochemical analysis, colon, ischemia–reperfusion, immunohistochemistry, verbascoside*

INTRODUCTION

Ischemia–reperfusion (I/R) injury is a common tissue injury. It might arise from various diseases or in various clinical cases with a pathophysiology of multifactorial processes. Conditions such as major traumas, acute mesenteric ischemia, septic and hypovolemic shock, abdominal aorta surgery, cardio-pulmonary bypass, and surgical applications may cause I/R injury.[1,2]

Colon I/R injury is a common clinical case, with significant risks of morbidity and mortality.[3] Colon ischemia might arise from cardiac (arrhythmia, heart failure, shock) vascular (emboli, thrombus, vasculitis, etc.), infectious (*Escherichia coli*, hepatitis B, cytomegalovirus) iatrogenic (surgical interventions to the aorta), physiological (long-distance run, etc.), and pharmacologic (antibiotics, diuretics, non-steroidal anti-inflammatory drugs, etc.) causes.[4,5]

Volvulus and trauma might result in ischemic injury in the colon.[2] Colon resection and anastomosis are common surgical applications in general surgery. Colon anastomoses are among the reasons for ischemic injury in the colon.

Ischemic preconditioning and controlled reperfusion antioxidant agents are used in the treatment of I/R injury.[2,5]

Corresponding author: ***Ayse Nur Deger****, e-mail:* ***ayhade@yahoo.com***
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I/R injury is related to multiple metabolic effects. Following ischemia, especially reactive oxygen species (ROS) and cytokine accumulation in the reperfusion phase result in tissue injury.[2] Various studies have confirmed the role of ROS and nitric oxide (NO) in I/R injury.[2,3] Reoxygenation occurring during reperfusion causes oxidative stress. Intracellular and extracellular ROS or its free radicals are produced, and they have damaging effects on the nucleic acid, protein, and lipids in the cell.[6,7] In the tissue, I/R injury results in neutrophil accumulation, increased myeloperoxidase (MPO) activity, apoptotic necrosis, accumulation of proinflammatory cytokines, and massive histopathological differences.[5]

Verbascoside, also known as acetonide, is structurally a phenylpropanoid glycoside. Like other phenolic components, it can be obtained primarily from plants like olive.[8]

Verbascoside has antihepatotoxic, neuroprotective, immunosuppressant, anti-nociceptive, and NO-scavenging effects.[8-12] Here, the corrective effect of verbascoside on histomorphological and biochemical differences in colon mucosa of rats in which colon I/R injury was induced, are assessed.

MATERIALS AND METHODS

This study was carried out at Dumlupinar University. The study experiments were approved by the Local Ethics Committee for Experiments on Animals of Dumlupinar University (Number: 2014.08.09).

All experiments were carried out by following the Guide for the Care and Use of Laboratory Animals published by the Institute of Laboratory Animal Resources Commission on Life Sciences National Research Council.

Animals

In the study, 50 Sprague Dawley adult male rats weighing 250-300 g were used. The animals were put into 10 cages, 5 in each cage, at constant temperature, with standard 12-hour periods of alternating dark and light, with ventilation. They were let free for food and water consumption. The rats were brought into the laboratory 1 week before the study for adaptation.

Chemicals

Verbascoside (Product Number: V4015 CAS Number: 61276-17-3 Formula: C29H36O15 Formula Weight: 624.59 g/mol was purchased from Sigma Aldrich Co LLC, St. Louis, MO). It was dissolved in absolute methanol (5 mg/mL) and diluted with physiological saline to a final methanol concentration of 1%.

Experimental Study Design

The rats included in the study were chosen randomly among the 50 rats and divided into 5 groups of 10 rats. Throughout the study, the rats were anesthetized with intraperitoneal 50 mg/kg ketamine (Ketalar 50 mg/ml, 10 mL flacon, Pfizer) and 20 mg/kg xylazine (Rompun 2% , 50 mL, Bayer).

The 5 groups of the study were group I, the control group (n = 10); group II, the sham group (n = 10); group III, the ischemia (I) group (n = 10); group IV, the ischemia–reperfusion (I/R) group (n = 10); and group V, the I/R+verbascoside group (n = 10).

Group I, **Control**: The anesthetized rats were placed in a recumbent position under a heating lamp. With a midline incision, the colon was removed. A part of the tissue obtained by cleaning the intestines was allocated for histopathological analysis, while a part of it was allotted for biochemical analysis.

Group II, Sham: The sham group was subjected to abdominal median laparotomy and not to I/R conditions. The rats were treated with intraperitoneal (ip) injections of 1 mL of methanol : saline (1 : 2, vehicle) 60 minutes before laparotomy, and the required amount of methanol to dissolve 10 mg verbascoside was used.

With a midline incision, the colon was removed. A part of the tissue obtained by cleaning the intestines was allocated for histopathological analysis, while a part of it was allotted for biochemical analysis.

Group III, Ischemia (I): The third group was planned as the I group. After the abdomens of the rats were opened, the superior mesenteric artery was ligated. Later, abdominal walls were closed one by one with 3/0 atraumatic silk. The rats were subjected to ischemia for 30 minutes. After this process, the abdomen was entered again and the colon was removed. A part of the tissue was allocated for histopathological analysis, while a part of it was allotted for biochemical analysis. To prevent hypothermia during the ischemia process, a hot moist dressing was applied to the rats.

Group IV, Ischemia–reperfusion (I/R): After the abdomens of the rats were entered into, the superior

mesenteric artery was ligated. After abdominal walls were closed with 3/0 atraumatic silk, the rats were subjected to ischemia for 30 minutes.

After this process, entering into the abdomen again, the superior mesenteric artery was opened and the ischemia operation was concluded. Mesenteric artery pulsation was seen. After the abdominal walls were closed one by one with 3/0 silk, the rats were subjected to reperfusion for 120 minutes. After this duration, the colon was removed and sectioned for histopathological and biochemical analyses. To prevent hypothermia during I/R process, a hot moist dressing was applied to the rats.

