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Grape Seed Extract Treatment Reduces Hepatic Ischemia-Reperfusion Injury in Rats

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This study was designed to determine the possible protective effect of grape seed extract (GSE), a widely used antioxidant dietary supplement, on hepatic ischemia/reperfusion (I/R) injury. Wistar albino rats were subjected to 45 min of hepatic ischemia, followed by a 60 min reperfusion period. GSE was administered in a dose of 50 mg/kg/day orally for 15 days before I/R injury and repeated before the reperfusion period. Liver samples were taken for histological examination or determination of hepatic malondialdehyde (MDA), glutathione (GSH) and myeloperoxidase (MPO) activity. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were determined to assess liver functions. Lactate dehydrogenase (LDH) and cytokines (TNF-α and IL-1β) were also assayed in serum samples for the evaluation of generalized tissue damage. Ischemia/reperfusion caused a significant decrease in hepatic GSH, and significant increases in MDA level, and MPO activity. Serum AST and ALT levels, as well as LDH activity and plasma TNF-α and IL-1β levels were also elevated in the I/R group. Treatment with GSE reversed all these biochemical parameters as well as histological alterations induced by I/R. In conclusion, GSE reduced I/R-induced organ injury through its ability to balance the oxidant–antioxidant status, to inhibit neutrophil infiltration and to regulate the release of inflammatory mediators. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: ischemia/reperfusion; grape seed extract; lipid peroxidation, glutathione, myeloperoxidase.

INTRODUCTION

Most surgical procedures involve a period of ischemia followed by reperfusion, as do many disease states such as shock, sepsis and pancreatitis. In the liver, ischemia/reperfusion (I/R) injury can occur in several clinical settings such as hepatic trauma, resection of large intrahepatic tumors and liver transplantation (Serracino-Inglott *et al.*, 2001). Deprivation of oxygen to the liver during ischemia induces severe damage, however, much more damaging reactive oxygen species (ROS) are generated during the reperfusion period (Parks and Granger, 1988). The organ dysfunction that accompanies this condition is generally associated with increased microvascular permeability, interstitial edema, impaired vasoregulation, inflammatory cell infiltration and parenchymal cell dysfunction and necrosis (Werns and Lucchesi, 1990; Granger and Korthuis, 1995). Thus, surgery is well designed to prevent and manage the tissue injury associated with reperfusion.

Free radical ablation for the treatment of reperfusion injury found its first clinical application in the prevention of postischemic tissue injury after organ transplantation (Amersi *et al.*, 2002; Seo and Lee, 2002). Thus, free radical scavengers and antioxidant agents are thought to be useful in the clinical setting of hepatic I/R damage.

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Oligomeric proanthocyanidins, naturally occurring antioxidants widely available in fruits, vegetables, nuts, seeds, flowers and bark, have been reported to possess a broad spectrum of biological, pharmacological and therapeutic activities against free radicals and oxidative stress (Bagchi *et al.*, 2000). Grape seed extract contains a number of polyphenols including procyanidins and proanthocyanidins and are powerful free radical scavengers (Bagchi *et al.*, 1997). Grape seed extracts have been reported to possess a broad spectrum of pharmacological, and therapeutic effects including antiinflammatory activity and reduced apoptotic cell death (Li *et al.*, 2001; Sato *et al.*, 2001). Grape seed extracts protect heart function and reduce infarct size in experimental cardiac ischemia (Shao *et al.*, 2003). It prevents acetaminophen-induced liver damage (Ray *et al.*, 1999) and puromycin-induced nephrosis (Mattoo and Kovacevic, 2003). In a previous study, the protective effects were demonstrated of GSE against bile duct ligation-induced hepatic fibrosis where oxidative stress takes place (Dulundu *et al.*, 2007). The present study, therefore, investigated the protective effect of GSE against oxidative stress during I/R injury of the liver, by measuring biochemical values and conducting histological examinations.

