# Grape seed protects cholestatic rats liver from ischemia/reperfusion injury[1]

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**ABSTRACT**

**PURPOSE:** To determine the effect of grape-seed extract against ischemia/reperfusion injury in cholestatic liver.

**METHODS:** Eighteen Wistar albino rats were divided into three groups. In control and study groups, cholestasis was provided by bile duct ligation. Seven days later, the rats were subjected to 30 min hepatic ischemia, followed by 60 min of reperfusion. Oral administration of 50 mg/kg/day grape-seed extract was started 15 days before bile duct ligation and continued to the second operation in the study group. Serum, plasma and liver samples were taken. Laboratory analysis, tissue gluthation, malondialdehyde, myeloperoxidase levels and histopathological examination were performed.

**RESULTS:** Significant decrease in liver gluthation level and significant increase in malondialdehyde level and myeloperoxidase activity were observed after ischemia/reperfusion in cholestatic rats. Serum and plasma levels for laboratory analysis were also significantly higher in cholestatic I/R group. Hepatic necrosis and fibrosis were detected in histopathological examination. Oral grape-seed extract administiration reversed all these parameters and histopathological findings except serum bilirubin levels.

**CONCLUSION:** Oral grape-seed extract treatment can improve liver functions and attenuate the inflammation and oxidative stress in cholestatic ischemia/reperfusion injury.

**Key words:** Grape Seed Extract. Ischemia. Injury. Liver. Reperfusion. Rats.

## Introduction

Hepatic ischemia and reperfusion (I/R) injury is a common problem after liver transplantation and major hepatectomies. After an ischemic period, reperfusion of the hepatocytes causes the release of toxic reactive oxygen species (ROS) that induce lipid peroxidation, mitochondrial damage, and apoptosis[1]. Although many compounds with antioxidant properties have been studied on experimental I/R injury models to decrease the harmful effects of ROS[2], there is still no reliable drug in clinical use for this purpose.

In the presence of cholestasis, the liver is more susceptible to reperfusion injury[3,4]. When the biliary flow comes to a standstill, a rapid injury to sinusoidal endothelial cells, accumulation of neutrophils, and activation of Kuppfer cells are induced[4]. The activated neutrophils and Kuppfer cells are the source of ROS and oxidative damage[5,6]. In real life, cholestasis is a common finding in hepatic or biliary pathologies requiring major hepatic surgery; therefore, cholestatic I/R injury models are more valuable for reflecting these clinical conditions.

The antioxidant features of grape-seed extract (GSE) have been known for a long time[7]. Procyanidines (or Proanthocyanidines), a class of derivatives of Flavan-3-ols that is the major biochemically active component of GSE, has powerful free radical scavenging capacity[7,8]. Hence, the success of GSE treatment against oxidative stress injury was previously demonstrated in I/R injury[9] and obstructive jaundice[10] models separately. In this study, a combined experimental model, cholestatic I/R injury model, was used to put forth the effect of oral GSE treatment in cholestatic rats that underwent I/R injury.

## Methods

Experimental procedures of this study were reviewed and approved by Akdeniz University, Local Committee of Animal Research and Ethics (decision number: 2014.09.16). The animals were obtained from the Laboratory of Experimental Animals of Akdeniz University. The number of animals was determined after power analysis. Eighteen Wistar Albino male rats (weighing 250-300 g; 5-7 months of age) were used in this study. The rats were kept at a 22°C constant temperature with a 12-h light/dark cycle and humidity at 60% and food and water available ad libitum. They were kept in plastic based production cages with plastic sides that were covered by wire knitted covers placed over the top of the cages.

### *Experimental design and study groups*

The rats were divided into three groups. In the sham group, an abdominal incision was closed without any other surgical manipulation after laparatomy in both operation days. In the cholestatic I/R injury group, I/R injury and cholestasis model were performed without any special treatment. In the I/R injury plus GSE treated (I/R+GSE) group, in addition to the I/R injury and cholestasis model, oral GSE treatment was administered. GSE in this study is an extract from Vitis vinifera (Mikro-Gen Pharmaceuticals, Istanbul, Turkey) and contains proanthocyanidins (oligomers of monomeric polyphenols; not less 70% polyphenolic compound).

