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Investigation of the Curative Effect of Urapidil on Intestinal Inflammation and Oxidative Damage: An Experimental Study

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Abstract

The antioxidant effects of urapidil were investigated histopathologically and biochemically on the intestinal tissue that underwent ischemia-reperfusion injury. 40 Wistar albino female rats were divided into sham operation (Group 1), ischemia-reperfusion (Group 2), ischemia-reperfusion plus dimethyl sulfoxide (Group 3), ischemia-reperfusion plus urapidil 0,5 mg/kg (Group 4), and ischemia-reperfusion plus urapidil 5 mg/kg (Group 5). All rats except group 1 had 1h ischemia followed by 2h reperfusion to investigate the effects of urapidil through TAS, TOS, MDA, MPO, SOD, NF-κB, caspase-3, and LC3B. In terms of immunohistochemical staining with caspase 3, NF-κB and LC3B there was a statistical difference in group 1 compared with other groups ($p < 0.05$). A statistically significant difference of MDA levels was only found between the treatment groups (groups 4 & 5) and Group 3. We suggest that urapidil decreases the oxidative damage in ischemia-reperfusion injury on the intestinal tissue of rats as a probable antioxidant.

Research Article

INTRODUCTION

Blood supply to the tissues should be maintained regularly in order to prevent tissue injuries. The interruption of blood, which is called ischemia, causes a lack of oxygen which leads to cell and tissue damage and even reperfusion. The conditions that damage the tissues and cells are called ischemia-reperfusion injury (IRI). The decrease of blood supply through the superior mesenteric artery is said to be the common reason for intestinal ischemia in humans according to USA reports [1].

The occurrence of IRI is common and reactive oxygen species (ROS), Ca^{2+} overload inside cells, mitochondrial injury, activated neutrophils, and some cytokines have roles in IRI pathogenesis [2-4]. During reperfusion, mitochondria and activated neutrophils produce ROS [5] that induce inflammation through TNF-α, IL-1β, and IL-6. The production of ROS during reperfusion harms DNA and mitochondrial membrane which leads to cell damage, apoptosis, and inflammatory processes contributing to the reperfusion injury [4,5]. Apoptosis induced by ROS is a way of elimination damaged cells and apoptosis is detected in histopathological specimens by using caspase-3 [6]. Some of the other known factors that increase ROS are infections, various toxins, and other pathological conditions [7].

Harmful ROS are scavenged by antioxidant enzymes endogenously in cytoplasm such as superoxide dismutase (SOD) [8]. The SOD and malondialdehyde (MDA) are measured to predict the activity of free radicals as the free radicals have short half-lives [9]. Other ways to detect the oxidative status biochemically are total oxidant status (TOS), total antioxidant status (TAS) and oxidative stress index (OSI) [10].

Urapidil is among cardiologic antihypertensive drugs with its sympatholytic effects [11]. Nevertheless, in this study, the histopathological and biochemical consequences of urapidil on intestinal IRI model was investigated.

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MATERIALS and METHODS

Animals and experimental protocols

40 adult Wistar albino female rats (12-16 weeks old / weighed 200 to 250 g) taken from Atatürk University Animal Laboratory to be used in our experiment whose protocols were in accordance with the international guidelines. Local Ethics Council of Animal Experiments of Atatürk University (No: 22, Date: 25.01.2018) approved our experiment.

Group 1 (sham control, n=8)

The animals of group 1 had 1-2 cm abdominal incisions which were then closed after the peritoneum was reached.

Group 2 (IRI, n=8)

The animals of group 2 had 1-2 cm abdominal incisions to reach peritoneum to clamp the superior mesenteric artery to make 1-hour ischemia. After 2 hours of reperfusion, animals were sacrificed.

Group 3 (IRI + 15% DMSO, n=8)

The animals of group 3 had 1-2 cm abdominal incisions to reach peritoneum to clamp the superior mesenteric artery to make 1-hour ischemia. Then 15% DMSO was administered intraperitoneally for 30 minutes. After intraperitoneal DMSO application, reperfusion was made for 2 hours. Then animals were sacrificed.

Group 4 (IRI + 0,5 mg/kg urapidil, n=8)

The animals of group 4 had 1-2 cm abdominal incisions to reach peritoneum to clamp the superior mesenteric artery to make 1-hour ischemia. Then 0,5 mg/kg urapidil was administered intraperitoneally for 30 minutes. After 0,5 mg/kg urapidil application, reperfusion was made for 2 hours. Then animals were sacrificed.