MATERIALS AND METHODS

Animals. Male Wistar albino rats (200–250 g) were housed in an air-conditioned room with 12 h light and

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dark cycles, with constant temperature (22 ± 2 °C) and relative humidity (65–70%) levels. All experimental protocols were approved by the Marmara University School of Medicine Animal Care and Use Committee. Rats were anesthetized with 100 mg/kg ketamine and 0.75 mg/kg chlorpromazine, given intraperitoneally (i.p.) during all surgical procedures.

Experimental protocol. Under anesthesia, a midline laparotomy was made using minimal dissection. Total hepatic ischemia was induced for 45 min by clamping the hepatic artery, the portal vein and the bile duct using a vascular clamp and the rats were then allowed to reperfuse for 60 min. Grape seed extract was dissolved in water and administered to sham-operated control (GSE group) and I/R groups (I/R + GSE group) for 15 days prior to I/R and repeated during ischemia period a daily dose of 50 mg/kg, orally. Grape seed extract used in this study comes as an extract from *Vitis vinifera* and contains proanthocyanidins (oligomers of monomeric polyphenols; not less 70% polyphenolic compound). The extract was kindly donated by Mikrogen Pharmaceuticals (Istanbul, Turkey). The sham-operated control (C) and I/R groups received equal amounts of saline for 15 days. None of the animals died during these procedures.

At the end of the reperfusion period, animals were decapitated and trunk blood samples were collected to determine serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels and lactate dehydrogenase (LDH) activity as indicators of liver functions and generalized tissue damage, respectively. The generation of proinflammatory cytokines due to I/R was evaluated by plasma TNF-α and IL-1β levels. The hepatic tissue samples were stored at –70 °C. Afterwards, tissue malondialdehyde (MDA) levels, an end product of lipid peroxidation, glutathione (GSH), a key antioxidant, and tissue-associated myeloperoxidase (MPO) activity, as indirect evidence of neutrophil infiltration, were measured in these samples. The hepatic tissue samples were also placed in formaldehyde (10%) for histological evaluation.

Assays. Serum AST, ALT (Moss *et al.*, 1987) and LDH levels (Martinek, 1972) were determined spectrophotometrically using an automated analyser. Plasma levels of tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1β, were quantified according to the manufacturer's instructions and guidelines using enzyme-linked immunosorbent assay (ELISA) kits specific for the previously mentioned rat cytokines (Biosource International, Nivelles, Belgium). These particular assay kits were selected because of their high degree of sensitivity, specificity, inter- and intraassay precision, and small amount of plasma sample required to conduct the assay.

Tissue samples were homogenized with ice-cold 150 mM KCl for the determination of malondialdehyde (MDA) and glutathione (GSH) levels. The MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as described previously (Beuge and Aust, 1978). Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and the results were expressed as nmol MDA/g tissue. GSH measurements were performed using a modification of the Ellman procedure (Beutler, 1975). Briefly, after centrifugation at 2000 × **g** for 10 min, 0.5 mL of supernatant was added to 2 mL of 0.3 mol/L $Na_2HPO_4.2H_2O$ solution. A 0.2 mL solution of dithiobisnitrobenzoate (0.4 mg/mL 1% sodium citrate) was added and the absorbance at 412 nm was measured immediately after mixing. GSH levels were calculated using an extinction coefficient of 1.36×10^4 M^{-1} cm^{-1}. The results were expressed in μmol GSH/g tissue.

Myeloperoxidase (MPO) is an enzyme that is found predominantly in the azurophilic granules of polymorphonuclear leukocytes (PMN). Tissue MPO activity correlates significantly with the number of PMN determined histochemically in inflamed tissues (Bradley *et al.*, 1982), and therefore, it is frequently utilized to estimate tissue PMN accumulation. MPO activity was measured in tissues in a procedure similar to that documented by Hillegas *et al.* (1990). Tissue samples were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0), and centrifuged at 41 400 × **g** (10 min); pellets were suspended in 50 mM PB containing 0.5% hexadecyltrimethylammonium bromide (HETAB). After three freeze–thaw cycles, with sonication between cycles, the samples were centrifuged at 41 400 × **g** for 10 min. Aliquots (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 present that caused a change in absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

Histopathological analysis. For light microscopic investigations, hepatic tissue specimens were fixed in 10% formaldehyde, dehydrated in alcohol series, clearing in toluene and embedding in paraffin. Paraffin sections (5 μm) were stained with hematoxylin and eosin (H&E) and examined under a photomicroscope (Olympus BX51, Tokyo, Japan). All tissue sections were examined microscopically for the characterization of histopathological changes by an experienced histologist in blind fashion.

