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***Basic Study***

**G****rape seed proanthocyanidin protects liver against ischemia/reperfusion injury by attenuating** **endoplasmic-reticulum stress**

Xu ZC *et al.* Grape seed proanthocyanidin attenuates ER-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 Affiiated 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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**Correspondance to: Guo-Qiang Li, MD, PhD,**Liver Transplantation Center, First Affiiated 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 modulation effect of grape seed proanthocyanidin(GSP) on protectin liver ischemia/reperfusion (I/R) injury and the alleviation of endoplasmic reticulum (ER) stress.

**METHODS**: Male Sprague–Dawley rats (220–250 g) were divided into three groups, namely, sham, I/R, and GSP groups (*n* = 8 in each group). A liver I/R (70%) model was established and reperfused for 6h. 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 (SOD) and methane dicarboxylic aldehyde (MDA) were detected. Western blot was conducted to analyze the expression of glucose-regulated protein78 (GRP78), C/EBP (CCAAT/enhancer binding protein) homologous protein (CHOP), activating transcription factor-4 (ATF4), inositol-requiring enzyme-1 (IRE1), (procaspase-12), and NF-κb. Apoptotic cells were detected by TUNEL staining.

**RESULTS**: We found that the serum aminotransferase, apoptotic cells, and Suzuki scores decreased in the GSP group compared with the I/R group. The MDA level was decreased in the GSP group, but the SOD level was reversed. Similarly, GSP downregulated the pro-inflammatory factors and upregulatedthe levels of anti-inflammatory factors. Western blot data showed that GSP increased GRP78 expression and suppressed CHOP, ATF4, IRE1, procaspase-12, and NF-κb expression compared with the I/R group.

**CONCLUSION**: In summary, GSP possesses anti-oxidation, anti-inflammatory, and anti-apoptosis effects by relieving ER stress through regulating relative signaling ways to achieve the defensive effect on liver injury.

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

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**Core tip:** Liver ischemia/reperfusion (I/R) injury induces the occurrence of endoplasmic reticulum (ER) stress. Numerous studies reported that immoderate ER stress aggravates I/R injury. Grape seed proanthocyanidin (GSP) is an effective protector in I/R injury. However, the detailed protective mechanisms remain unclear. Therefore, this study explored the modulation effect of GSP on protectin liver I/R injury and the alleviation of ER stress. From our study, we draw a conclusion that GSP possesses anti-oxidation, anti-inflammatory, and anti-apoptosis effects by relieving ER stress through regulating relative signaling ways to achieve the defensive effect on liver injury.

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**INTRODUCTION**

Ischemia/reperfusion (I/R) injury of the liver can occur in severalclinical settings, such as hepatic trauma, resection of large intrahepatic tumors, and liver transplantation[[1](#_ENREF_1)], resulting in cell death and tissue destruction[[2](#_ENREF_2)]. Increasing evidences have shown that both pro-inflammatory cytokines andreactive oxygen species (ROS) are key mediators of liverI/R injury[[3](#_ENREF_3)]. However, the exact mechanisms about endoplasmic reticulum (ER) stress on I/R is not clearly elucidated.

ER is a key organelle of 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 Ca2+ overload[[4](#_ENREF_4)].The ER protein folding capacity is reduced under stress, leading to accumulated unfolded proteins. A major response to ER stress is the activation of glucose-regulated protein78 (GRP78) through dissociation from its transmembrane receptor, which allows subsequent regulation of the levels of accumulated unfolded proteins[[5](#_ENREF_5)]. Slight and medium ER stress can protect cells from death, but severe ER stress induces caspase-12-dependent cell apoptosis[[6](#_ENREF_6)]. When severe ER stress occurs, ATF-4 can increase the expression of CHOP, promoting ER stress response through numerous mechanisms, and CHOP promotes oxidative stress inflammation and apoptosis[[7](#_ENREF_7)]. IRE1 is an important protein relative to ER stress and is vital to the occurrence of inflammation induced by ER stress, and the classical factor of NF-κb is activated simultaneously[[8](#_ENREF_8)]. Numerous studiesshow thatER stress plays a critical role in a variety of processes[[9](#_ENREF_9)] and is also important in the occurrence of I/R damage[[10](#_ENREF_10)]. Datahave suggested that attenuating ER stress-induced apoptosis can protect brain against I/R injury[[11](#_ENREF_11)]. Thus, ER stress is closely related toI/R injury.

