

Basic Study

Grape seed proanthocyanidin protects liver against ischemia/reperfusion injury by attenuating endoplasmic reticulum stress

Zhen-Chao Xu, Jie Yin, Bo Zhou, Yu-Ting Liu, Yue Yu, Guo-Qiang Li

Zhen-Chao Xu, Bo Zhou, Yu-Ting Liu, Yue Yu, Guo-Qiang Li, Liver Transplantation Center, First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China

Jie Yin, Department of Respiratory Medicine, Jinling Hospital, Nanjing 210002, Jiangsu Province, China

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Correspondence to: Guo-Qiang Li, MD, PhD, Liver Transplantation Center, First Affiliated Hospital of Nanjing Medical

University, Nanjing 210029, Jiangsu Province, China. liguoqiang@njmu.edu.cn
Telephone: +86-25-83672106
Fax: +86-25-83672106

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Abstract

AIM: To explore the effect of grape seed proanthocyanidin (GSP) in liver ischemia/reperfusion (IR) injury and alleviation of endoplasmic reticulum stress.

METHODS: Male Sprague-Dawley rats (220-250 g) were divided into three groups, namely, sham, IR, and GSP groups ($n = 8$ each). A liver IR (70%) model was established and reperused for 6 h. Prior to reperfusion, the GSP group was administered with GSP (100 mg/kg) for 15 d, and liver histology was then investigated. Serum aminotransferase and inflammatory mediators coupled with superoxide dismutase and methane dicarboxylic aldehyde were detected. Western blot was conducted to analyze the expression of glucose-regulated protein 78, CCAAT/enhancer-binding protein homologous protein, activating transcription factor-4, inositol-requiring enzyme-1, procaspase-12, and nuclear factor- κ b. Apoptotic cells were detected by TUNEL staining.

RESULTS: The serum aminotransferase, apoptotic cells, and Suzuki scores decreased in the GSP group compared with the IR group ($P_s < 0.05$). The methane

dicarboxylic aldehyde level was decreased in the GSP group, but the superoxide dismutase level was reversed ($P_s < 0.05$). Similarly, GSP downregulated the proinflammatory factors and upregulated the levels of anti-inflammatory factors ($P_s < 0.05$). Western blot data showed that GSP increased glucose-regulated protein 78 expression and suppressed expression of CCAAT/enhancer-binding protein homologous protein, activating transcription factor-4, inositol-requiring enzyme-1, procaspase-12, and nuclear factor- κ b compared with the IR group.

CONCLUSION: GSP possesses antioxidative, anti-inflammatory, and antiapoptotic effects by relieving endoplasmic reticulum stress through regulation of related signaling pathways to protect the liver against IR injury.

Key words: Anti-apoptosis; Endoplasmic reticulum stress; Grape seed proanthocyanidin; Inflammation; Ischemia/reperfusion injury

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Core tip: Liver ischemia/reperfusion (IR) injury induces endoplasmic reticulum (ER) stress. Numerous studies show that excessive ER stress aggravates IR injury. Grape seed proanthocyanidin (GSP) is an effective protector against IR injury. However, the detailed protective mechanisms remain unclear. Therefore, this study explored the effect of GSP in the liver for protection against IR injury and the alleviation of ER stress. The results indicate that GSP possesses antioxidative, anti-inflammatory, and antiapoptotic effects by relieving ER stress in the liver through regulation of related signaling pathways.

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INTRODUCTION

Ischemia/reperfusion (IR) injury of the liver can occur in several clinical settings, such as hepatic trauma, resection of large intrahepatic tumors, and liver transplantation^[1], resulting in cell death and tissue destruction^[2]. Increasing evidence has shown that both proinflammatory cytokines and reactive oxygen species (ROS) are key mediators of liver IR injury^[3]. However, the exact mechanisms regarding endoplasmic reticulum (ER) stress on IR has not been clearly elucidated.

The ER is a key organelle in eukaryotic cells, where lipid synthesis, protein folding (into tertiary and

quaternary structures), and protein maturation occur. The ER senses and responds to homeostatic changes with various stimuli, such as ischemia, hypoxia, elevated protein synthesis, and Ca^{2+} overload^[4]. The ER protein folding capacity is reduced under stress, leading to accumulation of unfolded proteins. A major response to ER stress is the activation of glucose-regulated protein 78 (GRP78) through dissociation from its transmembrane receptor, which allows subsequent regulation of the levels of accumulated, unfolded proteins^[5]. Slight and moderate ER stress can protect cells from death, but severe ER stress induces caspase-12-dependent cell apoptosis^[6]. When severe ER stress occurs, activating transcription factor (ATF)-4 can increase the expression of CCAAT/enhancer-binding protein homologous protein (CHOP), promoting ER stress response through numerous mechanisms, and CHOP promotes oxidative stress inflammation and apoptosis^[7]. Inositol-requiring enzyme (IRE)-1 is an important protein relative to ER stress and is vital to the occurrence of inflammation induced by ER stress and simultaneous activation of nuclear factor (NF)- κ b^[8]. Numerous studies show that ER stress plays a critical role in a variety of processes^[9], and is also important in the occurrence of IR damage^[10]. Data have suggested that attenuating ER stress-induced apoptosis can protect the brain against IR injury^[11]. Thus, ER stress is closely related to IR injury.