Statistics. Statistical analysis was carried out using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA). All data were expressed as mean ± SD. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Values of $p < 0.05$ were regarded as significant.

RESULTS

AST and ALT levels were significantly higher in the I/R group when compared with those of the control group ($p < 0.001$). GSE treatment reversed these values significantly. Similarly, in the I/R group, increased lactate dehydrogenase activity, as an index of generalized tissue damage, was reversed significantly by GSE treatment ($p < 0.001$) (Fig. 1).

Ischemia/reperfusion caused significant increases in plasma levels of proinflammatory cytokines, TNF-α and IL-1β ($p < 0.001$). GSE treatment significantly decreased both of these cytokine levels, however, they did not return to the control value ($p < 0.001$; Fig. 2).

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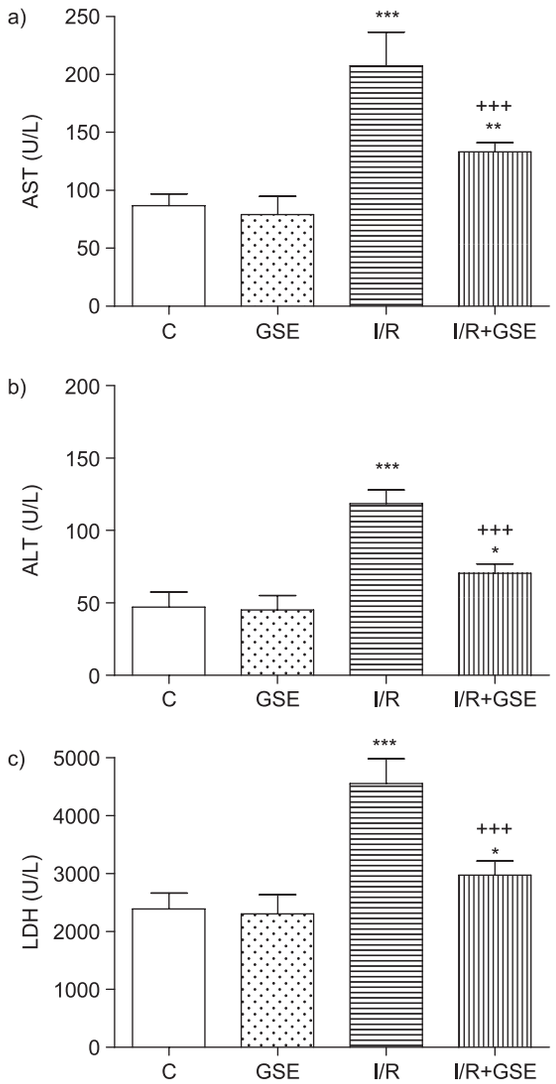

Figure 1. (a) Serum aspartate aminotransferase (AST), (b) alanine aminotransferase (ALT) and (c) lactate dehydrogenase activities (LDH) in the saline or GSE treated sham operated and I/R groups. Each group consists of eight animals. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ compared with control group. $^{+++}$ $p < 0.001$ compared with I/R group.