In the study group, GSE was dissolved in water and administered to I/R+GSE group for 15 days prior to first operation and repeated for more 7 days during cholestasis period at a daily dose of 50 mg/kg, orally. The sham and cholestatic I/R groups received equal amounts of saline for 21 days. None of the animals died during these procedures.

### *Surgical procedures*

Fist operation day: After 6 hours of fasting, anesthesia was provided by intraperioneal injection of 75 mg/kg ketamin HCL (Ketasol®, Richterpharma Ag, Wels Austria) and xylazin hydrochloride (Rompun, Bayer, Istanbul, Turkey). Antisepsis of incisional area was provided by application of povidone-iodine. Then a midline laparotomy was performed using 15-no surgical scalpel. In the sham group, the abdomen was closed in a running single layer with 3-0 silk suture. In the other two groups, the common bile duct was isolated, and ligated with 4-0 silk suture. Then, the abdomen was closed in the same manner. After the smooth ending of anesthesia, the rats had free access to food and water. The rats received postoperative analgesia with acetaminophen (Paracetamol; Sigma-Aldrich Chemistry, Steinheim, Germany) in a dose of 50 mg/kg/d by oral gavage.

Second operation day: One week later, the same midline incision was opened in all the groups. In the sham group, the incision was closed without any manipulation. In the other two groups, the partial liver ischemia was performed with an atraumatic clamp by occluding the portal vein and hepatic artery for 30 minutes. Later, the liver was reperfused with blood for 60 minutes by the release of the clamp. After that, the rats were sacrificed by decapitation, and the blood and liver tissue samples were obtained immediately.

*Laboratory examinations*

Blood was drawn from the abdominal aorta and centrifuged at 3.000 g for 10 min. Serum was collected and stored at −20°C. Serum levels of total bilirubin (Tbil), direct bilirubin (Dbil), alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) were measured with an auto biochemical analyzer (Hitachi, Japan).

Tissue samples were taken from the right lobe of the liver, washed in cold saline, quickly put into −196 °C liquid nitrogen during sampling, and then kept at −80°C until use. Before detection, samples were first thawed in 4°C condition and subsequently homogenized in ice-cold phosphate buffer (pH 7.4).

Plasma TNF-$\alpha$ and IL-1$\beta$ were analyzed using enzyme-linked immunosorbent assay (ELISA) kits (Biosource International, Nivelles, Belgium) in accordance with the manufacturer's instructions and guidelines. These assay kits were particularly selected because of their high degree of sensitivity, specificity and inter-assay and intra-assay precision, and because they require a small amount of plasma sample.

To determine Malondialdehyde (MDA) and Gluthatione (GSH) levels, hepatic tissue samples were homogenized in ice-cold 150 mM KCL. The MDA levels were assayed for the products of lipid peroxidation[11]. Results were expressed as nmol MDA/g tissue. GSH levels were measured by spectrophotometric method using Ellman's reagent[12]. Results were expressed as nmol GSH/g tissue. Serum MDA and GSH levels were measured using the same method.

Tissue-associated myeloperoxidase (MPO) activity was measured according to the procedure reported by Hillegas *et al*.[13]. Hepatic tissue samples were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0) and homogenates were centrifuged at 41 400g for 10 min; pellets were suspended in 50 mM PB containing 0.5% hexadecyltrimethylammonium bromide. After three cycles of freezing and thawing, with sonication between the cycles, the samples were centrifuged at 41 400g for 10 min. Volumes of 0.3 ml were added to 2.3 ml of reaction mixture containing 50 mM PB, o-dianisidine, and 20 mM $H_2O_2$ solution. One unit of enzyme activity was defined as the amount of MPO that caused a change in the absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

*Histapathological examination*

Tissues were harvested from the rats and preserved in 10% formalin. They were washed in tap water, dehydrated in an ascending series of ethanol, cleared in xylene and embedded in paraffin. Paraffin sections 5 μm in thickness were cut with a microtome (Thermo Scientific Inc, MA, USA). Paraffin sections for histological observations were deparaffinized, stained with hematoxylin and eosin (H&E), and observed with a bright field microscope (Olympus, New York Microscope Company Inc., NY, USA).