Group V, I/R+verbascoside: The rats in this group were administered verbascoside together with I/R (I/R+verbascoside). After the abdomens of the rats were opened, the superior mesenteric artery was ligated and then the abdomens were closed with 3/0 silk. The rats were subjected to ischemia for 30 minutes. At the end of this time, the suture on the superior mesenteric artery was removed, and immediately after the abdomen was closed with 3/0 silk, 10 mg/kg verbascoside was administered intraperitoneally. The reperfusion procedure lasted for 120 minutes. After this duration, the abdomen was opened and the colon was removed for histopathological and biochemical analyses.[2,13-15]

Histomorphological and Immunohistochemical Analysis

All the rats were sacrificed at predetermined durations. Colon samples 1-2 cm long were taken from the area 1 cm distal to the cecum–colon intersection. After the samples were washed with physiological saline solution, they were fixed with 4% paraformaldehyde. The tissue samples were dehydrated in ethanol and put into paraffin. For each histochemical and immunochemical analysis, sections of 4-5 μm were taken from the tissue samples.

Histomorphological assessment: The tissues were deparaffinized and stained with hematoxylin and eosin for histopathological and morphometric assessments. For the histochemical assessment, the Alcian blue staining method was used. The histomorphological differences in hematoxylin and eosin-stained sections were grouped between 0 and 4, and the indications were as follows[13]:

0: normal mucosa;
1: partial epithelial edema and necrosis;
2: diffuse distension in the epithelium, and necrosis;
3: necrosis and submucosal neutrophil infiltration; and
4: massive necrosis, massive neutrophil infiltration, and hemorrhage.

Detection of Goblet Cells with the Alcian blue Method: Using Alcian blue, the goblet cells producing mucus were detected. The goblet cells in 10 intestinal villi and the corresponding intestinal crypts were counted.[5]

Immunohistochemical assessment: Sections of 4-5 μm in size were prepared for immunohistochemical assessment for proliferating cell nuclear antigen (PCNA) and hypoxia–inducible factor 1 alpha (HIF1 α) assays. They were deparaffinized and rehydrated. Endogen peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol. Anti-PCNA antibody (ab18197-Abcam) and anti-HIF-1α antibody-ChIP Grade (ab2185-Abcam) were applied for 60 minutes. Peroxidase activity was released with diaminobenzidine (DAB). For contour staining, hematoxylin was applied. Cells giving positive reaction were counted in 400× enlargement. For each subject, enumeration was done in 60 intestinal villi and the corresponding crypts. The percentage of the cells giving positive reaction was computed.[16]

Detection of necrosis in situ: Apoptotic cells in the intestinal tissue were detected with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) method (In Situ Cell Death Detection Kit, POD, Roche 11684817910-50test). The samples were deparaffinized. Endogen peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol. TdT enzyme was applied at 37°C for 1 hour. Peroxidase activity was released with DAB. For contour staining, hematoxylin was applied. Cells giving positive reaction were counted in 400× enlargement. For each subject, enumeration was done in 60 intestinal villi and the corresponding crypts. The percentage of the cells giving positive reaction was computed.[16]

For all histomorphological and immunohistochemical assessments, the Olympus CX 41 light microscope was used.

Biochemical Analysis

Preparation of Colon Tissue Homogenates: For biochemical analysis, colon tissue samples were mixed with the cold working solution (50 mM phosphate buffer, pH 7.40), and homogenized with a mechanical homogenizer (Analytik Jena SpeedMill plus, Jena, Germany). The mixtures were then centrifuged at 10 000 × *g* for 15 minutes

at 4°C and the supernatants were preserved for biochemical analysis by storing on ice.

Measurement of Tissue Total antioxidant status (TAS), total oxidant status (TOS), and total thiol (TT) levels: TAS, TOS, and TT levels were measured on a Beckman Coulter AU680 analyzer (Beckman Coulter, Miami, FL, USA) using commercial reagents (Rel Assay Diagnostic, Gaziantep, Turkey) based on novel automated measurement methods developed by Erel.[17-19] The TAS levels were expressed as mmol Trolox Eq/mg protein. The TOS levels were expressed as μmol H_2O_2 Eq/mg protein. The TT levels were expressed as μmol/mg protein.

Calculation of Oxidative Stress Index (OSI): The percent ratio of TOS to TAS was accepted as the OSI, an indicator of the degree of oxidative stress. To perform the calculation, the unit of TAS, mmol Trolox Eq/mg protein, was converted to micromole Trolox equivalent/mg protein, and OSI was calculated as follows: OSI = [(TOS, μmol H_2O_2 Eq/mg protein)/(TAS, μmol Trolox Eq/mg protein) × 100].[20]

Measurement of Tissue SOD and GSH-PX Activities: Tissue SOD activities were measured on a Beckman Coulter AU680 analyzer (Beckman Coulter, Miami, FL, USA) using a Ransod kit (Randox Laboratories Ltd., Crumlin, UK). SOD activities were expressed as U/mg protein. Tissue glutathione peroxidase (GSH-PX) activities were measured on a Beckman Coulter AU680 analyzer (Beckman Coulter, Miami, FL, USA) using a Ransel kit (Randox Laboratories Ltd., Crumlin, UK). GSH-PX activities were expressed as U/mg protein.

Measurement of Tissue MDA Levels: Tissue MDA levels were measured based on the thiobarbituric acid reactive substances method, using commercial enzyme-linked immunosorbent assay kits (Cayman Inc, Ann Arbor, MI, USA) on a microplate reader (BMG Labtech Spectrostar Nano, GmbH, Ortenberg, Germany).[21] MDA levels were expressed as μM/mg protein.

Measurement of Tissue Protein levels: Tissue protein levels were measured based on the Bradford method on a Beckman Coulter AU680 analyzer (Beckman Coulter, Miami, FL, USA).[22]

Statistical Analysis

Statistical Analysis of Histomorphological Data: The data were analyzed using IBM SPSS (Statistical Package for Social Sciences) Statistics 20 statistical program, with descriptive statistics including the test of normality, homogeneity of variance test, one-way analysis of variance and post hoc (multiple comparison) tests, Tukey HSD, Tamhane`s test, and the Kruskal–Wallis test. A *P* value less than .05 was considered statistically significant.