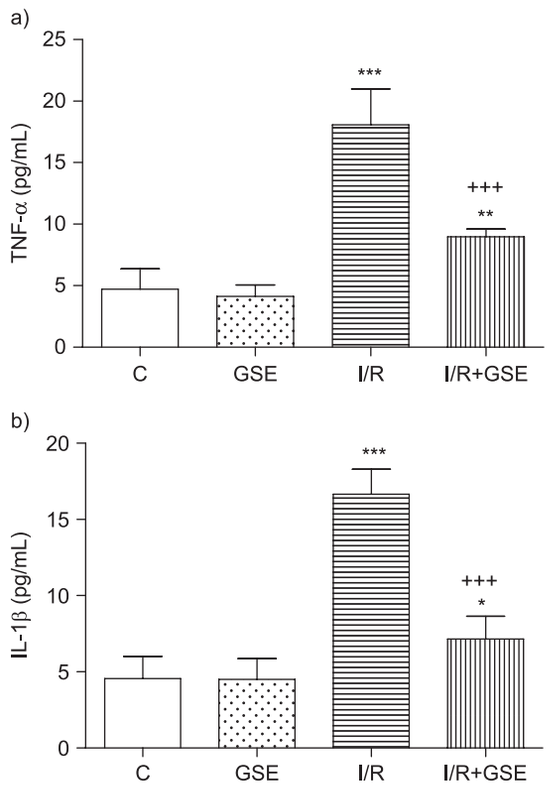

Figure 2. (a) TNF-α and (b) IL-1β levels in the plasma samples of saline or GSE treated sham operated and I/R groups. Each group consists of eight animals. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ compared with control group. $^{+++}$ $p < 0.001$ compared with I/R group.

The liver MDA, which is an index of tissue lipid peroxidation, was found to be significantly higher in the I/R group (58.1 ± 7.5 nmol/g), however treatment with GSE decreased the elevated MDA level significantly back to the control level (32.5 ± 4.0 nmol/g) (Fig. 3A).

The endogenous antioxidant, GSH, level in the hepatic tissue was decreased significantly after I/R (0.87 ± 0.2 μmol/g). On the other hand GSE treatment significantly reversed this I/R-induced GSH reduction (1.87 ± 0.2 μmol/g) (Fig. 3B).

When compared with the control group (11.0 ± 2.7 U/g), hepatic MPO activity was increased significantly in the I/R group (24.1 ± 4.2 U/g), indicating increased neutrophil infiltration to the tissue. This elevation in the MPO activity induced by I/R was reversed back to the control level with GSE treatment (13.8 ± 2.3 U/g) (Fig. 3C).

Light microscopic investigation of the control group (either given saline or GSE) revealed a regular morphology of liver parenchyma with intact hepatocytes and sinusoids (Fig. 4A). In the I/R group, severe sinusoidal congestion and hemorrhage, dilation of central vein, subendothelial edema and degenerated hepatocytes with perinuclear vacuolization were observed (Fig. 4B). In the GSE treated I/R group, histological analysis demonstrated a well-preserved liver parenchyma. Despite the mild sinusoidal dilatation and hemorrhage, which were in localized areas, the usual appearance of the central vein and hepatocytes was observed in most areas (Fig. 4C).

DISCUSSION

The current data demonstrate that temporary blockade of hepatic blood supply yielded structural and functional alterations in the liver with a concomitant increase in proinflammatory cytokines in the blood. A dietary antioxidant, GSE, on the other hand, depressed the concentration of these cytokines and reduced the severity of injury.

I/R injury is a complex process involving numerous intracellular signaling pathways, mediators, cells and pathophysiological disturbances; and its prevention during surgery is of the utmost importance (Marubayashi and Dohi, 1996). Considerable evidence suggests that oxygen derived free radicals are involved in the hepatic injury caused by ischemia and reperfusion (Sener *et al.*, 2003, 2005; Ofluoglu *et al.*, 2006; Zhu *et al.*, 2006). Thus, therapeutic strategies are designed to reduce free radical-induced damage, either by intervening in the process by which free radicals are formed or by scavenging the free radicals that have already been formed. Different