Group 5 (IRI + 5 mg/kg urapidil, n=8)

The animals of group 5 had 1-2 cm abdominal incisions to reach peritoneum to clamp the superior mesenteric artery to make 1-hour ischemia. Then 5 mg/kg urapidil was administered intraperitoneally for 30 minutes. After 5 mg/kg urapidil application, reperfusion was made for 2 hours. Then animals were sacrificed.

Biochemical assay

The tissues kept in deep freeze were taken and weighed just before making a 10% homogenate with phosphate buffer through its vehicle (IKA, Germany) at 12,000 rpm for 1–2 min on ice. The prepared homogenates had centrifugation operation lasting thirty minutes (5000 rpm, at +4°) in order to get supernatant from each subject for the measurements of biochemical parameters mentioned. The measurement of MDA was based on the description of Ohkawa et al [12]. The commercial kit was used to detect TAS (Rel Assay Diagnostics, Ref. No. RL0024) and TOS (Rel Assay Diagnostics, Ref.No.: RL0005). The OSI was calculated as follows: OSI = ([TOS, mmol H_2O_2 equivalent/L] / [TAS, mmol Trolox equivalent/L] ×10). MPO and SOD enzyme were analyzed as described in our previous article [13].

Histopathological examination

The intestines which were preserved in ten per cent of neutral formalin for 24 -48 hours to get paraffin-embedded blocks. 5-µm sections obtained from those blocks were stained with hematoxylin-eosin to detect the histopathological findings under a microscope with grading scores in number from 0 to 3 meaning none, mild, moderate, and severe respectively.

Immunohistochemical examination

The slides were immersed in antigen retrieval solution (pH 6.0) following paraffin was uncovered. Then in order to unmask the antigens, the slides were put in microwave for 15 minutes. After heating the slides were dipped in 3% H_2O_2 for 10 min to have the endogenous peroxidase blocked. Then the sections were kept at room temperature with cleaved caspase 3 antibodies (cat no. NB600-1235, dilution 1/200; Novus Biological, USA), NFkB (Cat no. ab7971, dilution 1/200; Abcam) and LC3B (Cat. no.sc-376404, dilution 1/200; Santa Cruz). All histopathological analyses were completed according to the kit procedure. Immunoreactivity in the sections was graded in number from 0 to 3 meaning none, mild, moderate, and severe respectively.

Statistical analysis

Tissue biochemical molecule concentration was indicated in the table as mean value and (±) standard deviation (S.D.). Data were analyzed with the SPSS package program ($p < 0.05$). One-Way ANOVA test, Kruskal Wallis test and Mann Whitney U test were used for analyzing.

RESULTS

Biochemical results

Table 1 shows the biochemical concentrations of the intestinal tissue among the different treatment groups. TAS and SOD are antioxidant indicators and the others are oxidant indicators. According to the results of this study, the TOS and MPO levels were found to be significantly higher in ischemia-reperfusion groups (2 and 3) than those in the group-1, whereas the TAS levels were significantly lower in groups 2 and 3 compared to the control group. The concentration of SOD reduced in the ischemia-reperfusion groups compared to the group-1 but no difference was found statistically. Nevertheless, the MDA levels were found to be higher in ischemia-reperfusion groups compared to group-1. The TAS levels were found to have increased statistically in the treatment groups (groups 4 and 5) when compared with the ischemic groups (groups 2 and 3). But the TOS and OSI levels were found to have decreased statistically in the treatment groups when compared with the ischemic groups. The MPO levels of the treatment groups were seen to have dropped statistically when compared to Group 2, however, a statistical difference was seen in Group 3 when compared to the high dose urapidil group (Group 5). The MDA levels were found to have increased in the ischemic groups (groups 2 and 3) compared to the other groups (groups 1, 4 and 5). But a statistically significant difference in MDA levels was only found between the treatment groups (groups 4 and 5) and Group 3.

IRI= Ischemia reperfusion injury; DMSO=dimethyl sulfoxide; TAS = Total Antioxidant Status; TOS = Total Oxidant Status; OSI = Oxidative Stress Index; SOD=Superoxide Dismutase; MPO=Myeloperoxidase; MDA=Malondialdehyde. Data are presented as mean ± S.D.