*Statistical analysis*

SPSS 20.0 (IBM SPSS Statistics for Windows, IBM Corporation, Armonk, NY, USA) software was used for statistical analysis. Normally distrubuted continuous variables were expressed as mean (±standard deviation), and variables not normally distributed were expressed as median (range). For comparison of the three groups, the Kruskal-Wallis test was used, and the Mann-Whitney U test was used if any statistical significance was found. All tests were two-sided, and $p < 0.05$ was accepted as statistically significant.

**Results**

*Results of biochemical analysis*

Serum levels of ALT, AST, LDH, ALP, GGT, and total and direct bilirubin were significantly higher in both I/R and I/R+GSE groups compared to the sham group. Of them, serum ALT, AST, LDH, ALP, and GGT levels were significantly higher in I/R group than in I/R+GSE group ($p < 0.01$ for all of them). However, total and direct bilirubin levels did not differ between I/R and I/R+GSE groups ($p=0.148$ and $p=0.108$ respectively). The detailed results are presented in Table 1.

Tissue MDA and MPO levels were higher in both I/R and I/R+GSE groups compared to the sham group. The difference was not significant for tissue MDA level between I/R+GSE and sham groups ($p=0.055$), while there wasa significant difference both between sham vs I/R and I/R vs I/R+GSE ($p=0.004$). Tissue GSH levels were lower in both I/R and I/R+GSE groups compared to the sham group. When the I/R and I/R+GSE groups were compared, tissue MDA and MPO levels were significantly higher and tissue GSH level was significantly lower in I/R group than in I/R+GSE group. Plasma levels of proinflammatory cytokines, TNF-α and IL-1β, were significantly higher in both I/R and I/R+GSE groups compared to the sham group ($p < 0.02$ for both). Plasma levels of these cytokines were signifcantly higher in I/R group than in I/R+GSE group ($p=0.004$ for TNF-α and $p=0.006$ for IL-1β). The detailed results are summarized in Table 1.

**TABLE 1 -** Labaratory Parameters and Comparison of the Groups (mean±SEM, n=6).

| | Sham | Cholestatic I/R | Cholestatic I/R+GSE | p value overall | p value sham vs. I/R | p value sham vs. I/R+-GSE | p value I/R vs.I/R+-GSE |
|---|---|---|---|---|---|---|---|
| ALT (U/L)† | 100.6±20.5 | 371.7±42.7 | 262.5±48.6 | *0.001* | *0.000* | *0.000* | *0.002* |
| AST (U/L)† | 125.2±37.9 | 1808±199.5 | 1069±226 | *0.001* | *0.000* | *0.000* | *0.000* |
| LDH (U/L)† | 652.2±104.9 | 3919±401 | 2295±425 | *0.001* | *0.000* | *0.000* | *0.000* |
| ALP (U/L)† | 204.7±25.3 | 714.5±53.8 | 487±64.7 | *0.001* | *0.000* | *0.000* | *0.000* |
| GGT (U/L)† | 4.1±0.4 | 55.4±8.2 | 39.3±4.5 | *0.001* | *0.000* | *0.000* | *0.002* |
| DBIL (mg/dl)† | 0.4±0.01 | 7.0±0.3 | 6.6±0 | *0.002* | *0.000* | *0.000* | 0.148 |
| TBIL (mg/dl)† | 2.0±0.2 | 10.9±0.8 | 10.2±0.6 | *0.002* | *0.000* | *0.000* | 0.108 |
| TNF α (pg/ml)‡ | 9.9±1.5 | 22.3±4.7 | 13.1±2.1 | *0.001* | *0.000* | 0.012 | 0.001 |
| IL-1β (pg/ml)‡ | 208.5±26.8 | 410.0±37.7 | 306.2±40.1 | *0.001* | *0.000* | *0.001* | *0.001* |
| MDA (nmol/g)ʃ | 28.5±5.1 | 59.1±7.5 | 36.7±7.4 | *0.001* | *0.000* | 0.049 | *0.000* |
| GSH (nmol/g)ʃ | 1468±65 | 780.7±37.8 | 1231±99 | *0.001* | *0.000* | *0.001* | *0.000* |
| MPO (U/g)ʃ | 20.6±1.3 | 35.3±4.6 | 29.1±2.6 | *0.001* | *0.000* | *0.000* | *0.016* |