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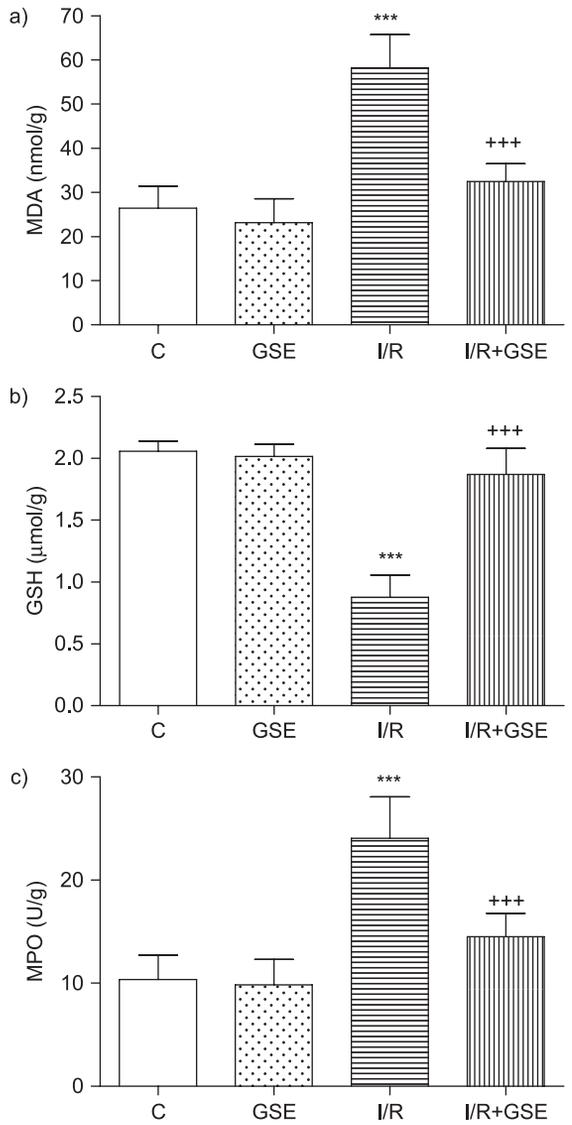

Figure 3. (a) Malondialdehyde (MDA), (b) Glutathione (GSH) levels and (c) Myeloperoxidase activity (MPO) in the hepatic tissues of saline or GSE treated sham operated and I/R groups. Each group consists of eight animals. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ compared with control group. $^{+++}$ $p < 0.001$ compared with I/R group.

