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The healing effects of *Ganoderma lucidum* on intestinal ischemia-reperfusion damage in rats

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Kırıkkale University Animal Experiments Local Ethics Committee, 01.03.2017, 17/02.
All procedures in this study involving human participants were performed in accordance with the 1964 Helsinki Declaration and its later amendments.

Conflict of Interest
No conflict of interest was declared by the authors.

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Abstract

Background/Aim: Intestinal ischemia-reperfusion (I/R) injury causes serious clinical problems and carries high morbidity and mortality risks. *Ganoderma lucidum* (GL) is known as an anti-inflammatory immunomodulator and antioxidant. This study aimed to investigate the curative effects of GL on intestinal I/R injury in rats.

Methods: Twenty-four Wistar-Albino male rats were randomly divided into three groups. After 30/90 minutes of I/R, intestinal and hepatic tissue examples were histologically examined. Biochemical analysis, serum, intestinal and hepatic tissue malondialdehyde (MDA), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) levels were measured.

Results: The I/R group had significantly elevated leukocyte, thrombocyte, serum, and intestinal and hepatic tissue MDA levels compared to the sham group ($P < 0.05$). Serum, intestinal tissue, and liver tissue SOD levels were significantly lower in the I/R group than in the control group ($P < 0.05$). In addition, GSH-Px levels measured in serum and liver tissue were lower in the I/R group. However, the use of GL prevented these decreases due to I/R damage. Prior administration of GL considerably alleviated histopathologic changes due to I/R injury in the intestinal and liver tissue samples.

Conclusions: Our experimental study showed that I/R injury led to significant oxidative stress by inducing free oxygen radicals in intestinal and hepatic tissues. Serum and tissue MDA, SOD, and GSH-Px levels were considerably useful laboratory parameters in identifying oxidative stress. The protective effects of GL on intestinal I/R injury were promising considering these parameters.

Keywords: Antioxidative, Anti-inflammatory, Immunomodulator, Intestinal ischemia-reperfusion, *Ganoderma lucidum*

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Introduction

The intestinal ischemia-reperfusion (I/R) injury may result from a variety of clinical conditions, such as intestinal obstruction, mesenteric arterial occlusion, hemodynamic shock, and surgical interventions. Factors such as free oxygen radicals, neutrophils activation, and xanthine oxidase enzyme systems seem to be related to tissue damage due to I/R injury [1]. Leukocyte-endothelial interactions lead to excessive overproduction of pro-inflammatory cytokines and free oxygen radicals. Intestinal tissue damage caused by I/R injury triggers local and systemic inflammatory responses. Multiorgan failure is one of the most catastrophic outcomes of intestinal tissue damage [2].

Ganoderma lucidum (GL) has been used for various purposes for more than 2,000 years [3]. It has been demonstrated to be effective in cerebral I/R injury [4], renal I/R injury [5], and cardiac I/R injury [6]. It has also been reported that there are effective immunomodulators, antioxidants, as well as more than 100 isolated molecules with chemopreventive and tumoricidal properties in GL [7-9]. Our experimental study aimed to evaluate the possible ameliorative effects of GL on intestinal I/R injury.

Materials and methods

After obtaining approval from the ethics committee (Kırıkkale University Animal Experiments Local Ethics Committee, 01.03.2017, 17/02), 24 healthy males (250-300 gr) Wistar-Albino rats were randomly divided into three groups: control, I/R injury, and I/R injury with GL treatment (I/R+GL). All rats were kept under a 12-hour cycle (12 hours of dark and light), fed ad libitum for two weeks, and deprived of food 12 hours before the experiment but allowed to drink water. Seven days before the I/R injury, GL treatment group rats were administered with 250 mg/kg body weight GL dissolved in 2 ccs of saline by orogastric lavage. Before the surgical procedure, all rats were anesthetized by intramuscular administration of 5-10 mg/kg xylazine hydrochloride (Rompun®, Bayer-Istanbul), and 50-70 mg/kg dose of ketamine hydrochloride (Ketalar®, Pfizer Istanbul). After shaving the abdomen of all rats, a midline incision was performed, and the abdomen was entered after local cleaning with a 10% povidone-iodine solution and sterile dressing.