Proanthocyanidins are highly bioavailable and provide a significantly greater protection against damage from oxidative stress than vitamin C, vitamin E, or β -carotene^[12]. ER stress intensifies various types of damage, leading to inflammation, oxidative stress injury, and abnormal cell apoptosis. Thus, the botanical ingredients previously mentioned can effectively inhibit the occurrence of injury^[13] induced by ER stress. Grape seed proanthocyanidin (GSP) is abundant in phenolic compounds and exerts antibacterial, antiviral, anticarcinogenic, antimutagenic, anti-inflammatory, antiallergic, and vasodilatory effects^[14]. Various animal studies have also shown its antiapoptotic effect^[15]. Several natural botanical ingredients have been reported to effectively alleviate the injury owing to their benefits on anti-inflammatory, antioxidant, and other pharmacologic properties, but the relevant mechanism remains unclear. Therefore, this study aimed to investigate whether regulation of ER stress is one of the mechanisms by which GSP protects the liver against IR.

MATERIALS AND METHODS

Animal care and use

Male Sprague-Dawley rats weighing 220-250 g obtained from Beijing Vital River Experimental Animal Technology Co. Ltd (SCXK2010-0001) were maintained at room temperature with a 12 h light/dark cycle. The rats were allowed to move freely, and food and water were available *ad libitum*. All procedures involving animals were reviewed and approved by the Institutional

Table 1 Suzuki scores for the assessment of liver damage after liver ischemia/reperfusion

Score	Congestion	Vacuolization	Necrosis
0	None	None	None
1	Minimal	Minimal	Single cell necrosis
2	Mild	Mild	< 30%
3	Moderate	Moderate	30%–60%
4	Severe	Severe	> 60%

Table 2 Primer sequences

Gene	Sequence
Interleukin-6	F: 5'-CCCTGCGTTCTCTGCAAAC-3' R: 3'-TTTCAGGGTGAAGGCAGAC-5'
Tumor necrosis factor- α	F: 5'-CATCCGTTCTCTACCCAGCC-3' R: 3'-AATTCGTGAGCCCGGAGTTGG-5'
Interleukin-10	F: 5'-CCTCTGATACAGCTGCGAC-3' R: 3'-GTAGATGCCGGTGGTTCAA-5'
Tumor growth factor- β 1	F: 5'-AGGGCTACCATGCCAACTTC-3' R: 3'-CCACGTAGTAGACGATGGGC-5'

Animal Care and Use Committee of the Nanjing Medical University (IACUC protocol number: NJMU08-092).

Surgery and experimental design

The rats were randomly divided into three groups: sham, IR, and GSP groups ($n = 8$ each). GSP (purity > 95%; Tianjin Jianfeng Natural Product R&D Co., Ltd., Tianjin, China) was dissolved in distilled water and administered to the GSP group (a daily dose of 100 mg/kg)^[16] by oral gavage for 15 d prior to surgery. The sham and IR groups received equal amounts of saline (0.9%) for 15 d. None of the animals died during the procedure.

Surgery was performed on rats after a 10-h abrosia. Under the intraperitoneal injection of hydrate (10%, 3 mL/kg) anesthesia, a midline laparotomy was made using minimal dissection. Hepatic ischemia (70%) was induced for 60 min by clamping the portal vein, hepatic artery, and bile duct of the left and median, and the rats were then reperused for 6 h after the surgery. The rats were anesthetized, and tissue blood samples were collected. Parts of the hepatic tissue samples were stored at -80 °C for Western blot and reverse transcription (RT)-PCR analyses. Other parts of the hepatic tissue samples were placed in formaldehyde (10%) for histologic evaluation^[17] and TUNEL staining^[18]. Blood samples were collected from the rats' vena cavae using a Bioclean injector (5 mL) and centrifuged at 3000 r/min for 10 min to obtain the serum, which was stored at -80 °C until further examination.

Hepatocellular function assay

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT), the indices of hepatocyte damage, were determined spectrophotometrically

using an automated analyzer in the clinical biochemical room of the First Affiliated Hospital of Nanjing Medical University.

Histopathologic analysis

For light microscopic detections, hepatic tissue specimens were fixed in 10% formaldehyde, dehydrated in alcohol series, cleared in toluene, and embedded in paraffin. Paraffin sections (5 μ m) were stained with hematoxylin and eosin and examined under a photomicroscope (Olympus BX51; Olympus Corp., Tokyo, Japan). Sections were scored from 0 to 4 for sinusoidal congestion, vacuolization of hepatocyte cytoplasm, and parenchymal necrosis as described by Suzuki *et al.*^[19] (Table 1).