Statistical Analysis of Biochemical Data: Statistical analyses were performed using GraphPad Prism, version 6.05 (GraphPad Software, Inc., CA, USA). All data were expressed as means ± standard error of the mean (SEM). Because of the small experimental groups, we used nonparametric statistical tests. The differences among the multiple groups were analyzed using the Kruskal–Wallis analysis of variance on ranks. Comparisons between 2 groups were analyzed using the Mann–Whitney *U*-test. A *P* value less than .05 was considered statistically significant.

RESULTS

Assessment of Histomorphological and Immunohistochemical Data

It can be said that there is a significant difference between the control, sham, I, I/R, and I/R+verbascoside groups in the histomorphological assessment with the hematoxylin and eosin-stained sections.

As for Alcian blue, there is a significant difference between the mean values of control–I/R, control–I, sham–I, sham–I/R, I–I/R+verbascoside, and I/R–I/R+verbascoside binaries. There is no significant difference between the mean values of control–sham, control–I/R+verbascoside, sham–I/R+verbascoside, and the I–I/R binaries.

As for PCNA, there is a significant difference between the mean values of control–I/R, control–I, control–I/R+verbascoside, sham–I/R, I–I/R, and I/R–I/R+verbascoside binaries. There is no significant difference between the mean values of control–sham, sham–I, sham–I/R+verbascoside, and I–I/R+verbascoside binaries.

As for TUNEL, there is a significant difference between the mean values of control–IR, control–I, control–I/R+verbascoside, sham–I, sham–I/R, I/R–I/R+verbascoside, sham–I/R+verbascoside, and I–I/R+verbascoside binaries. There is no significant difference between the mean values of control–sham and I–I/R binaries.

As for HIF-1α, there is a significant difference between the mean values of control–I/R, control–I, sham–I, sham–I/R,

I/R–I/R+verbascoside, and I–I/R+verbascoside binaries. There is no significant difference between the mean values of control–sham, sham–I/R+verbascoside, I–I/R, and control–I/R+verbascoside binaries.

In the I/R+verbascoside group, compared with the I/R group, histopathological signs such as edema, necrosis, and inflammatory infiltration were improved in the hematoxylin–eosin-stained sections, and mucin loss improved in the histochemical examination with Alcian blue.

In the I/R+verbascoside group, compared with the I/R group, PCNA and cell proliferation increased, TUNEL and apoptosis decreased. In the IR+verbascoside group, compared with the I/R group, HIF-1α expression, an indicator of oxidative stress, decreased (Figure 1-5).

Assessment of Biochemical Data

According to the table and graphs above, it is seen that compared with the I/R group, in the I/R+verbascoside group, as indicators of antioxidant activity, SOD, GSHPX, TAS, and TT levels increased, while as indicators of oxidative stress, TOS, OSI, and MDA levels decreased (Figure 6).

DISCUSSION

Ischemic injury in the colon is the most common injury type in the gastrointestinal system. Mild disease can be corrected with conservative medical treatment. However, at more severe levels, surgical intervention might be required, and it might result in mortality.[4] In the literature, there are numerous studies on I/R injury in different organs and the treatment of I/R injury.[6-8,23] However, we found few studies on I/R injury in the colon and no studies on the effect of verbascoside on I/R injury in the colon.[1-3,5]