The only laparotomy was performed to examine the superior mesenteric artery in the control group of rats. In the I/R+GL and I/R groups, the superior mesenteric artery was clamped for 30 minutes and released for 90 minutes to induce I/R injury. Tissue and blood samples were harvested at the end of 90 minutes, and all animals were decapitated.

Blood analysis: The blood samples taken from the rats were centrifuged at 3,000 xg for 10 minutes. The blood samples were sampled in the Eppendorf tubes and kept at -80 C for use on the next working day. On the next working day, the blood samples were brought to room temperature, and the frozen serums were allowed to melt. SOD, MDA, and GSH-Px levels in serum samples were measured by the ELISA method. Also, blood thrombocyte, leukocyte, and hemoglobin levels were studied.

Biochemical tissue analysis: Intestinal and liver tissue samples taken for the study were homogenized with PBS (Phosphate Buffer Saline, pH: 7.4) solution. The amount of total protein in all tissue samples was measured by the Bradford method with the aid of a spectrophotometer. The values of MDA, GSH-Px, and SOD parameters in tissue homogenates were measured by the ELISA method in a plate reader (Thermo Scientific Multiskan FC, 2011-06, USA).

Histopathologic examination of intestinal and liver tissue samples: Tissue samples taken from the intestine and liver were fixed in 10% formaldehyde. Samples were kept in fixation for 24 hours. After determination, for the evaluation of the ileum, a 1-cm longitudinal section was taken from each resection material and placed on blotter paper, and the tissue was followed. Approximately a 1-cm sample was taken from the liver tissues and dried. It was embedded in paraffin after routine tissue procedures. Sections of 5-micron thickness were taken from paraffin blocks. They were then stained with hematoxylin-eosin. All preparations were examined by the same pathologist under the same light microscope (Olympus BX53F-Japan). Intestinal tissue samples were evaluated histopathologically according to the classification mentioned in the study of Chiu et al. [10]. Histopathologic changes in the liver tissue, such as obstruction, leukocyte infiltration, sinusoidal dilatation, and vacuolar degeneration were evaluated. Histopathologic examination of the samples using the same light microscope was performed by the same pathologist unaware of the study groups.

Statistical analysis

All results were statistically analyzed using SPSS 18.0 windows package program. Numeric variables were expressed as mean (standard deviation). The differences among the groups in terms of parameters were analyzed using analysis of variance (ANOVA) with the Tukey post hoc test. In paired comparisons of the groups, the Mann Whitney U and Kruskal Wallis tests were used. $P < 0.05$ was accepted as significant.

Results

Hematologic parameters

The mean leukocyte levels were $6.21x10^3$ uL in the control group, $8.55x10^3$ uL in the I/R group, and $5.56x10^3$ uL in the I/R+GL group. All groups were compared in terms of leukocyte levels. The leukocyte level of the I/R group was significantly higher than that of the control group ($P = 0.001$). When the I/R+GL group and the control group were compared, the difference was found to be statistically insignificant. When the I/R group and I/R+GL were compared, the leukocyte level of the I/R group was found to be significantly higher than that of the I/R+GL group ($P = 0.001$). Platelet mean levels were $894.75x10^3$ uL in the control group, $1066.63x10^3$ uL in the I/R group, and $960.38x10^3$ uL in the I/R+GL group, with the I/R group having significantly high levels ($P = 0.024$). When the I/R group and the I/R+GL group were compared, platelet levels of the I/R+GL group were found to be significantly lower than those of the I/R group ($P = 0.044$) (Figure 1).