Enzyme-linked immunosorbent assay

Serum levels of interleukin (IL)-6, tumor necrosis factor (TNF)- α , IL-10, and transforming growth factor (TGF)- β 1 were detected using ELISA according to the manufacturer's protocols (DL goat anti-rat TNF- α enzyme-linked immunosorbent assay (ELISA) kit, ADL goat anti-rat IL-6 ELISA kit, ADL goat anti-rat IL-10 ELISA kit, and ADL goat anti-rat TGF- β 1 ELISA kit) and are expressed as pg/mL.

RT-PCR

Quantitative analysis of the mRNA expression of IL-6, TNF- α , IL-10, and TGF- β 1 was performed by RT-PCR using 96-well optical reaction plates in the ABI Prism 7500 System (Applied Biosystems of Thermo Fisher Scientific, Waltham, MA, United States). The primers used in the current study (Table 2) were selected from the PubMed database. The measurements of each sample were performed in triplicate. The real-time PCR data were analyzed using the relative gene expression (*i.e.*, $\Delta\Delta$ CT) method. In brief, the data are presented as the fold change in gene expression normalized to the endogenous reference gene (GAPDH) and relative to a calibrator.

Serum superoxide dismutase and methane dicarboxylic aldehyde concentration assays

The serum superoxide dismutase (SOD) activity and the methane dicarboxylic aldehyde (MDA) concentration were determined using assay kits (Nanjing Jiancheng Corp., China) following the manufacturer's recommendations. The amount of MDA was measured by reaction with thiobarbituric acid at 532 nm with a Perkin Elmer Lambda 20 spectrophotometer (Norwalk, CT, United States). The values were calculated using the molar extinction coefficient of chromophore (1.56 \times 10 mol/L per cm). The SOD assay was conducted using a modified pyrogallol autoxidation method, and the activity was measured at 420 nm^[20].

TUNEL staining

The anesthetized animals were perfused with formaldehyde, and the livers were removed and processed