Table-1 Comparisons of biochemical parameters among the experimental groups.

Groups n=8	**TAS (mmol/L)**	**TOS (µmol/L)**	**OSI (arbitrary unit)**	**SOD (U/mg protein)**	**MPO (U/g protein)**	**MDA (µmol/g protein)**
Sham operation (1)	1.53 ± 0.20	4.30 ± 0.32	0.28 ± 0.034	307.02 ± 93.46	265176.69 ± 261062.19	66.45 ± 22.84
IRI (2)	0.65 ± 0.17[a]	6.71 ± 1.26[a]	1.08 ± 0.37[a]	233.09 ± 105.95	640806.43 ± 110211.66[a]	89.15 ± 35.69
IRI+DMSO (3)	0.69 ± 0.11[a]	6.62 ± 1.81[a]	0.94 ± 0.155[a]	229.15 ± 55.66	633553.60 ± 288355.77[a]	91.90 ± 14.42
IRI+urapidil 0.5mg/kg (4)	1.21 ± 0.24[abc]	4.75 ± 0.38[abc]	0.40 ± 0.07[abc]	296.49 ± 72.20	352085.19 ± 241463.19[b]	69.61 ± 15.82[c]
IRI+urapidil 5 mg/kg (5)	1.38 ± 0.27[bc]	4.49 ± 0.66[bc]	0.33 ± 0.07[abc]	300.81 ± 94.49	277142.97 ± 50083.39[bc]	68.03 ± 16.59[c]

[a] $p < 0.05$ compared to sham operation group.
[b] $p < 0.05$ compared to I/RI group.
[c] $p < 0.05$ compared to IRI+DMSO group.

Histopathological examination

A statistically significant difference was found when the sham group and experimental groups were compared ($p < 0.05$). Histopathologic lesions seen in groups 2 and 3 were observed to be more severe than those in groups 4 and 5. While severe haemorrhages were seen in the lamina propria of the intestinal tissue of rats in groups 2 and 3, it was observed that the severity of these haemorrhages decreased in groups 4 and 5, but severe hyperemia continued (Fig.1).

Immunohistochemical examination

A statistically significant difference was found in immunohistochemical staining with caspase 3, NF-κB and LC3B antibodies when the sham group was compared with the experimental groups ($p < 0.05$). The immunopositivity of Caspase 3 and NF-κB was found to be at severe levels in groups 2 and 3, but lower levels in groups 4 and 5. The immunopositivity of these antibodies were seen in the inflammatory cells in the lamina propria and in desquamated cells (Figures 2 and 3). The immunopositivity of LC3B was found to be at severe levels in groups 2 and 3 but slightly lower levels in groups 4 and 5. The immunopositivity of this antibody was seen in the inflammatory cells in the lamina propria and the crypt epithelium (Fig.4).

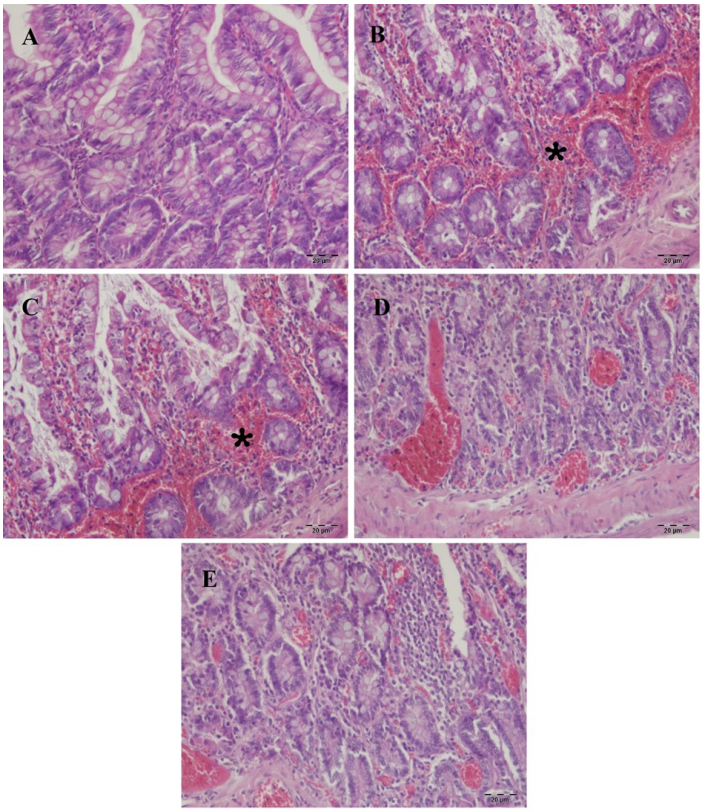

Fig.1. (A) Group 1, (B) Group 3, (C) Severe haemorrhage in lamina propria of groups 2&3 (*), (D) Mild haemorrhage in groups 4&5. H&E