Figure 1: A-The leukocyte levels of the study groups. B- The hemoglobin levels of the study groups C- The platelet levels of the study groups. D- Serum MDA levels of the study groups E- Serum GSH-Px levels of the study groups. F- Serum SOD levels of the study groups

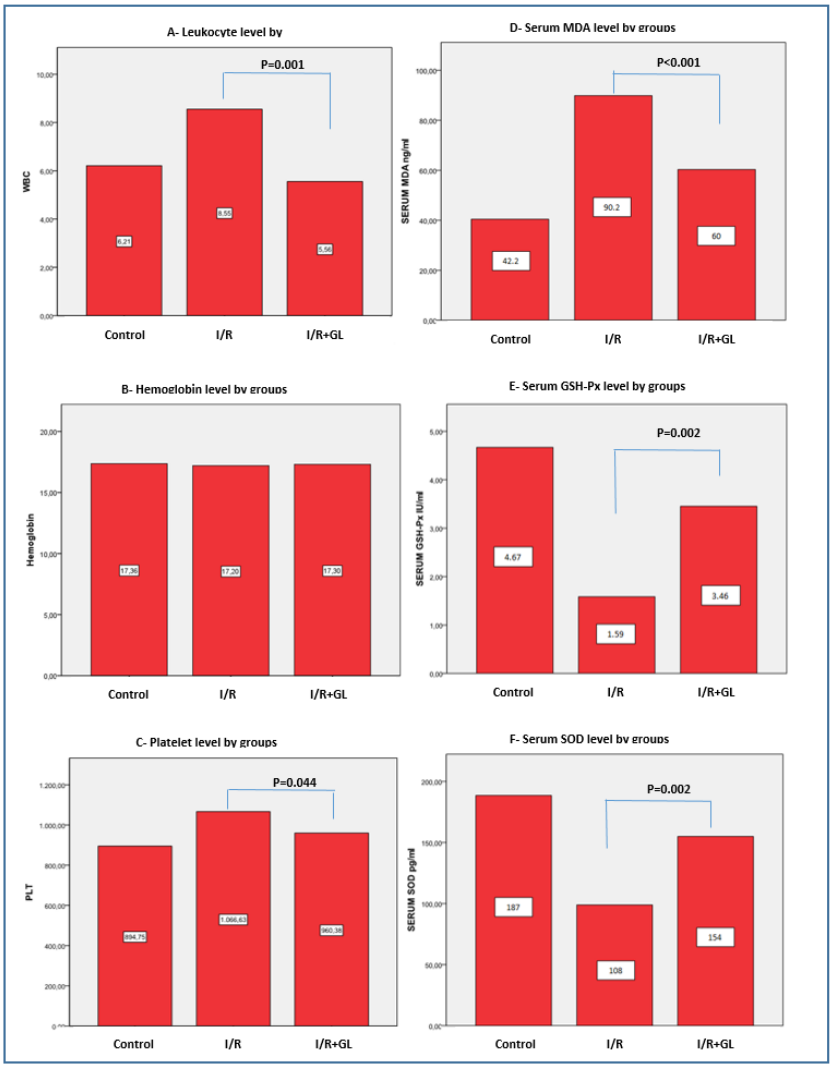
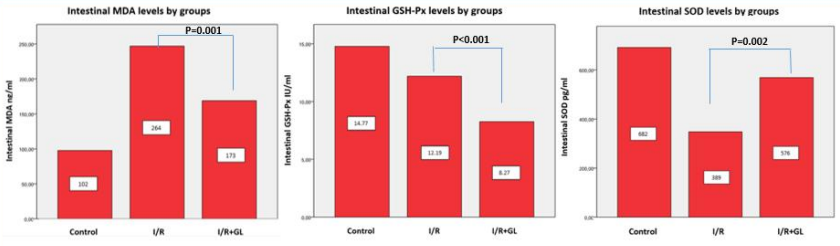
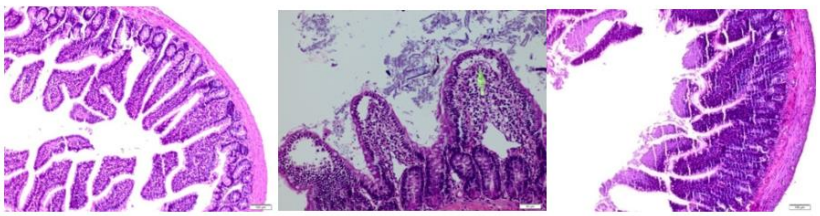


Figure 2: Intestinal tissue MDA, GSH-Px, and SOD levels of the study groups and histopathologic changes according to Chiu's classification of the study groups.