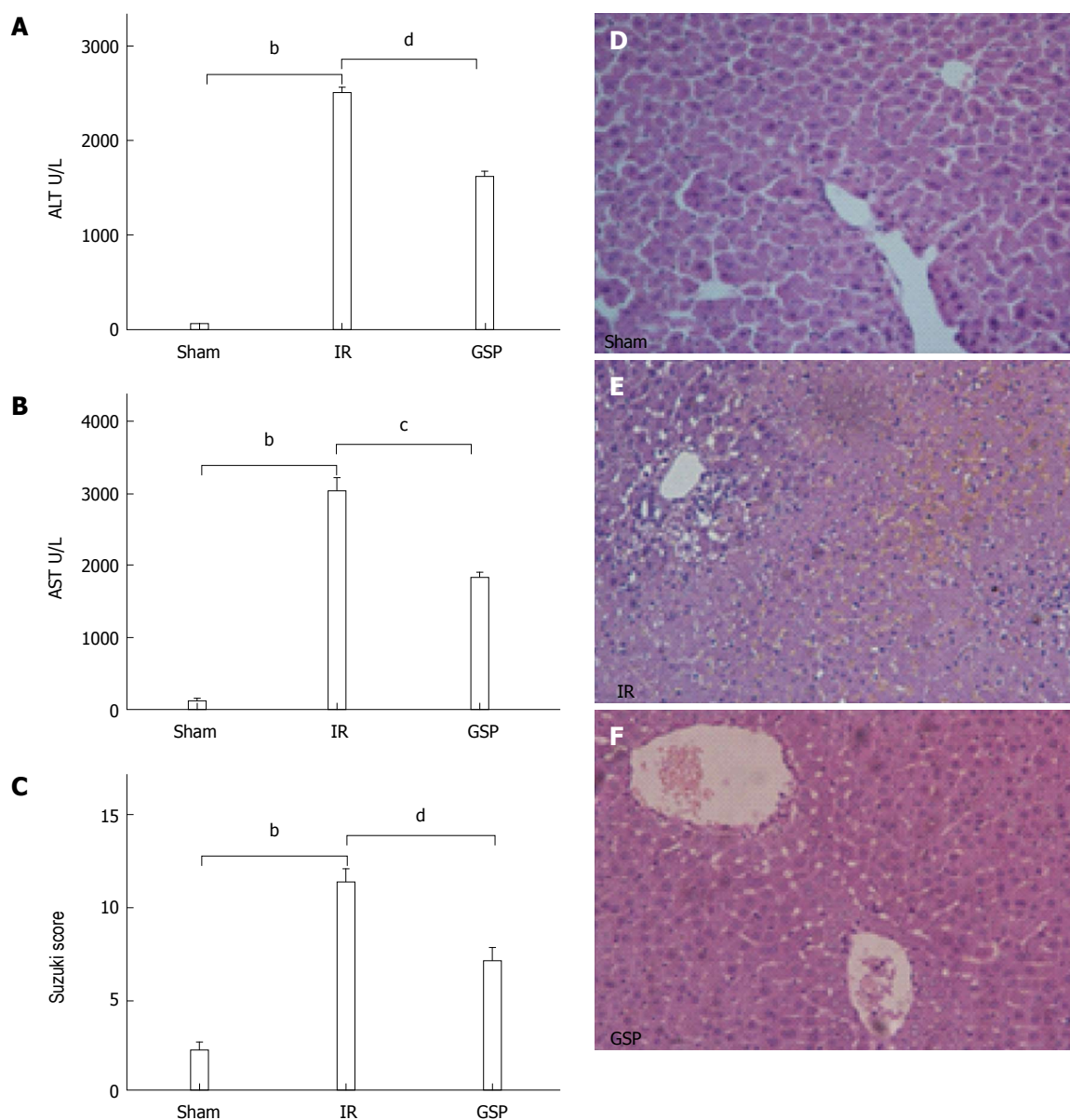


Figure 1 Effects of grape seed proanthocyanidin on serum aminotransferase levels after reperfusion. The levels of A: Alanine aminotransferase (ALT); and B: Aspartate aminotransferase (AST) after ischemia/reperfusion (IR) and with grape seed proanthocyanidin (GSP) treatment. C: Histopathologic changes were scored (Suzuki score) in tissues collected 6 h after reperfusion; D: The sham group showed normal liver structure; E: IR induced severe lobular distortion, apparent edema, hemorrhage, neutrophil infiltration, and cell apoptosis; F: GSP administration improved the condition (HE, 200 ×); *n* = 8 each; ^a*P* < 0.05, ^b*P* < 0.01 vs IR.

as previously described^[21]. The KlenowFragEL DNA Fragmentation Detection Kit (EMD Chemicals, Gibbstown, NJ, United States) was used to detect the DNA fragmentation characteristic of apoptosis in formalin-fixed, paraffin-embedded liver sections^[22]. Results were scored semi-quantitatively by averaging the number of TUNEL⁺ apoptotic cells/microscopic field at 200 × magnification. Ten fields were evaluated per tissue sample.

Western blot analysis

Proteins (40 μg/sample) from frozen liver samples were subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-

Rad Laboratories Inc., Hercules, CA, United States). Anti-GRP78, anti-CHOP, anti-ATF-4, anti-IRE-1, anti-procaspase-12, anti-NF-κb, and anti-GAPDH were obtained from Abcam (Cambridge, United Kingdom). The relative quantities of proteins were determined by a densitometer and expressed in absorbance units.

Statistical analysis

Data analysis was carried out using GraphPad Prism 5.0 (GraphPad Software Inc. La Jolla, CA, United States). All data are expressed as mean ± SE. Differences between groups were statistically analyzed by analysis of variance and Dunnett’s test for unpaired data when appropriate. A *P* < 0.05 was considered statistically

significant.

RESULTS

GSP treatment protects liver against IR injury

Serum aminotransferase level is an important indicator of liver injury, which significantly increases after IR and peaks 6 h after reperfusion^[20]. Figure 1 shows that the detected serum ALT (Figure 1A) and AST (Figure 1B) levels were significantly increased in the IR group compared with the sham group ($P_s < 0.05$). Interestingly, GSP treatment significantly decreased these levels compared to the IR group ($P_s < 0.05$).

Whether GSP treatment could alter the liver pathology after IR was also examined. After reperfusion, the pathologic features of IR liver tissue displayed severe lobular distortion with widespread necrosis, apparent edema, hemorrhage, and neutrophil infiltration in the IR group (Figure 1E). However, the GSP treatment considerably relieved the aforementioned pathologic changes. Mild architectural damage characterized by interstitial edema and less neutrophil infiltration was observed (Figure 1F). A significantly higher Suzuki score was observed in the IR group, which was attenuated with GSP treatment ($P_s < 0.05$) (Figure 1C).

GSP treatment increase anti-inflammatory cytokines but decreases proinflammatory cytokines

To detect the effect of GSP on the involvement of inflammatory cytokines in liver IR, the serum concentrations (by ELISA) and mRNA expression levels (by RT-PCR) of TGF- β 1, IL-10, TNF- α , and IL-6 were measured. Compared with the IR group, the serum levels of TGF- β 1 and IL-10 were significantly increased in the GSP group ($P_s < 0.05$) (Figure 2A and B). GSP treatment significantly reduced the secretion of TNF- α and IL-6 ($P_s < 0.05$) (Figure 2C and D). To further analyze whether the increase in serum cytokines was caused by intrahepatic production, the expression of hepatic cytokines was measured by RT-PCR, which showed a similar trend of change in mRNA expression. GSP treatment reversed the decrease in the anti-inflammatory cytokines TGF- β 1 (Figure 2E) and IL-10 (Figure 2F) and increased the proinflammatory cytokines IL-6 (Figure 2G) and TNF- α (Figure 2H) induced by IR.