†serum level, ‡plasma level, ʃtissue level
I/R: ischemia and reparfusion injury, GSE: grape seed extract, ALT: alanine transaminase, AST: aspartate transaminase, LDH: lactate dehydrogenase, ALP: alkaline phosphatase, DBIL: direct bilirubin, TBIL: total bilirubin, TNF: tumor necrosis factor, IL: interleukin, MDA: malondialdehyde, GSH: glutathione, MPO: myeloperoxidase

*Results of histopathological analysis*

In histopathological examination, there were some changes in liver sections, including necrosis, periportal fibrosis, and vascular and sinusoidal congestion in cholestatic I/R group (Figure 1). The histopathological appearances in cholestatic I/R+GSE group were similar that in the sham group except for sinusoidal diatations (Figures 2 and 3) (Table 2).

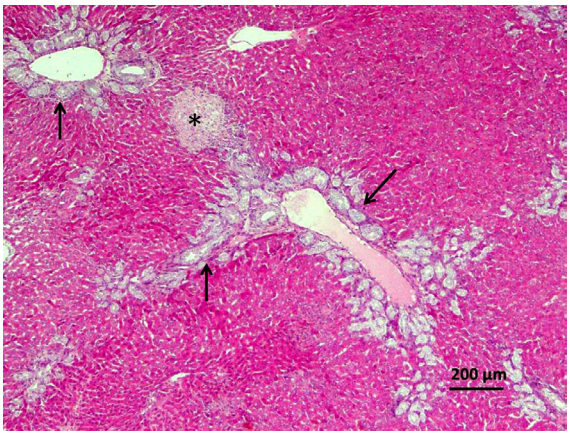


**FIGURE 2** - Hepatic necrosis, increased prominency of bile duct, and fibrotic changes (*arrows*) in cholestatic I/R group (H&E, x50).

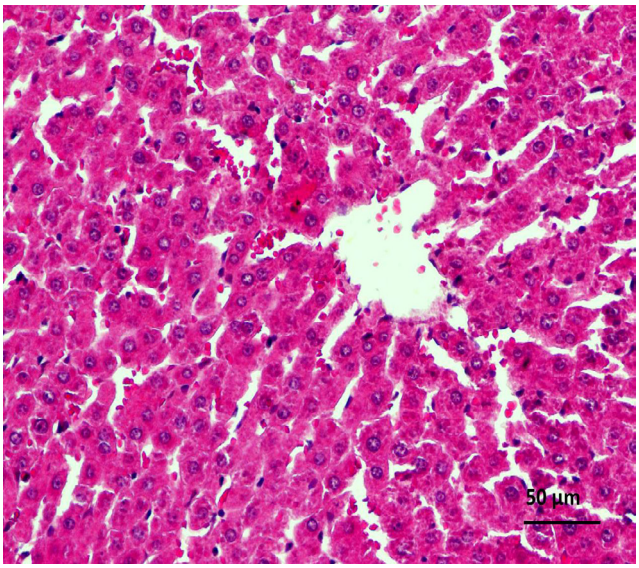


**FIGURE 1** - Normal histopathological appearance of liver tissue in the sham group (H&E, x200).

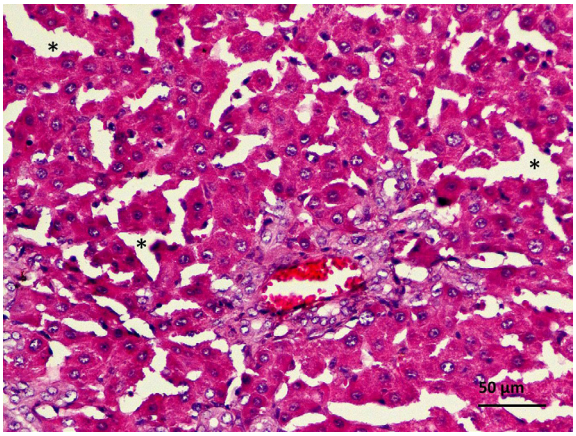


**FIGURE 3** - Histopathological appearance of liver in grape-seed extract treated group. Almost normal histopathological findings, except some sinusoidal dilatations (H&E, x200).