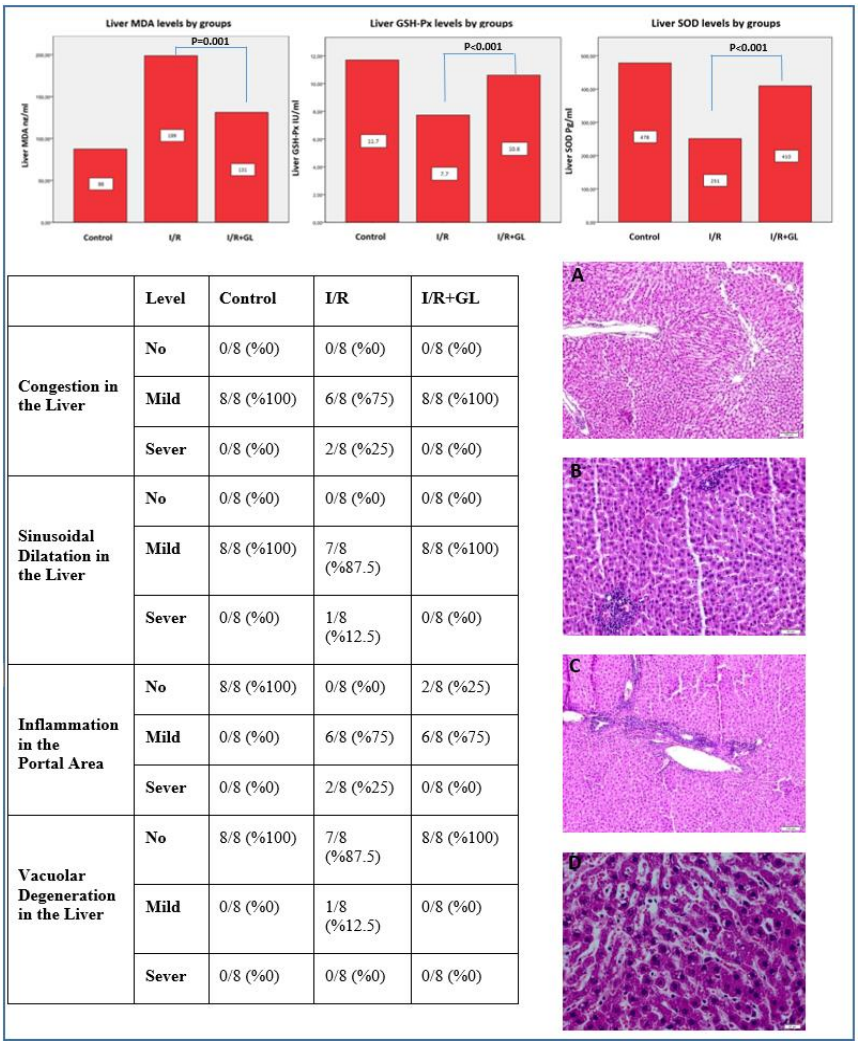
Groups	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5	Total
Countrol	8						8
I/R	0	6(%75)	2(%25)				8
I/R+GL	4(%50)	4(%50)					8

Chiu	Mucosal Changes
Grade 0	Normal mucosal villi.
Grade 1	Development of subepitelial Gruenhangen's space, usually at the apex of the villus; often with capillary congestion.
Grade 2	Extension of the subepithelial space with moderate lifting of epithelial layer from the lamina propria.
Grade 3	Massive epithelial lifting down the sides of villi. A few tips may be denuded.
Grade 4	Denuded villi with lamina propria and dilated capillaries exposed. Increased cellularity of lamina propria may be noted.
Grade 5	Digestion and disintegration of lamina propria; hemorrhage and ulceration.