GSP treatment increases SOD level and decreases MDA concentration

SOD and MDA are two indices of oxidative stress. The SOD level was decreased in the IR group compared with the sham group, but the SOD level in the GSP group was significantly increased compared with the IR group (all $P < 0.01$) (Figure 3A). However, the MDA concentration was significantly increased in the IR group, which was significantly attenuated by GSP (all $P < 0.01$) (Figure 3B).

GSP reduces apoptosis induced by ER stress

The result of TUNEL staining is shown in Figure 4. TUNEL⁺ cells were barely observed in the GSP group (Figure 4C), but were abundantly observed in the IR group (Figure 4B). Figure 4D shows the decrease in the number of apoptotic cells in the GSP group compared with the IR group. Protein procaspase-12 (Figure 5D), one of the apoptosis-regulated proteins, increased abundantly in the IR group compared with the GSP group as detected by Western blot.

GSP treatment attenuates the protein expression of GRP78, IRE-1, and ATF-4 and downregulates IRE-1-NF- κ b and ATF-4-CHOP signaling

GRP78, IRE-1, and ATF-4 are markedly expressed proteins when severe ER stress occurs. As shown in Figure 5, the expression of ER stress protein GRP78 was increased in the IR group compared with the sham group, whereas the rats treated with GSP showed a much higher GRP78 expression compared with the IR group. However, the proteins IRE-1 and ATF-4 exhibited reversed results: the IR group showed a higher expression level than the GSP group. This phenomenon indicates that ER stress was successfully induced in the animal models, and GSP treatment could moderately increase the GRP78 expression and decrease the expression of IRE-1 and ATF-4.

NF- κ b is a classical factor involved in the generation of the inflammatory response. NF- κ b can be regulated by the IRE1-NF- κ b signaling pathway^[2]. The expression of NF- κ b and IRE-1 was increased in the IR group compared with the sham group (Figure 5). However, this was attenuated by GSP treatment.

Previous studies reported that ATF-4-CHOP^[2,23] is one of the signaling pathways of ER stress. Thus, ATF-4 expression in the liver tissues was analyzed by Western blot. The expression of ATF-4 and CHOP were increased with IR, whereas the rats treated with GSP showed comparatively lower levels (Figure 5).

DISCUSSION

This study shows that GSP protects the liver against IR injury with anti-inflammatory, antioxidative, and antiapoptotic effects by alleviating ER stress.

The increased ALT and AST coupled with the pathologic changes in the IR group revealed the severe damage induced by IR. Reduced ALT and AST levels and the minor pathologic changes in the GSP group confirmed the protective effect of GSP on IR. Meanwhile, the high level of ER stress proteins (*e.g.*, GRP78, IRE-1, and ATF-4) indicate that ER stress was successfully induced in the animal models in the IR group compared with the sham group. GSP treatment effectively decreased the expression of those proteins, indicating that ER stress may be relieved by GSP treatment. The relationship between the subdued ER stress and the IR