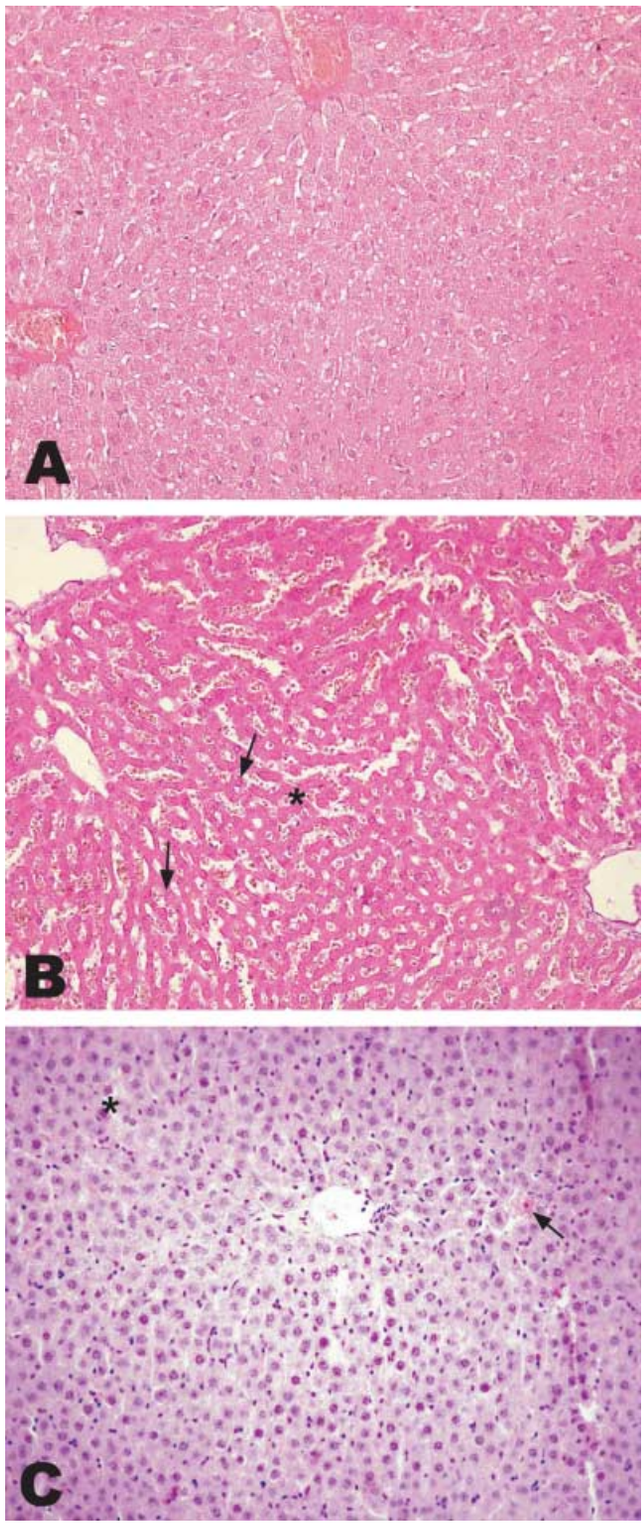

Figure 4. (A) Control group of rats show regular liver parenchyma with normal appearance of hepatocytes and sinusoids. (B) I/R group of rats show severe sinusoidal congestion (→) and haemorrhage, dilated central vein and degenerated hepatocytes (*) showing perinuclear vacuolization. (C) I/R+ GSE group of rats show mild sinusoidal dilatation (*) and haemorrhage in localized areas, with the usual appearance of central vein and hepatocytes (→) in most areas. H&E staining, original magnifications: ×200.

degrees of protection were obtained with numerous compounds; however, the structure-activity relationship, bioavailability and therapeutic efficacy of these compounds differ extensively. Thus clinical application of these agents is limited in respect of their side effects, toxicity, solubility, membrane penetration etc.

The biological, pharmacological and medicinal properties of bioflavonoids and proanthocyanidins have been extensively reviewed (Shahidi and Wanasundara, 1992; Rice-Evans *et al.*, 1996). Besides the free radical scavenging activity, proanthocyanidins exhibit vasodilatory, anticarcinogenic, antiallergic, antiinflammatory, antibacterial, cardioprotective, immune-stimulating, antiviral and estrogenic activities, as well as being inhibitors of the enzymes phospholipase A2, cyclooxygenase and lipoxygenase (Rice-Evans *et al.*, 1996; Salah *et al.*, 1995). The chemical properties of proanthocyanidins in terms of the availability of the phenolic hydrogens as hydrogen donating radical scavengers and singlet oxygen quenchers predicts their antioxidant activity (Rice-Evans *et al.*, 1996; Chen *et al.*, 1996). Thus, having antioxidant activity suggests that GSE is beneficial in various cardiovascular, cerebrovascular and neurological disorders in which oxidants are involved.

Oxygen radical-initiated lipid peroxidation may contribute to the impaired cellular function and necrosis associated with reperfusion of ischemic tissues (Stark, 2005). In the present study, I/R caused significant increases in the hepatic malondialdehyde levels, end products of lipid peroxidation. This observation is in agreement with the previous studies, where elevated levels of lipid peroxidation products were increased from 40% to 80% above basal values (Sener *et al.*, 2003; Ofluoglu *et al.*, 2006; Zhu *et al.*, 2006). Furthermore, our results demonstrated that GSE treatment abolished these increases in malondialdehyde, probably in part by scavenging the very reactive hydroxyl and peroxyl radicals. El-Alfy *et al.* (2005) demonstrated that grape seed proanthocyanidins, when administered to alloxan-induced diabetic rats, decreased the oxidant generation and lipid peroxidation by its antioxidant effect. Similarly, in the neonatal rats GSE suppresses lipid peroxidation and reduces hypoxic ischemic brain injury by reducing 8-isoprostaglandin $F_{2\alpha}$ and thiobarbituric acid reacting substances (Feng *et al.*, 2005).