**TABLE 2 -** The average degree of histopathologic injury was expressed as medians within each liver section of groups were shown.

| Parameters | Sham | Cholestatic I/R | Cholestatic I/R+GSE |
|---|---|---|---|
| Vascular congestion | 0 | 1 | 0 |
| Degeneration and dilatation of the sinusoids | 0 | 2 | 1 |
| Hydropic degeneration (cellular swelling) | 0 | 1 | 0 |
| Degeneration of hepatocytes, necrosis | 0 | 1 | 0 |
| Increase in the number of activated Kupffer cells | 0 | 1 | 0 |
| Periportal fibrosis | 0 | 1 | 0 |
| Total score | 0 | 7 | 1 |

## Discussion

Ischemia, especially normothermic form, causes metabolic changes in hepatocytes especially in the mitochondria[14]. Failure of oxydative phosphorylation due to lack of oxygen leads to cellular ATP depletion, increase in glycolysis and formation of lactate. Additionally, the $Na^+/K^+$ pump becomes non-functional, resulting in intracellular $Na^+$ accumulation and acceleration in function of $Ca^{++}/Na^+$ pump. The overall result is an increase in intracellular $Ca^{++}$ level that leads to cellular damage[14,15]. After the reintroduction of oxygen to the ischemic tissues, toxic reactive oxygen species (ROS) are released especially from mitochondria, that are the major causes of the I/R injury[14].

Cholestasis is another cause of oxidative stress injury. Yoshidome *et al*.[4] observed a decrease in hyaluronic acid clearence in a cholestatic liver, which is evidence of the injury of sinusoidal endothelium. Additionally, obstruction of biliary flow results in accumulation and activation of polymorphonuclear neutrophils and Kuppfer cells[4,10] which trigger the oxidative stress injury[5,6]. The liver is more susceptible to I/R injury in the presence of obstructive jaundice[3,4]. Although Georgiev *et al*.[16] asserted the reverse arguments, most studies have shown a further increase in neutrophil infiltration and depending oxidative stress injury in postischemic cholestatic liver[3,4,10]. Therefore, we thought that the power of an antioxidant drug should be tested on a condition more compatible with clinical practice.

The major antioxidant component of GSE is procyanidines (or proanthocyanidins), a subtype of flavan-3-ols. Procyanidines behave as antioxidants by scavenging the ROS, chelating the transition metals, or inhibiting some enzymes[8]. The electronic configuration of flavan-3-ols provides easy release of electrons to ROS, by which the harmful character of the ROS is transferred to the flavan-3-ol[8]. In a relatively old study, Bagchi *et al.*[7] revealed that the GSE had significantly higher dose-dependent free radical scavenging capability than vitamin E.

In a previous study, premature mortality in the first 24 hours was very high after a 45-minute ischemic period in the cholestatic I/R injury model[3]. Therefore we have preffered thirty minutes of ischemia time for this animal model.

Reduced form of glutathion (GSH) underwent oxidation with the enzymatic effect of gluthatione peroxidase to eliminate the ROS and release oxidized glutathion (GSSG) in hepatocytes[14]. Accordingly, GSH has an important mission against oxidative stress injury[14]. Hepatic GSH production decreases during normothermic ischemia, and discharge of ROS during reperfusion phase causes rapid consumption of GSH[19,20]. These two mechanisms cause decreases in both intracellular and extracellular GSH levels, which causes oxidation and degration of important structures in the cell such as proteins, lipids, or double strand structure of DNA[20]. Therefore, the tissue GSH level has been accepted as a marker for oxidative stress injury. Less tissue GSH level means more oxidative stress injury[21]. In our study, GSE treatment provides attenuation of oxidative stress injury in a cholestatic I/R injury model. The decrease in tissue GSH level is significantly less in I/R+GSE group than in I/R group in the setting of cholestasis ($p < 0.0001$).