Chiu Grade 0 Chiu Grade 1 Chiu Grade 2

Figure 3: The MDA, GSH-Px, and SOD levels in the liver tissue samples of the study groups, and histopathologic changes in the liver tissue samples of the study groups. A-İntense sinusoidal dilatation and mild congestion with no portal inflammation (Hematoxylin & eosin staining, x100, original magnification), B-Mild sinusoidal dilatation, congestion, and portal inflammation (Hematoxylin & eosin staining, x200, original magnification), C-Intense portal inflammation, (Hematoxylin & eosin staining, x100, original magnification), D-Vacuolar changes (Hematoxylin & eosin staining, x400, original magnification)



	Level	Control	I/R	I/R+GL
Congestion in the Liver	No	0/8 (%0)	0/8 (%0)	0/8 (%0)
	Mild	8/8 (%100)	6/8 (%75)	8/8 (%100)
	Sever	0/8 (%0)	2/8 (%25)	0/8 (%0)
Sinusoidal Dilatation in the Liver	No	0/8 (%0)	0/8 (%0)	0/8 (%0)
	Mild	8/8 (%100)	7/8 (%87.5)	8/8 (%100)
	Sever	0/8 (%0)	1/8 (%12.5)	0/8 (%0)
Inflammation in the Portal Area	No	8/8 (%100)	0/8 (%0)	2/8 (%25)
	Mild	0/8 (%0)	6/8 (%75)	6/8 (%75)
	Sever	0/8 (%0)	2/8 (%25)	0/8 (%0)
Vacuolar Degeneration in the Liver	No	8/8 (%100)	7/8 (%87.5)	8/8 (%100)
	Mild	0/8 (%0)	1/8 (%12.5)	0/8 (%0)
	Sever	0/8 (%0)	0/8 (%0)	0/8 (%0)

Oxidative stress-related parameters: Serum and tissue MDA levels

The mean serum MDA level was 42.2 ng/ml in the control group, 90.2 ng/ml in the I/R group, and 60 ng/ml in the I/R+GL group (Table 1). When the control group and the I/R group were compared, the difference was found to be significant ($P < 0.05$). When the control group and the I/R+GL group were compared, the difference was also found to be significant ($P < 0.05$). Serum MDA levels were found to be significantly lower in the GL treatment group ($P < 0.05$). Mean intestinal tissue MDA levels were measured as 101.87 ng/ml in the control group, 263.6 ng/ml in the I/R group, and 173.3 ng/ml in the I/R+GL group (Table I). Intestinal MDA levels were found to be significantly ($P < 0.05$) higher in the I/R group. Intestinal MDA levels of the GL treatment group were significantly ($P < 0.05$) lower than those of the I/R group. Mean MDA levels measured in liver tissues were 87.57 ng/ml in the control group, 198.84 ng/ml in the I/R group, and 131.23 ng/ml in the I/R+GL group (Table 1). The liver MDA level of the I/R group was significantly higher than that of the control group ($P < 0.05$). The liver MDA level of the I/R+GL group was significantly higher than that of the control group ($P = 0.023$). When the I/R group and the I/R+GL group were compared, it was seen that the liver MDA level of the I/R+GL group was lower than that of the I/R group, and this difference was statistically significant ($P < 0.05$) (Figure 1, 2, 3).