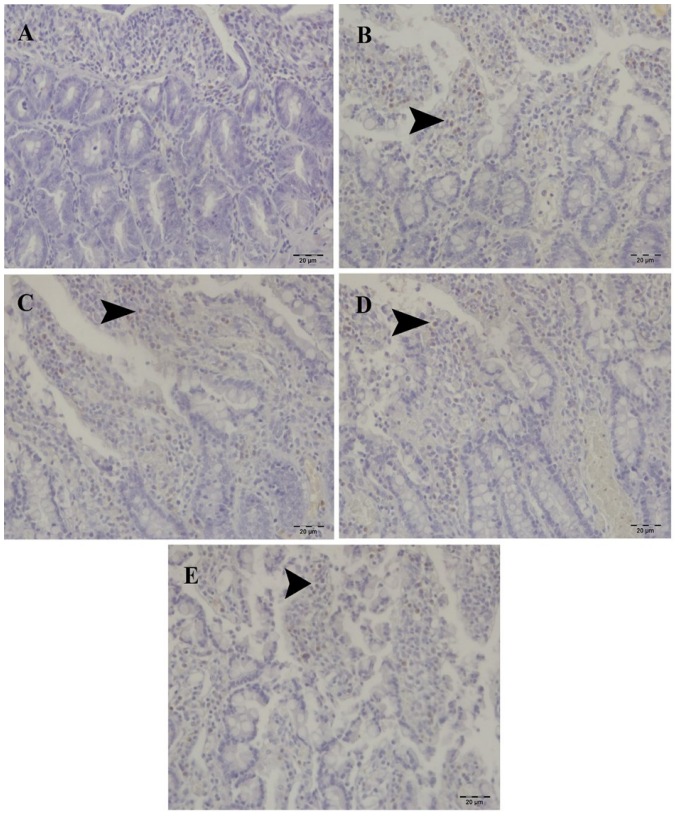

Fig.2. (A) Group 1, (B) Group 3, (C) Severe caspase 3 immunopositivity in lamina propria in groups 2&3, (D) Mild caspase-3 immunopositivity in groups 4&5, (arrowhead). IHC

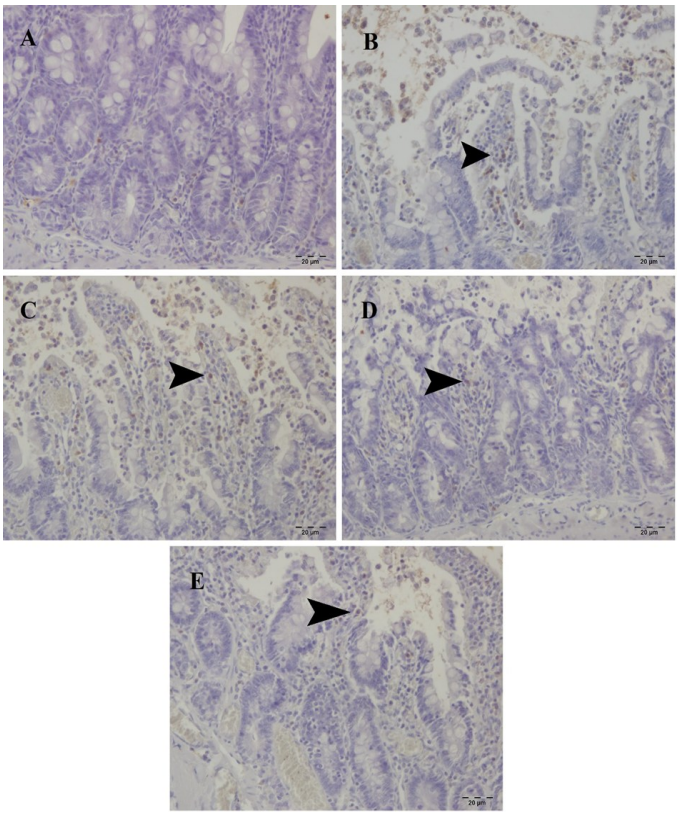

Fig.3. (A) Group 1, (B) Group 3, (C) Severe NF-κB immunopositivity in lamina propria in groups 2&3, (D) Mild NF-kB immunopositivity in groups 4&5 (arrowhead). IHC