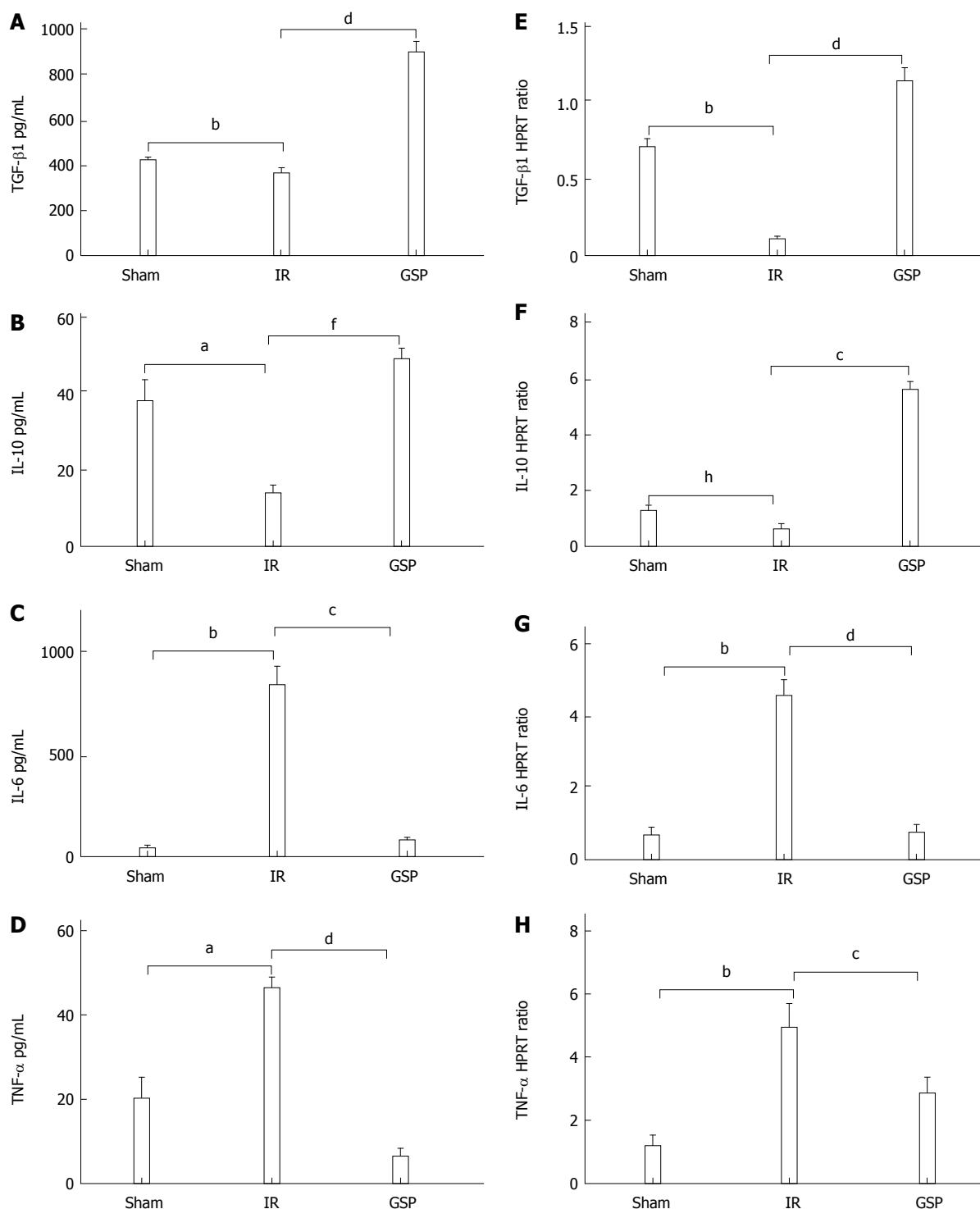


Figure 2 Effects of grape seed proanthocyanidin on inflammatory factors after reperfusion. A-D: ELISA; and E-F: Reverse-transcription PCR were performed to evaluate release and expression of transforming growth factor (TGF)-β1, interleukin (IL)-10, IL-6, and tumor necrosis factor (TNF)-α after ischemia/reperfusion (IR) and with grape seed proanthocyanidin (GSP) treatment; *n* = 8 each; ^a*P* < 0.05, ^b*P* < 0.01 vs IR.

injury is unknown.

The inhibition of inflammation to alleviate IR injury was also investigated. While IL-6 and TNF-α decreased in the GSP group, TGF-β1 and IL-10 increased. TGF-β1 and IL-10 inhibit the production of proinflammatory cytokines, such as IL-1, IL-6, IL-8, and TNF-α^[24]. Thus, we speculate that GSP relieves the inflammation response by decreasing proinflammatory factors and

increasing anti-inflammatory factors. Another study indicated that GSP inhibits the production of TNF-α and IL-17a from T cells by inhibiting NF-κB^[25]. The ROS-NF-κB signaling pathway is one of the downstream pathways involved in ER stress. Antioxidants reduce NF-κB activation^[26]; in the present study, GSP treatment decreased MDA and NF-κB and increased SOD. Thus, GSP likely inhibited inflammation through the ROS-

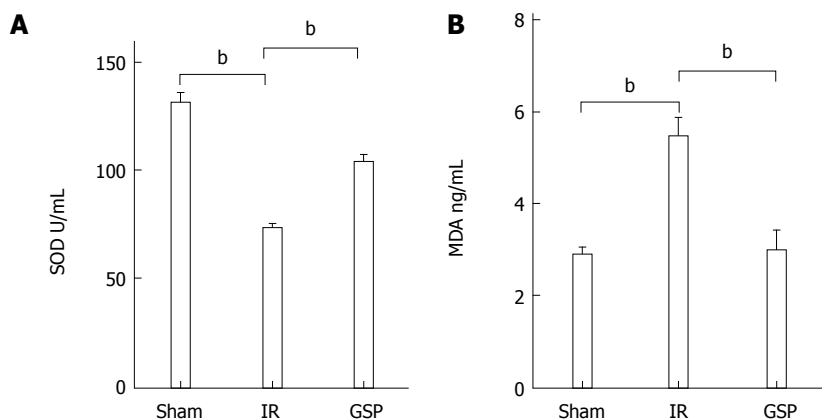


Figure 3 Effects of grape seed proanthocyanidin on superoxide dismutase and methane dicarboxylic aldehyde levels. Levels of A: superoxide dismutase (SOD); and B: Methane dicarboxylic aldehyde (MDA) after ischemia/reperfusion (IR) and with grape seed proanthocyanidin (GSP) treatment; $n = 8$; $^bP < 0.01$ vs IR.