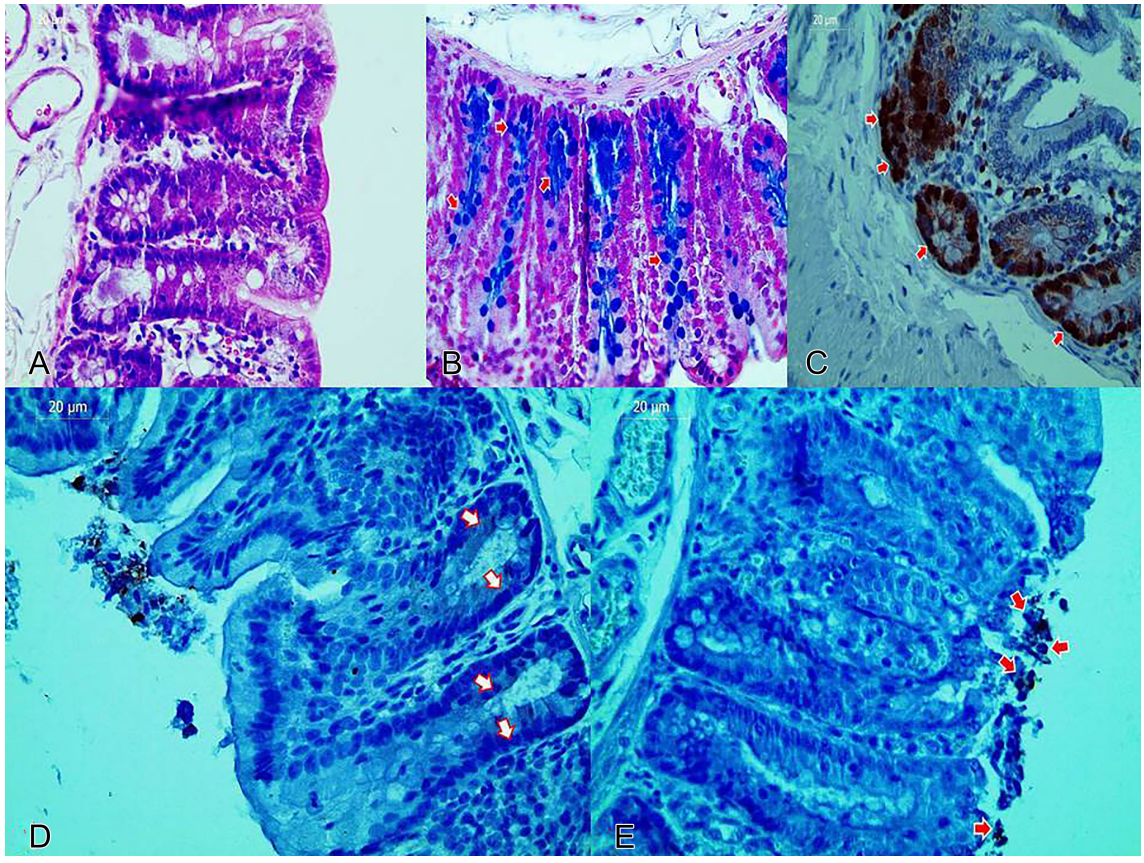

Figure 1. Representative photomicrographs exhibiting histomorphological, histochemical, and immunohistochemical appearance of the normal colon mucosa in control groups. (A) Normal tissue architecture of colon mucosa. H&E ×40. (B) Histochemical detection of goblet cells by the Alcian blue method in normal colon mucosa (positive cytoplasms are noted with arrows). Alcian blue ×40. (C) PCNA expression in normal colon epithelial cells (positive staining is noted with arrows). PCNA ×40. (D) HIF-1α expression in normal colon epithelial cells (positive staining is noted with arrows). HIF-1α ×40. (E) TUNEL expression in normal colon epithelial cells (positive staining is noted with arrows). TUNEL ×40.

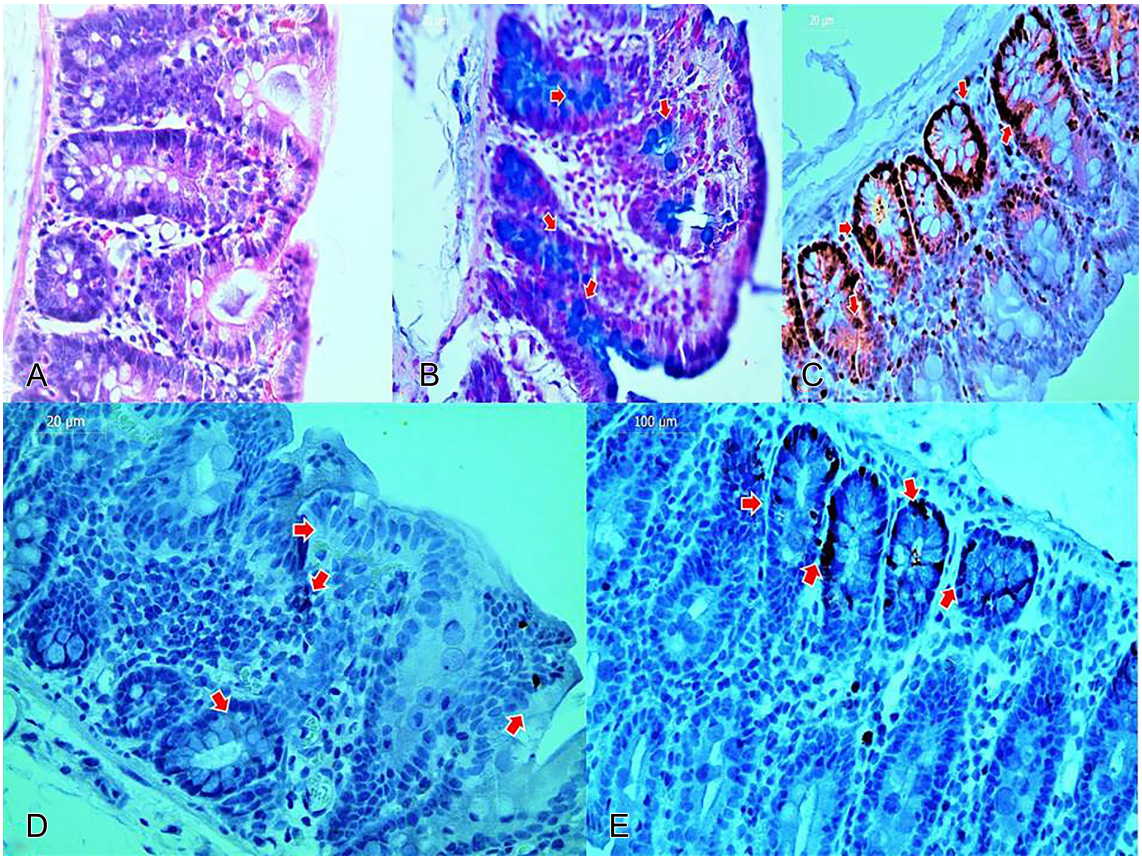

Figure 2. Representative photomicrographs exhibiting histomorphological, histochemical, and immunohistochemical appearance of the colon mucosa in the sham group. (A) Normal tissue architecture of colon mucosa. H&E ×40 (B) Histochemical detection of goblet cells by the Alcian blue method in colon mucosa (positive cytoplasms are noted with arrows). Alcian blue ×40. (C) PCNA expression in colon epithelial cells (positive staining is noted with arrows). PCNA ×40. (D) HIF-1α expression in colon epithelial cells (positive staining is noted with arrows). HIF-1α ×40. (E) TUNEL expression in colon epithelial cells (positive staining is noted with arrows). TUNEL ×40.

In this study, we aimed to see the corrective effect of verbascoside on rats in which colon I/R injury was induced. To see I/R injury and the corrective effect of verbascoside on I/R injury, we used histomorphological, immunohistochemical, and biochemical parameters.

I/R injury is a fundamental form of tissue injury; disruption of blood flow results in ischemic injury in all body systems. Restoration of blood flow is essential to prevent irreversible cellular injury in the ischemic organ, but reperfusion increases injury in ischemic tissue by releasing toxic mediators.[1-3] In tissues, I/R injury occurs via oxidative stress and free oxygen radicals. Necrosis, neutrophil accumulation, and apoptotic necrosis are seen in ischemic tissues due to the detrimental effect of cytokines. Cellular regeneration and proliferation are slowed down by I/R injury.[2,5,24] In the literature, studies assess basic histomorphological differences in hematoxylin–eosin-stained sections. Shailesh Solanki et al. assessed inflammatory differences and collagen content in the colonic anastomosis site and showed that aminoguanidine decreased ischemia-based histomorphological differences.[2]

Oğuzhan Karatepe et al. showed the corrective effect of adrenomedullin on ischemic injury using histomorphological parameters such as granulocyte cell infiltration in ischemic left colon anastomosis, fibroblast cell infiltration, necrosis, and the formation of new capillaries.[24] We showed the healing effect of verbascoside in the colon with I/R injury by using histomorphological parameters such as edema in the epithelium, and necrosis, submucosal neutrophil infiltration, and hemorrhage.