Antioxidant system-related parameters: Serum and tissue SOD and GSH-Px levels

The mean serum GSH-Px level was measured as 4.67 IU/ml in the control group, 1.59 IU/ml in the I/R group, and 3.46 IU/ml in the I/R+GL group (Table 1). The serum GSH-Px level of the I/R group was significantly ($P < 0.05$) lower, while it was significantly higher in the I/R+GL group ($P < 0.05$). Mean intestinal tissue GSH-Px levels were measured as 14.77 IU/ml in the control group, 12.19 IU/ml in the I/R group, and 8.27 IU/ml in the I/R+GL group (Table I). The I/R group presented significantly lower intestinal tissue GSH-Px levels than the control group ($P < 0.05$). When the control group and the I/R+GL group were compared, $P < 0.05$ was found: the intestinal tissue GSH-Px level of the I/R+GL group was found to be significantly lower than that of the control group. When the I/R group and the I/R+GL group were compared, it was observed that the serum GSH-Px level of the I/R+GL group was lower than that of the I/R group, and the difference was statistically significant ($P < 0.05$). The mean liver tissue GSH-Px levels were measured as 11.7 IU/ml in the control group, 7.7 IU/ml in the I/R group, and 10.6 IU/ml in the I/R+GL group (Table 1). Liver Tissue GSH-Px levels were significantly lower in the I/R and I/R+GL groups compared to the control group. However, liver tissue GSH-Px levels were significantly lower in the I/R group than in the I/R+GL group ($P < 0.05$) (Figure 1, 2, 3)

Table 1: The MDA, GSH-Px, and SOD levels in serum, intestinal tissue, and liver tissue by groups (Control, I/R, I/R+GL)

	Serum			Intestinal Tissue			Liver Tissue		
	Control	I/R	I/R+GL	Control	I/R	I/R+GL	Control	I/R	I/R+GL
SOD (pg/ml)	187	108	154	682	389	576	478	251	410
GSH-Px(IU/ml)	4.67	1.59	3.46	14.77	12.19	8.27	11.7	7.7	10.6
MDA (ng/ml)	42.2	90.2	60	102	264	173	88	199	131

I/R: Ischemia-reperfusion, GL: Ganoderma lucidum, SOD: Superoxide dismutase, GSH-Px: Glutathione peroxidase, MDA: Malondialdehyde.

Mean serum SOD levels were 187 pg/ml in the control group, 108 pg/ml in the I/R group, and 153 pg/ml in the I/R+GL group (Table 1). The serum SOD level of the I/R group was found to be significantly lower than that of the control group ($P < 0.05$). When the control group and the I/R+GL group were compared, $P < 0.05$ was found: a significant difference was noted. The serum SOD level of the I/R+GL group was noted as significantly ($P < 0.05$) higher than that of the I/R group. Mean intestinal tissue SOD levels were measured as 682 pg/ml in the control group, 389 pg/ml in the I/R group, and 576 pg/ml in the I/R+GL group (Table 1). When the control group and the I/R group were compared, a significant difference was noted ($P < 0.05$). When the I/R group and the I/R+GL group were compared, it was observed that the intestinal SOD level of the I/R+GL group was significantly higher ($P < 0.05$). Mean liver tissue SOD levels were measured as 478 pg/ml in the control group, 251 pg/ml in the I/R group, and 410 pg/ml in the I/R+GL group (Table 1). When the control group and the I/R group were compared, a significant difference was observed ($P < 0.05$). When the I/R group and the I/R+GL group were compared, it was observed that the liver SOD level of the I/R+GL group was significantly ($P < 0.05$) higher. (Figures 1, 2, 3)

Histopathologic changes in the intestinal tissue samples of the study groups

Changes in the intestinal tissue of the groups were evaluated according to Chui's classification [10]. The villi in all rats in the control group were evaluated as normal (Chui Grade 0). Enlargement of the subepithelial area and capillary congestion at the apex of villi (Chui Grade 1) were observed in six (75%) rats in the I/R group. In two (25%) rats, it was observed that the subepithelial area was enlarged, and the epithelial layer was separated from the lamina propria moderately (Chui Grade 2). While villi were evaluated as normal in four (50%) rats in the I/R+GL group (Chui Grade 0), enlargement in the subepithelial area and capillary congestion in the apex of the villi (Chui Grade 1) were detected in four (50%) rats in the I/R+GL group (Figure 2).