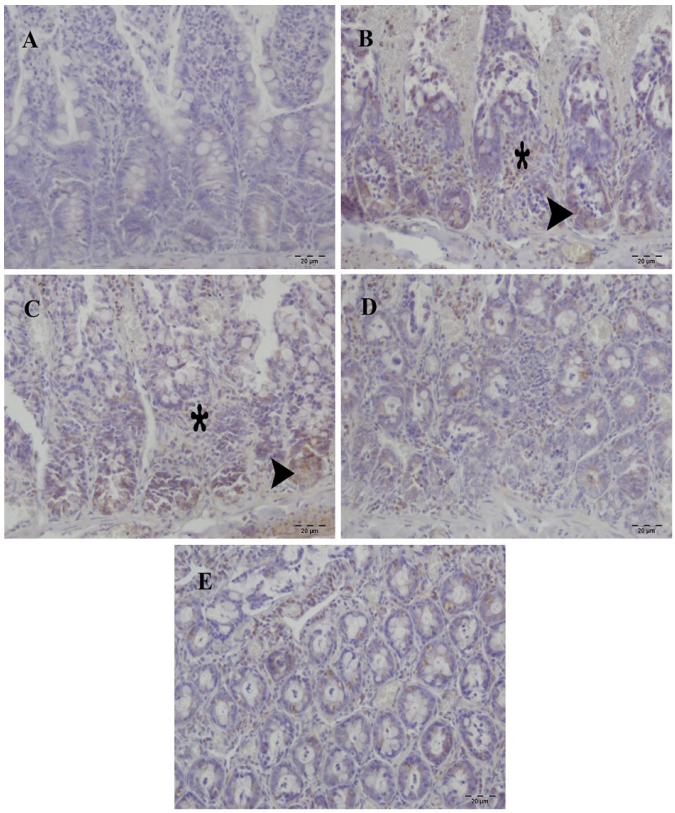

Fig.4. (A) Group 1, (B) Group 3, (C) Severe LC3B immunopositivity in lamina propria (*) and in the crypt epithelium (arrowhead) in groups 2&3, (D) Mild LC3B immunopositivity in groups 4&5. IHC

DISCUSSION

In this study, the antioxidant activity of urapidil was biochemically tested and histopathological examinations were carried out on the intestinal tissue of rats subjected to intestinal IRI model using parameters such as TAS, TOS, OSI, SOD, MPO, MDA.

Urapidil is widely used in antihypertensive therapies due to its sympatholytic and alpha-adrenergic antagonist effects that decrease the arterial resistance and increase cardiac output, but there are few studies in the literature that are similar to the present study, which was conducted on the antioxidant effects of urapidil [11].

Intestinal IRI has always been an important challenge for clinicians due to the fact that it is associated with abdominal surgery, infectious disorders and organ transplantation [12,14]. Haemorrhage in the mucosa is seen in the reversible phase of an acute intestinal ischemia [15, 16]. Parallel to the literature haemorrhage was seen in the lamina propria of the ischemic groups at severe levels (groups 2 and 3) in this study. However, according to the findings of this study in the groups treated with urapidil (groups 4 and 5) the level of haemorrhage was mild.

Pathological apoptosis, which is a way to eliminate damaged cells, is induced by many factors including IRI. Caspase 3 is a marker of apoptosis and is used to detect many apoptotic changes [17]. According to the histopathological findings of this study, the lamina propria of the intestinal tissue had severe levels of caspase 3 immunopositivity in the groups subjected to IRI (groups 2 and 3), which shows that intestinal IRI induces apoptosis. The groups treated with urapidil (groups 4 and 5) had caspase 3 immunopositivity at mild levels indicating that urapidil decreased the level of apoptosis. The decreased of caspase 3 indicates lowering of apoptosis according to literature [18].