It was observed that intraperitoneally-applied verbascoside corrected the above mentioned histomorphological differences. Compared with the I/R group, significant

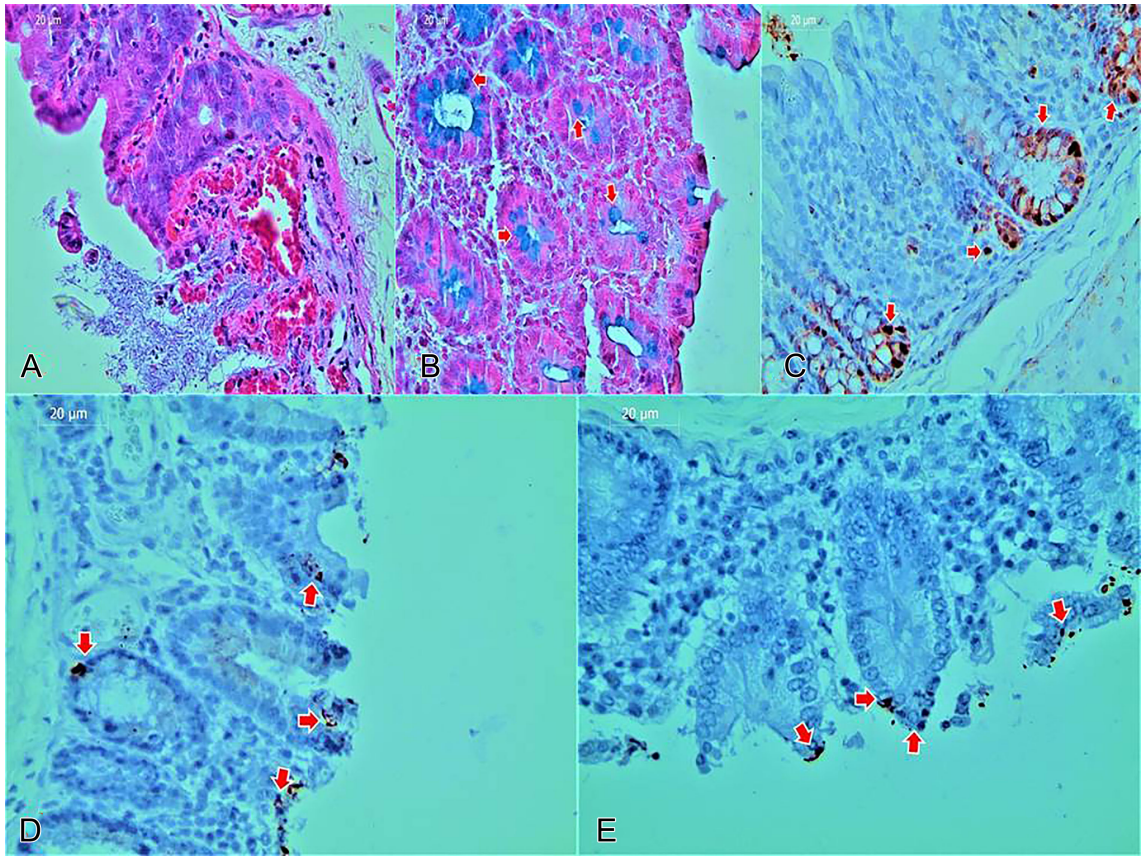

Figure 3. Representative photomicrographs exhibiting histopathological, histochemical, and immunohistochemical changes of the ischemic colon mucosa in the ischemia groups. (A) Massive destruction, tissue necrosis and submucosal neutrophil infiltration in colon mucosa. H&E ×40. (B) Massive mucin loss in ischemic colon mucosa is seen with Alcian blue method (positive cytoplasms are noted with arrows). Alcian blue ×40. (C) Decreased PCNA expression in ischemic colon epithelial cells (positive staining is noted with arrows). PCNA ×40. (D) Increased HIF-1α expression in ischemic colon epithelial cells (positive staining is noted with arrows). HIF-1α ×40. (E) Increased TUNEL expression in ischemic colon epithelial cells (positive staining is noted with arrows). TUNEL ×40.

improvement was determined in the histomorphological parameters of the I/R+verbascoside group ($P < .05$).

The Alcian blue histochemical method can be used to assess the goblet cell population that produces mucus in the intestinal epithelium displaying I/R injury.[5,25] Milan Marette et al. showed that goblet cell number decreased in the jejunal epithelium in rats after I/R injury. In the same study, it was also shown that alanyl–glutamine application has a protective effect in I/R injury. It was seen that in the group in which alanyl–glutamine was applied, goblet cell number in the jejunal epithelium increased.[5]

We determined goblet cell loss in the colon epithelium due to I/R injury. Compared with the control group, goblet cell number decreased in the I group and the I/R group ($P < .05$). We determined that verbascoside restored goblet cell loss in to I/R injury. Compared with the I/R group, the goblet cell number increased in the I/R+verbascoside group ($P < .05$).