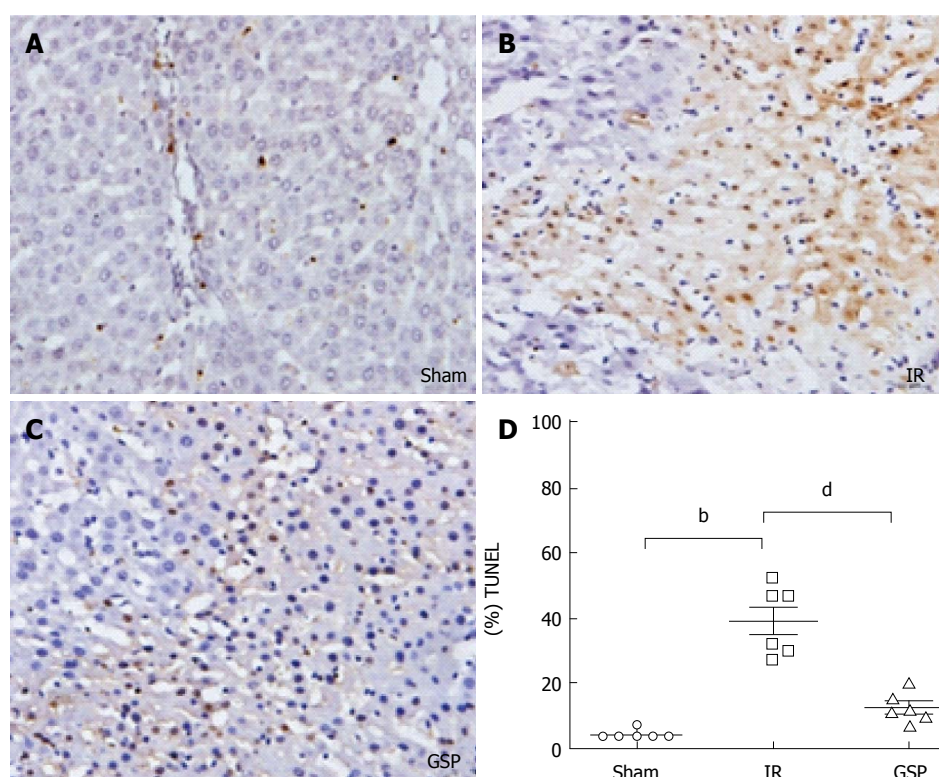


Figure 4 Effects of grape seed proanthocyanidin on hepatocytic apoptosis. TUNEL staining was performed and 100 cells per field were counted in three fields (400 \times). A: Sham; B: Ischemia/reperfusion (IR); and C: IR with grape seed proanthocyanidin (GSP) treatment; D: Quantification of TUNEL staining; $n = 8$ each; $^bP < 0.01$ vs IR.

NF- κ b signaling pathway. However, NF- κ b could also be relieved by GSP through the IRE-1-NF- κ b signaling pathway, as IRE-1-mediated TNF receptor-associated factor 2 can promote NF- κ b-mediated inflammation^[8]. In the present study, IRE-1 and NF- κ b were significantly decreased as detected by Western blot. In summary, GSP relieved the inflammation induced by ER stress in the IR rats.

The antiapoptotic role of GSP in IR rats *via* relief of ER stress was explored. In the GSP group, the antiapoptotic effect of GSP was affirmed by decreasing procaspase-12 expression and reduced numbers of apoptotic cells compared with the IR group. The

mechanism involved may include the following. First, GSP can affect caspase-12-dependent cell apoptosis induced by ER stress. When ER stress occurs, procaspase-12 is activated after its dissociation from the ER membrane^[27], thus initiating downstream apoptotic pathways^[27]. Caspase-12-deficient mice are resistant to ER stress-induced apoptosis^[28], which supports this finding. The current results demonstrate that the level of procaspase-12 is downregulated with GSP treatment, which inevitably alleviates activation of the pathway. Second, the proapoptotic transcription factor CHOP, downstream of the ATF-4 pathway in UPR^[29], is vital in ER stress-induced apoptosis^[30]. A