ROS generation after reperfusion leads to the activation of NF-κB that regulates the expressions of TNF-α, IL-6, and IL-1β. This pro-inflammatory transcription factor helps to predict inflammatory status because in inflammatory situations NF-κB expression increases and plays an antiapoptotic role through complicated mechanisms [19]. In this study, the levels of NF-κB were similar to the levels of caspase 3. While the NF-κB immunopositivity levels in the lamina propria of groups 2 and 3 were found to be at severe levels, groups 4 and 5 had NF-κB immunopositivity at mild levels.

Autophagy, which eliminates the damaged structures by autophagosomes, can be detected by LC3B [20]. The fact that groups 2 and 3 had LC3B immunopositivity in the lamina propria and in the crypt epithelium, proved that the autophagy processes occurred after ischemia-reperfusion injury in this study. Furthermore, the mild level LC3B immunopositivity in groups 4 and 5 suggests that urapidil had protective effects in terms of autophagy on intestinal tissue after ischemia-reperfusion injury.

Among the parameters used to estimate oxidative stress, TAS is negatively correlated with ROS but positively correlated with TOS and OSI. Ischemia, drugs, ageing, infections, traumas and haemorrhage may alternate the levels of TAS, TOS and OSI [13]. The levels of TOS and OSI in the ischemic groups significantly increased compared to the sham group, however, the TAS levels decreased (Table 1). Also, the difference was found between the treatment groups (groups 4 and 5) and the ischemic groups (groups 2 and 3) according to the statistical results.

IL-6, IL-8, and TNF-α are the cytokines associated with intestinal IRI [21]. The resident neutrophil activation is said to be the most harmful factor on intestinal mucosal function during IRI [22] as the activated neutrophils increase ROS in the tissues [5]. Myeloperoxidase (MPO), which is a product of activated neutrophils, damages tissue by converting hydrogen peroxide (H_2O_2) into ROS during IRI [23]. In the findings of this study, MPO which is positively correlated with ROS generation significantly increased in the ischemic groups (groups 2 and 3) compared to the sham group (p=0,002). In various studies conducted on ischemia, sepsis also increased MPO levels [24]. Furthermore, the levels of MPO significantly decreased in the urapidil treated groups (groups 4 and 5) compared to the ischemic groups (groups 2 and 3). This suggests that urapidil treatment decreased the amount of ROS generation.

Malondialdehyde (MDA), a product of lipid peroxidation, is used as a biomarker of oxidation which helps to make predictions on oxidative status [25]. MDA is positively correlated with TOS and MPO. The results of this study are parallel with those in the literature in terms of the groups subjected to IRI (groups 2 and 3) having higher levels of MDA compared to the sham group (Table 1). However, the treatment groups had similar results with the controls. Also, a significant

decrease occurred in the urapidil treated groups compared to Group 3, which had ischemia without treatment in this study.

Superoxide dismutase (SOD) is one of the endogenous antioxidant enzymes in cells and converts superoxide radicals into H_2O_2 which is then converted into water by CAT. Because ROS is scavenged by SOD, which decreases during ischemia, SOD is used as a biochemical marker to predict the level of ischemic activity [8]. Parallel to the literature, the levels of SOD decreased in the ischemic groups, but the SOD levels of the treatment groups (groups 4 and 5) were found to be similar to the sham group. However, in our study, the difference was not found between the groups regarding SOD concentrations.

According to the results of this study, urapidil played a protective role in intestinal tissue after intestinal IRI. Similar results in the literature are available but there are few studies on the antioxidant effects of urapidil. For example, the study by Meštrović (2017) showed that urapidil prevented cellular damage in testicular torsion induced ischemia [26]. This study is the first in the literature to demonstrate that urapidil has antioxidant and antiapoptotic effects in intestinal IRI models.

Intestinal ischemia-reperfusion injury has fatal complications. Further studies need to be conducted in order to protect patients from their mortal outcomes. Using histopathological and biochemical parameters, novel drugs were tested through intestinal IRI models. While oxidant parameters such as TOS, MDA were expected to increase, the antioxidants like SOD and TAS were expected to decrease. In IRI apoptosis and autophagy occur. For the detection of apoptosis on histopathological specimens caspase 3 is used and to detect autophagy LC3B is used. In this study, urapidil was tested in order to protect the intestinal tissue from IRI. According to the results, it was found that urapidil reduced the oxidative effects of IRI on the intestinal tissue of rats. Additional studies are required to improve treatment patterns of urapidil in order to treat patients suffering from intestinal IRI.

Conflict of interest

The authors declare that there is no conflict of interest.

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