PCNA is an immunohistochemical marker capable of cell proliferation and nuclear repair. PCNA is a significant cell cycle-regulated nuclear protein for DNA polymerase. PCNA-labeled nuclei identify cells in the late G1 and early S phase of the cell cycle, as well as cells undergoing DNA repair. PCNA protein was mainly located in the nuclei of proliferative cells in crypts.[16]

PCNA has been used in different studies to show loss of cell proliferation in I/R injury, and to assess the effect of pharmacological agents used against I/R injury.[5,16,26]

Yujiao Cai et al. made use of PCNA while assessing the corrective effect of keratinocyte growth factor in intestinal I/R injury.[27] Weichen et al. also made use of PCNA to

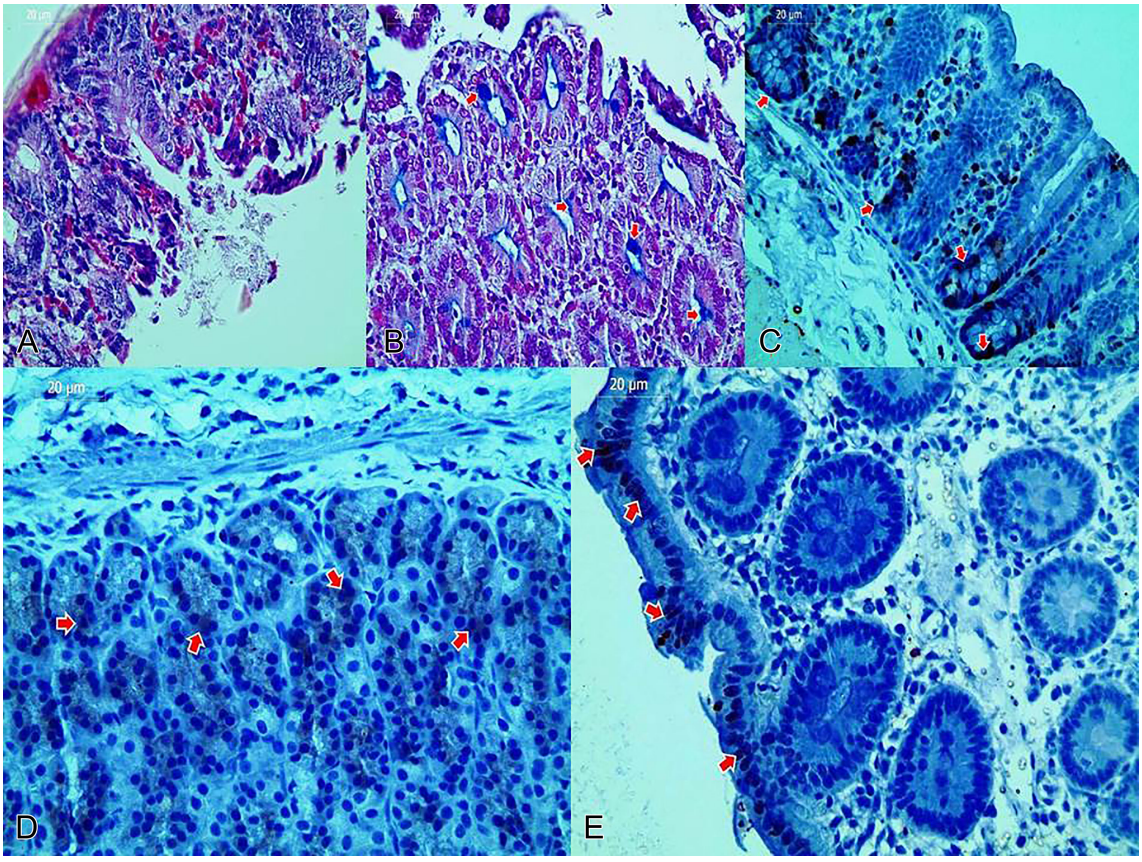

Figure 4. Representative photomicrographs exhibiting histopathological , histochemical, and immunohistochemical changes of the ischemic colon mucosa in I/R groups. (A) Massive destruction, tissue necrosis, and submucosal neutrophil infiltration in colon mucosa, H&E ×40. (B) Massive mucin loss in ischemic colon mucosa is seen with Alcian blue method (positive cytoplasms are noted with arrows), Alcian blue ×40. (C) Decreased PCNA expression in ischemic colon epithelial cells (positive staining is noted with arrows), PCNA ×40. (D) Increased HIF-1α expression in ischemic colon epithelial cells (positive staining is noted with arrows), HIF-1α ×40. (E) Increased TUNEL expression in ischemic colon epithelial cells (positive staining is noted with arrows), TUNEL ×40.

show the corrective effect of fibroblast growth factor on cell proliferation in intestinal I/R injury.[16]

Milan Maretta et al. used PCNA while assessing the protective effect of alanyl–glutamine in jejunal ischemia.[5] We saw that compared with the control group, the number of cells that give a positive reaction with PCNA in the colon epithelium decreased in the I and I/R groups. After verbascoside application, cell proliferation in the colon with I/R injury increased ($P < .05$).

TUNEL analysis is a method used for analyzing DNA fragmentation. DNA fragmentation occurs via apoptotic cascade activation.[25,26,27] In I/R injury, the number of TUNEL-positive cells increases. The increase in the number of TUNEL-positive cells is related to the activation of various paths for programmed cell death and injured epithelial cells.[25]

In the tissues with I/R injury, apoptotic cell death and the number of TUNEL-positive cells increase. Maria Merassantos et al., Yujiao Cai et al., and Stefan Toth Jr et al. used the TUNEL method to assess apoptotic cell death in I/R injury.[25,26,27] Using the TUNEL method, we saw that the number of apoptotic cells increased in rats in which colon I/R injury was induced, and we determined that verbascoside restored I/R injury and decreased the number of TUNEL-positive cells in the colon ($P < .05$).

The main factor causing lesions in ischemic tissues is hypoxia. Hypoxia initiates an intracellular signal path and causes HIF1 activation.