Lipid peroxidation is another important part of oxidative stress injury. Lipids in cellular membranes underwent lipid peroxidation, leading cellular damage. The end product of this reaction is MDA[17]. In the current study, I/R injury caused a huge amount of MDA accumulation in the liver. The mean value of liver MDA level was more than 2-fold that of normal liver tissue. In the previous studies about I/R injury, 1.5- to 4-fold of basal level of MDA was obtained[9,22-24]. Therefore, it is certain that the tissue MDA level can reflect exactly the severity of the oxidative stress injury. According to our results, GSE treatment attenuated the lipid peroxidation effectively; thus, liver MDA level was very close to the basal level in normal liver (sham vs. cholestatic I/R+GSE; $p=0.049$).

Kupffer cells and polymorphonuclear neutrophils are the other participants in I/R injury reactions, and they are the main sources of the ROS during I/R injury[6]. Therefore, the oxidative activity should be proportional with the activity and number of these cells. MPO level in a tissue is directly proportional with the number of activated neutrophils and Kupffer cells that was previously used to evaluate the I/R injury in both liver and

small intestine[18,25]. Furthermore, activated Kupffer cells release cytokines, especially TNFα and IL-1β, in addition to ROS[14]. Increases in polimorphonuclear neutrophils and activity of Kupffer cells, reflected by tissue MPO, plasma TNFα, and IL-1β levels, are indicators of both oxidative stress and inflammatory reaction[5,8,14]. In the current study, cholestatic I/R injury caused a significant increase in the levels of tissue MPO, and plasma TNFα and IL-1β. With the GSE treatment, all were significantly lowered in comparison to the cholestatic I/R injury group ($p = 0.016$ for MPO; $p < 0.001$ for TNFα and IL-1β).

According to the results of the current study, although GSE treatment significantly decreased the oxidative stress in cholestatic I/R model, the reduction was not enough to reach the basal level of the normal liver. Thus, there were statistically significant differences for all oxidative stress parameters between the cholestatic I/R+GSE group and the sham group. In contrast, Sehirli et al.[9] reported that the liver GSH, MPO and MDA levels reversed to the levels in the control group with oral GSE treatment in I/R injury model without cholestasis. Likewise, Dulundu *et al*.[10] revealed that GSE treatment returned the liver tissue GSH, MPO, and MDA levels to almost-normal basal level in the cholestatic model without I/R. Relatively less success in our study may be related to differences in experimental models; hence, in our study, the combination of cholestatsis and I/R might deepen the oxidative stress injury. In another study, significant antioxidant effect of intraperitoneal grape seed proanthocyanidin administiration was demonstrated, but the level of oxidative stress parameters, including GSH, MDA, TNFα and IL-1β, did not reach the basal level of normal liver even with ischemic postconditioning[26]. According to these data, although GSE treatment decreases the oxidative stress injury after reperfusion, it seems to be infufficient to reach the normal hepatic status. However, the present effect may be reasonable for clinical improvement.

In this study, cholestatic I/R damaged the hepatocytes, sinusoidal endothelial cells, and biliary ductal epithelium. The presence of these damages was proven by a significant increase in AST, ALT, ALP, and GGT in cholestatic I/R group. Additionally histopathological examination revealed a hepatic necrosis, endothelial swelling and fibrosis. GSE treatment provides significant improvement in the levels of these parameters, although the level of bilirubin did not improve. The succcess of GSE tretment was seen in the histopthological examination. Hence, almost normal liver tissue appearance except for some sinusoidal dilatations was seen in histopathological examination of liver tissue of the cholestatic I/R+GSE group.

## Conclusions

Reperfusion of the liver after a period of ischemia in a cholestatic model causes considerable increase in proinflammatory cytokines in bloodstream and oxidative stress in liver tissue. The overall results are hepatocellular and sinusoidal endothelial damage. Oral GSE treatment can improve the liver functions and attenuate the inflammation and oxidative stress in the cholestatic I/R injury. These experimental findings demonstrated that GSE appears to be an effective agent against hepatic I/R injury in a cholestatic liver.

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