Histopathologic changes in the liver tissue samples of the study groups

There was severe and mild congestion in the liver of two (25%) and six (75%) rats, respectively, in the I/R group. All the rats in the I/R+GL group showed mild congestion. In the I/R group, mild sinusoidal dilatation was observed in seven (87.5%) rats, while severe dilatation was observed in one (12.5%) rat. In the I/R+GL group, mild sinusoidal dilatation was observed in all rats. In the I/R group, inflammation in the portal area was severe in only two (25%) rats and mild in six (75%) rats. In the I/R+GL group, two (25%) rats had no signs of inflammation, while 6 (75%) had mild signs of inflammation (Figure 3).

Discussion

Complement activation, activation of pro-inflammatory and inflammatory cytokines, and the emergence of free oxygen radicals are important steps in the chain of events that trigger each other and can lead to organ damage during an I/R injury. Especially toxic release due to the endothelial relationship with neutrophil activation, xanthine oxidase enzyme system, cytokines, and free oxygen radicals are the main factors responsible for tissue damage [11]. The intestinal tissue is one of the tissues sensitive to I/R injury. An I/R injury in the intestinal tissue carries high morbidity and mortality risks [12, 13]. Many experimental studies have been conducted to explain the complex interactions and reduce the unfavorable effects of I/R injuries. We preferred the intestinal I/R experimental model to evaluate the protective effects of GL in our study.

Ischemia and reperfusion times vary in different studies. Laurens et al. performed a 60-minute ischemia duration followed by a 60-minute reperfusion duration in their experimental study [14]. Yoshida et al. applied a 20-minute and 40-minute ischemia duration followed by 180-minute reperfusion in their experimental I/R model [15]. In our study, we applied 30-min ischemia and 90-min reperfusion to the superior mesenteric artery in rats to induce the intestinal I/R injury.

GL is represented with anti-inflammatory, antidiabetic, immunomodulatory, anti-inflammatory, antibacterial, neuroprotective, antioxidative, and antitumor properties in the literature [16]. It has also been shown as an effective agent against I/R injury with, particularly, anti-inflammatory and immunomodulatory properties. The healing effects of GL were reported in the studies focusing on I/R injuries in tissues such as renal [5], cardiac [6], and cerebral [4]. Our study is the first study to investigate the possible protective effects of GL on intestinal I/R injury in rats. GL was administered orally at a dose of 250 mg/kg in the treatment group of rats. The effectiveness of this dose has been shown in the literature [17].

Hematologic changes such as blood platelet, leukocyte, and hemoglobin levels are commonly used in both experimental intestinal I/R models and clinical follow-up and investigation of the efficacy of therapeutic agents. It has been reported that leukocyte and platelet levels increase in rats with I/R injury [18]. Our findings in our I/R injury group rats were consistent with these findings. WBC levels were found to be significantly high in the I/R group rats, whereas WBC levels in the GL treatment group rats were close to normal levels. Normal WBC levels in the rats administered with GL before I/R injury seem to be related to the immunomodulatory and anti-inflammatory properties of GL. Increased platelet level is an important marker in terms of identifying the degree of tissue damage after I/R injury.

Previous studies showed that I/R injury led to an increase in platelet levels in the early period of the injury. Increased platelet levels seem to be responsible for the damage to the intestinal villi [19]. However, the mechanism of these changes is still unknown. Our results showed that the platelet levels of the GL treatment group animals were lower than those of the I/R group animals.