HIF1 is a critical regulator of the transcriptional response to low oxygen (O_2) conditions (hypoxia/anoxia) in mammalian cells. Heterodimeric protein is composed of a

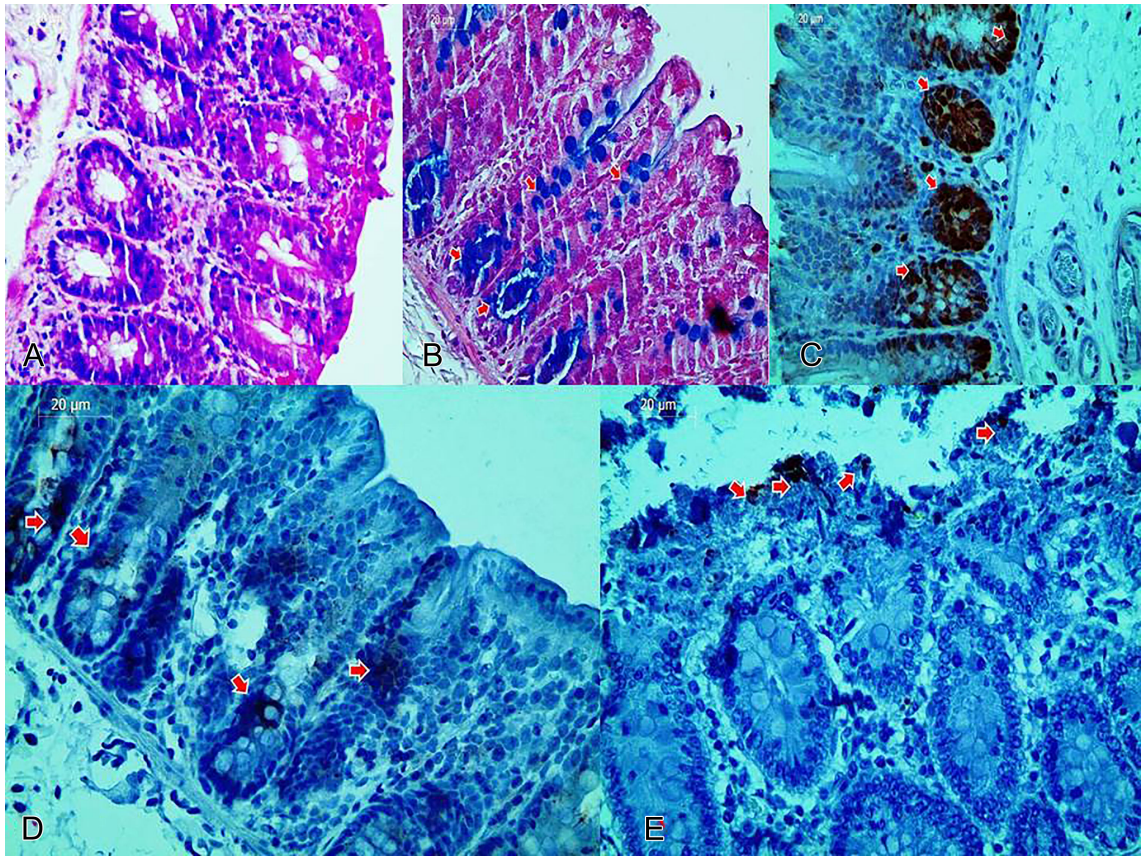

Figure 5. Representative photomicrographs exhibiting histomorphological, histochemical, and immunohistochemical appearance of the colon mucosa in the I/R+verbascoside groups. (A) Improved histopathological changes in colon mucosa (partial epithelial edema and minimal necrosis are seen), H&E ×40. (B) Improved mucin loss in colon mucosa is seen with Alcian blue method (positive cytoplasms are noted with arrows), Alcian blue ×40. (C) Improved PCNA expression in colon epithelial cells (positive staining is noted with arrows), PCNA ×40. (D) Improved HIF-1α expression loss in colon epithelial cells (positive staining is noted with arrows), HIF-1α ×40. (E) Improved TUNEL expression loss in ischemic colon epithelial cells (positive staining is noted with arrows), TUNEL ×40.

constitutively expressed HIF-1 β subunit and O_2-regulated HIF1α subunit.

HIF1α is a nuclear transcription factor and is critical for initiating a cellular response to hypoxia. Many studies have demonstrated that there is a very close relationship between the expression of the HIF1α and the formation of ROS in ischemia.[28]

We observed that compared with the control group, HIF1α expression in the colon epithelium in the I and I/R groups. We also determined that verbascoside decreased HIF1α expression in colon epithelium in rats in which colon I/R injury was induced ($P < .05$). I/R injury leads to ROS formation, followed by lipid peroxidation and injury in the membrane of cells and subcellular organelles.[6,28] During lipid peroxidation, MDA is produced. An increase in MDA indicates ischemic injury.

One of the most important antioxidant enzymes, SOD, converts superoxide radicals to hydrogen peroxide. Then, hydrogen peroxide is converted to water molecules by GSH-Px. In I/R injury, SOD and GSH-Px activities fall, depending on the depletion of these 2 enzymes.[6,7,23,24,29-33]

Besides MDA, GSH-Px, and SOD values, TAS, TOS, OS, and OSI were also used while assessing I/R injury in various studies in the literature.[16,28,30,31] We also used these parameters to assess in I/R injury in the colon and the effect of verbascoside in I/R injury.

Thiol groups are sulfur-containing compounds that are important members of the antioxidant cascade, because they destroy ROS and other free radicals by enzymatic or non-enzymatic mechanisms.[20] Total thiols are composed of both intracellular and extracellular thiols. Both