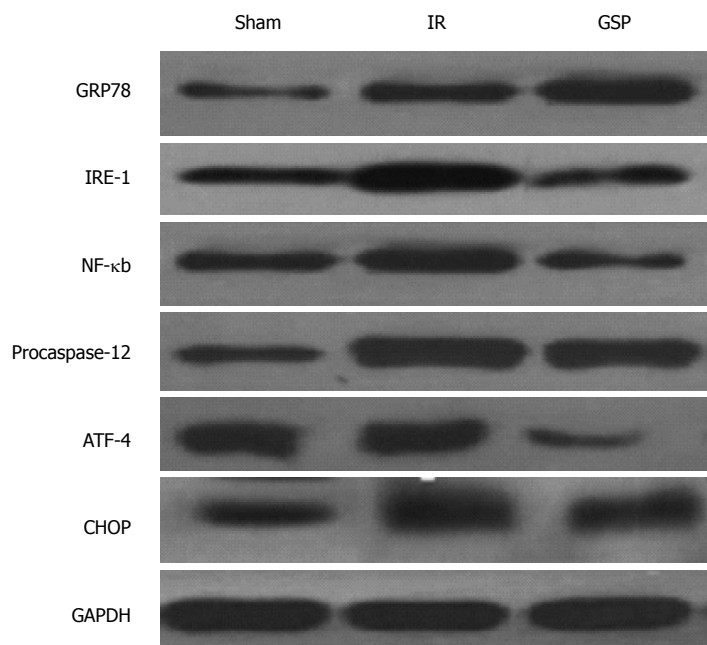


Figure 5 Effects of grape seed proanthocyanidin on the protein expression of endoplasmic reticulum stress proteins in the liver. The expression of glucose-regulated protein (GRP)78, inositol-requiring enzyme (IRE)-1, nuclear factor (NF)- κ B, procaspase-12, activating transcription factor (ATF)-4, and CCAAT/enhancer-binding protein homologous protein (CHOP) was assessed by Western blotting after ischemia/reperfusion (IR) and with grape seed proanthocyanidin (GSP) treatment. GAPDH was used as a loading control.

component of this pathway, P-eIF2, plays a key role in cell death signaling and results in increased activation of ATF-4 and CHOP^[31] when ER stress is initiated. The transcription factor ATF-4 induces CHOP transcription through the branch signal mediated by p-eIF2^[32]. The results of the present study show that the expression of ATF-4 and CHOP is significantly decreased in the GSP group compared with the IR group. Therefore, downregulation of the ATF-4-CHOP signaling pathway may be one of the protective mechanisms. Moreover, the higher increase of GRP78 protein in the GSP group than in the IR group suggests that GSP may effectively relieve ER stress by increasing GRP78. The increase in proper protein folding by upregulation of GRP78 was shown in an experiment where tunicamycin treatment easily induced ER stress in GRP78-depleted cells^[26,33]. Upregulation of GRP78 reduced the expression of CHOP and apoptosis during the ER stress^[30]. Nevertheless, pathologic development in the liver after IR involves a variety of complicated mechanisms, though the primary cause has not yet been completely clarified. Thus, the relationship between the effects of GSP (such as antiapoptotic, anti-inflammatory, and antioxidative effects) and the attenuation of ER stress should be further explored. In addition, further research is needed to identify an effective drug within the ER-stress pathway that hastens the fight against liver and other systemic diseases.

In conclusion, this study demonstrates that GSP possesses antioxidative, anti-inflammatory, and antiapoptotic effects by relieving ER stress to achieve a protection against liver IR. This protective mechanism may be a result of the botanical ingredients that

comprise GSP. This finding may serve as a guide to prevent the damage induced by IR and possesses important clinical significance.

COMMENTS

Background

Liver ischemia/reperfusion (IR) injury induces the occurrence of endoplasmic reticulum (ER) stress. Numerous studies report that excessive ER stress aggravates IR injury. Grape seed proanthocyanidin (GSP) is an effective protector in IR injury. However, the detailed protective mechanisms remain unclear. Therefore, this study explored the modulation effect of GSP on protection against liver IR injury and the alleviation of ER stress.

Research frontiers

Previous studies have demonstrated that GSP possess antibacterial, antiviral, anticarcinogenic, antimutagenic, anti-inflammatory, and antiallergic effects. Furthermore, they showed that GSP relieves the IR injury via its botanical effects. However, the relative mechanism needs to be explored.

Innovations and breakthroughs

The authors explored the mechanism that GSP protects the liver against IR injury and provided a new finding of the occurrence of IR injury. This finding may serve as a guide to prevent the damage induced by IR.

Applications

This study shows that GSP possesses antioxidative, anti-inflammatory, and antiapoptotic effects by relieving ER stress to achieve protection in the liver against IR. This finding may serve as a guide to prevent the damage induced by IR and possesses important clinical significance.

Terminology

The ER regulates protein folding, calcium storage, and the biosynthesis of macromolecules such as steroids, lipids, and carbohydrates. Various stimuli increase the accumulation of unfolded proteins in the lumen of ER, leading to ER stress. GSP is abundant in phenolic compounds and exerts antibacterial, antiviral, anticarcinogenic, antimutagenic, anti-inflammatory, antiallergic, and vasodilatory effects.

Peer-review

This is a good descriptive study in which the authors suggest that GSP can

protect rat liver from IR injury by its anti-inflammatory, antioxidative, and antiapoptotic effects and attenuating liver ER stress. The authors present GSP as a potent reagent.

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