We used MDA levels as one of the biochemical parameters of I/R injury in our study. Zhong et al. [5] examined

kidney MDA levels as an indicator of I/R injury in a study that investigated the oxidative stress preventive role of GL polysaccharides (GL-PS) in kidney I/R injuries. They found that MDA levels in the group with I/R injury were higher than in the group administered with GL-PS. They reported the positive effects of GL-PS on kidney I/R injury. A study by Zhonghui et al. [20] investigated the effects of GL-PS on I/R injury in skeletal muscles. To evaluate the antioxidant activity of GL-PS, they studied tissue MDA levels as an indicator of lipid peroxidation resulting from ischemia in skeletal muscles, and SOD, GSH-Px, and catalase levels in tissue. They found that GL-PS effectively reduced MDA levels in the skeletal muscle of mice and showed that high dose GL (200 mg/kg) showed a better effect. The authors concluded that GL reduced lipid peroxidation and prevented exercise-induced oxidative damage. In this study, we studied MDA levels in serum, intestinal, and liver tissue samples. The fact that MDA values increased significantly in serum, intestinal, and liver samples in the I/R group compared to the control group showed that I/R injury was fully developed in our experimental model. Serum MDA levels were normal in the GL treatment group rats, and these results showed that GL considerably reduced lipid peroxidation and had a protective effect against tissue damage due to I/R injury. Our study also examined the antioxidant activity of GL treatment by measuring GSH-Px and SOD levels in intestinal and liver tissue samples and serum. Significantly decreased GSH-Px and SOD levels were noted in tissue samples and serum of I/R injury group animals. Oxidative stress was achieved with our experimental model in our study. When all groups were compared in terms of SOD and GSH-Px levels, it was observed that the SOD levels of the GL treatment group were higher than those of the I/R group and closer to those of the control group. The antioxidant activity of GL was attributed to an increase in the antioxidant system enzyme of the SOD level. Our results showed that the administration of GL before I/R injury considerably alleviated the decrease in GSH-Px and SOD levels, especially in the liver tissue and serum.

It has been shown that the release of lysosomal hydrolase enzymes and increased microvascular permeability occur after intestinal I/R injury and lead to edema, bleeding, necrosis, flattening of the villi, and disruption in mucosal integrity [11]. The nature of the injury is related to the type of tissue. Intestinal tissue is the most vulnerable tissue to I/R injury [13, 21]. Our findings were consistent with these findings in the I/R injury group rats. The intestinal venous system drains into the portal venous system. An important part of the oxygenation of the liver tissue is provided by the portal venous system. It is known that mesenteric ischemia can affect the liver by reducing portal vein blood flow [22]. Therefore, in this study, we examined histopathological changes in intestinal tissue as well as serum and liver samples.

It has been reported that GL treatment reduces lipid peroxidation and has a healing effect on tissue ischemic damage. Chen et al. [23] investigated intestinal MDA levels as an indicator of oxidative stress and found that GL-PS reduced intestinal MDA levels in a study in which they examined the healing effects of GL-PS on small intestinal damage in mice developed after the use of methotrexate. Our results showed that GL treatment reduced intestinal tissue damage due to I/R injury.

Lin et al. [24] investigated the healing effects of GL on carbon tetrachloride-induced hepatic fibrosis in rats. They found that carbon tetrachloride induced hepatic fibrosis in rats and significantly increased MDA and hydroxyproline concentrations. They also noted that the GL extract reduced the hepatic MDA and that hydroxyproline levels increased by carbon tetrachloride. In our study, histopathologic examination of the liver tissue samples of the GL treatment group rats showed that liver tissue was considerably preserved from I/R injury. MDA levels were also consistent with these findings.

Conclusions

Early diagnosis and treatment of acute mesenteric ischemia remain a challenging problem due to the lack of a reliable, sensitive, and specific parameter. Additionally, there is no therapeutic agent that can be effective in the progression of organ failure caused by a delay in diagnosis.

Our results showed that GL, with its anti-inflammatory, immunomodulatory, and antioxidant properties, had significant healing effects on preventing I/R injury damage in the intestinal tissue and eliminating its unfavorable effects on the liver tissue. Our experimental study is the first to examine the effects of GL on intestinal tissue damage resulting from I/R injury. There is a need for further investigations to identify the biochemical mechanisms of the healing effects of GL on I/R injury.

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