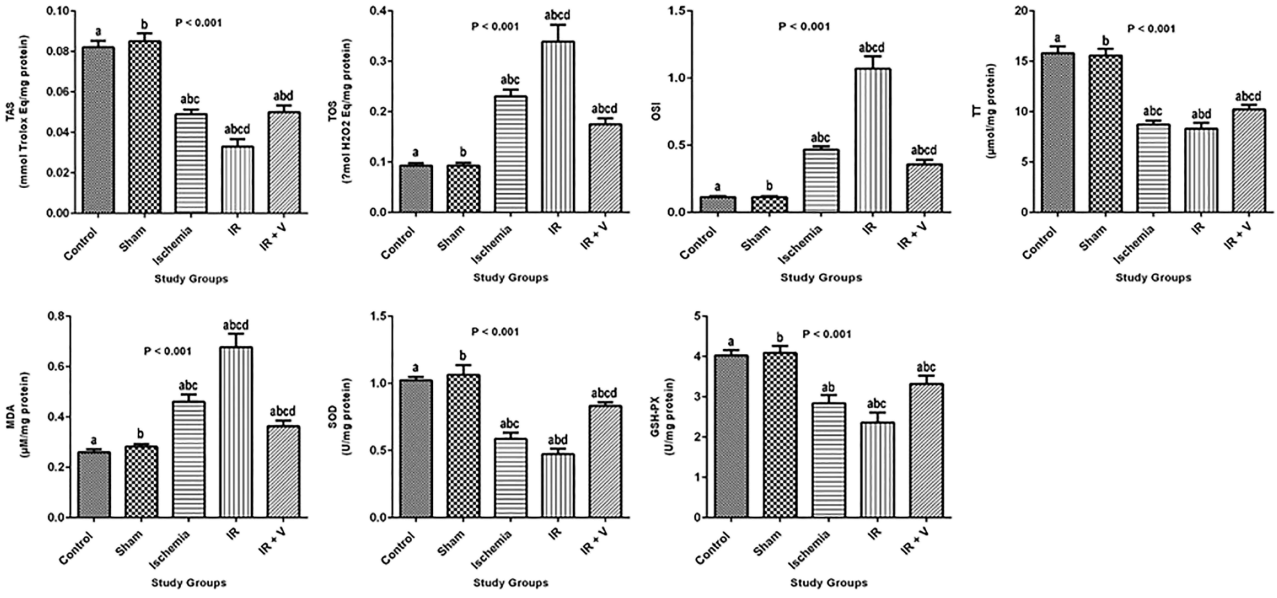

Figure 6. Tissue TAS, TOS, OSI, TT, MDA, SOD, and GSH-PX levels in assay groups. *P*: shows the differences between all groups (one-way ANOVA). [a, b, c, d, e]In each line, the differences between the mean values with same letters are significant, $P < .05$ (Tukey HSD post hoc test). a, control group versus I/R+V; b, sham group versus I/R+V; c, ischemia group versus I/R+V; d, I/R group versus I/R+V.

Table 1. Comparison of Tissue TAS, TOS, OSI, TT, and MDA levels, and Tissue SOD and GSH-PX Activities Between Assay Groups

Parameters (Mean ± SEM)	Control (*N* = 10)	Sham (*N* = 10)	Ischemia (*N* = 10)	I/R (*N* = 10)	I/R + V (*N* = 10)	*P*
TAS (mmol Trolox Eq/mg protein)	0.082 ± 0.003^{a}	0.085 ± 0.003^{b}	0.049 ± 0.002^{abc}	0.033 ± 0.004^{abcd}	0.050 ± 0.003^{abd}	<.001
TOS (µmol H_2O_2 Eq/mg protein)	0.093 ± 0.005^{a}	0.091 ± 0.006^{b}	0.23 ± 0.013^{abc}	0.34 ± 0.033^{abcd}	$0.18 \pm 0.0.12^{abcd}$	<.001
OSI	0.11 ± 0.007^{a}	0.12 ± 0.008^{b}	0.47 ± 0.024^{abc}	1.07 ± 0.093^{abcd}	0.36 ± 0.033^{abcd}	<.001
SOD (U/mg protein)	1.023 ± 0.026^{a}	1.064 ± 0.072^{b}	0.59 ± 0.05^{abc}	0.47 ± 0.04^{abd}	0.83 ± 0.03^{abcd}	<.001
GSH-PX (U/mg protein)	4.02 ± 0.14^{a}	4.09 ± 0.17^{b}	2.84 ± 0.20^{ab}	2.36 ± 0.25^{abc}	3.32 ± 0.21^{abc}	<.001
TT (µmol/mg protein)	15.8 ± 0.7^{a}	15.6 ± 0.6^{b}	8.7 ± 0.4^{abc}	8.3 ± 0.6^{abd}	10.2 ± 0.4^{abcd}	<.001
MDA (µM/mg protein)	0.26 ± 0.01^{a}	0.28 ± 0.01^{b}	0.46 ± 0.03^{abc}	0.68 ± 0.05^{abcd}	0.36 ± 0.02^{abcd}	<.001

P shows the differences between all groups (one-way ANOVA).
[a, b, c, d, e]In each line, the differences between the means with same letters are significant, $P < .05$ (Tukey's HSD post hoc test).
a, control group versus I/R+V; b, sham group versus I/R+V; c, ischemia group versus I/R+V; d, I/R group versus IR+V.
I/R, ischemia/reperfusion; SEM, standard error of mean; TAS, total antioxidant status; TOS, total oxidant status; OSI, oxidative stress index; SOD, superoxide dismutase; GSH-PX, glutathione peroxidase; TT, total thiol; MDA, malondialdehyde, V, verbascoside.

intracellular and extracellular redox states of thiols play critical roles in maintaining protein structure and function, regulating enzymatic activity, and providing antioxidant protection.[34] Total thiol groups are very sensitive to oxidation. When the cells are exposed to oxidative stress, thiol groups are the first affected antioxidants. Total thiol groups of proteins are responsible for their antioxidant response, and they can serve as a sensitive indicator of oxidative stress.[35] Oxidative protein damage is characterized by decreased protein thiol levels; thus TT levels may reflect the extent of ROS-mediated protein oxidation.[36]

We observed that compared with the I/R group, in the I/R+verbascoside group, SOD, GSH-Px, TAS, and TT levels increased, while TOS, OSI, and MDA levels decreased. Verbascoside led to restoration in the biochemical parameters of I/R injury in the colon.

CONCLUSION

This study showed that as a pharmacological agent with multiple effects, verbascoside decreased I/R injury in the colon epithelium and restored histomorphological differences. It also decreased goblet cell loss. Verbascoside led to restoration in the biochemical parameters indicating I/R injury.

Ethics Committee Approval: Ethics committee approval was received for this study from the Local Ethics Committee for Experiments on Animals of Dumlupinar University (Number: 2014.08.09).

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