Oxidative stress-induced tissue damage can be prevented or ameliorated by favoring the balance towards a lower oxidative status. Glutathione is an important constituent of intracellular protective mechanisms against various noxious stimuli, including oxidative stress (Ross, 1988). The results of the present study support the notion that depletion of tissue GSH, as observed in the I/R-induced hepatic injury, is one of the major factors that permit lipid peroxidation and subsequent tissue damage. On the other hand, the decrease in hepatic GSH effect was reversed by the administration of GSE. A possible explanation for this effect is that GSE function as free radical scavengers and therefore increase the available free GSH which detoxify the reactive intermediary oxygen products of lipid peroxidation induced by I/R.

It has been demonstrated by various investigators that circulating proinflammatory cytokines, such as TNF-α, interleukin-1beta (IL-1beta) and IL-6 which trigger hepatic injury, were increased, at least in part, by a free radical-mediated apoptotic mechanism. Therefore, it seems reasonable to propose that hepatic ischemia/reperfusion (I/R) injury leads to the release of the cytokines. In the study of Hato *et al.* (2001) neutrophil accumulation and the content of cytokines, including TNF-α and IL-1β, were increased in the reperfused liver. Similarly, Tsuchihashi *et al.* (2006) who studied the role of cytokines in I/R injury of the liver tissue, suggested that I/R led to the expression of TNF-α, IL-1β, IL-6 in the reperfused rat liver model, and the expressed cytokines are expected to aggravate I/R injury. In accordance with these findings, in the present study, the plasma levels of the pro-inflammatory cytokines TNF-α, and IL-1β, were significantly elevated in I/R-induced hepatic injury, which was verified using both biochemical and histological assessments. Furthermore, GSE treatment reversed all the injury parameters and the levels of inflammatory mediators while protecting the liver tissue against reperfusion-induced oxidative injury.

Proinflammatory cytokines play important roles in the induction of polymorphonuclear neutrophil (PMN) activation and infiltration. Although it is not certain whether neutrophil accumulation and activation are the causes or the result of reperfusion injury, increasing evidence suggests that mesangial cells and neutrophils release chemotactic substances (e.g. interleukin 8), which increase the damage (Zimmerman *et al.*, 1990). The activity of neutrophil-specific enzyme MPO, is used to define the role of neutrophils in reperfusion tissue injury (Hillegas *et al.*, 1990). In the present study, the presence of elevated MPO activity in the liver indicates that I/R-induced injury involves the contribution of neutrophil infiltration. Grisham *et al.* (1986) have demonstrated a significant increase in this enzyme activity. On the other hand the neutrophil accumulation initiated by reperfusion was shown to be significantly attenuated by pretreatment with either SOD, CAT, allopurinol, hydroxyl radical scavenger (dimethylthiourea) or desferoxamine (Zimmerman *et al.*, 1990). In our study, increased MPO activity due I/R injury was effectively reversed by GSE treatment.

The results of this study clearly demonstrated that temporary blockade of hepatic blood supply yielded structural and functional alterations in the liver with a concomitant increase in proinflammatory cytokines in the blood. On the other hand GSE treatment improved I/R-induced impairment in the liver functions, significantly decreased I/R-induced elevations in hepatic lipid peroxidation, myeloperoxidase activity and plasma cytokines, while decreased GSH levels were replenished by GSE treatment. These protective effects of grape seed extract on reperfusion-induced injury can be attributed, at least in part, to its ability to inhibit neutrophil infiltration, to balance oxidant–antioxidant status, and to regulate the generation of inflammatory mediators, suggesting a future role in the treatment of organ failures due to ischemia reperfusion.

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