Proanthocyanidins are highly bioavailable and provide a significantly greater protection against damage from oxidative stress than vitamin C, vitamin E, and β-carotene[[12](#_ENREF_12)]. ER stress intensifiesdifferent types of emergency damages in the fields leading to inflammation, oxidative stress injury, and abnormal cell apoptosis. Thus, the botanical ingredientspreviously mentioned can effectively inhibit the occurrence of injury[[13](#_ENREF_13)] induced by ER stress. Grape seed proanthocyanidin (GSP) is abundant in phenolic compounds and exerts anti-bacterial, anti-viral, anti-carcinogenic, anti-mutagenic, anti-inflammatory, anti-allergic, and vasodilatory effects[[14](#_ENREF_14)]. Various animal studies have also shown its anti-apoptotic effect[[15](#_ENREF_15)]. Several natural botanical ingredients have been reported to effectively alleviate the injury owing to their benefits on anti-inflammatory,antioxidant,and other pharmacological properties, but the relevant mechanismremains unclear. Therefore, this study aimed to investigate whether regulated ER stress by GSP is one of the mechanisms that protectsliver against I/R.

**MATERIALS AND METHODS**

***Animal care and use***

Male Sprague–Dawley ratsweighing 220–250g obtained from Beijing Vital River Experimental Animal Technology Co. Ltd (SCXK2010-0001) weremaintained at room temperature with a 12h 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 Animal Care and Use Commitee of the Nanjing Medical University (IACUC protocal number: NJMU08-092).

***Surgeryandexperimental design***

The rats were randomly divided into three groups: sham, I/R, and GSP groups (*n* = 8 in each group). GSP (purity > 95%; Tianjin Jianfeng Natural Product R&D, Co. Ltd) was dissolved in distilled water and administered to the GSP group (a daily dose of 100 mg/kg)[[16](#_ENREF_16)] by oral gavage for 15 d prior to surgery. The sham and I/R groups received equal amounts of saline (0.9%) for 15 d. None of the animals died during the procedure.

Surgery was performedon rats after a 10-hour abrosia. Under the intraperitoneal injection of hydrate (10%, 3 mL/kg) anesthesia, a midlinelaparotomy 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 reperfusedfor 6h after the surgery. The rats wereanesthetized, and tissueblood samples were collected. Parts of the hepatic tissue samples were stored at -80 °C forWesternblot and RT-PCR analyses.Other parts of the hepatic tissue samples were placed in formaldehyde (10%) for histological evaluation[[17](#_ENREF_17)] and TUNNEL staining[[18](#_ENREF_18)]. Blood samples were collected from the rats vena cava 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.

***Histopathological 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 μm) were stained with hematoxylin and eosin and examined under a photomicroscope (Olympus BX51; 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](#_ENREF_19)] (Table2).

***Enzyme-linked immunosorbent assay***

Serumlevels of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interleukin-10 (IL-10), and transforming growth factor-β1 (TGF-β1) were detected by an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s protocol (ADL goat anti-rat TNF-α ELISA kit, ADL goat anti-rat IL-6 ELISA kit, ADL goat anti-rat IL-10 ELISA kit, and ADL goat anti-rat TGF-β1 ELISA kit) and expressed as pg/mL.

***Real-time reverse-transcriptase polymerase chain reaction***

Quantitative analysis of themRNA expression of IL-6, TNF-α, IL-10, and TGF-β1genes was performed by RT-PCR by subjecting thecDNAgenerated from theaforementioned reaction to PCRamplification using 96-well optical reaction plates in theABI Prism 7500 System (Applied Biosystems). The primersused in the current study (Table 1) were selected fromthe PubMed database.The measurements of each sample were performed in triplicate. The real-time PCR data were analyzed using the relative geneexpression (*i.e.,* ΔΔCT) method. In brief, the data are presented as the fold change in gene expressionnormalized to the endogenous reference gene (GAPDH) and relative to a calibrator.

***Serum SOD and MDA concentration assay***

The serum SOD activity and the MDA concentration were determined using an assay kit (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 co-efficient of chromophore (1.56 × 10 mol/L/cm). SOD assay was conducted using modified pyrogallolautoxidation method, and the activity was measured at 420 nm[[20](#_ENREF_20)].

***TUNEL staining***

The anesthetized animals were perfused with paraformaldehyde, and the livers were removed and processed as previously described[[21](#_ENREF_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](#_ENREF_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 membrane (Bio-Rad, Hercules, CA, United States). Anti-GRP78, anti-CHOP, anti-ATF4, anti-IRE1, anti-procaspase-12, anti-NF-κb, and anti-GAPDH were obtained from Abcam (Kendall Square Suite B2304, Cambridge, MA 02139-1517, United States).The relative quantities of proteins were determined by a densitometer and expressed in absorbance units.

***Statistical analysis***

Data analysis was carried out using GraphPad Prism5.0 (GraphPad Software, San Diego, CA, United States). All data were expressed as mean ± SE. Differences between groups were statistically analyzed by ANOVA andDunnett test for unpaired data when appropriate. A *P* < 0.05 was considered statistically significant.

**RESULTS**

***GSP treatmentprotects liver against I/R injury***

Serum aminotransferase level is an important indicator of liver injury, which significantly increases after I/R and peaks 6 h after reperfusion[[20](#_ENREF_20)]. Figure 1 shows that the detected serum ALT (Figure 1A) and AST (Figure 1B) levels were considerably increased in the I/R group compared with the sham group.Interestingly, GSP treatment could significantly decrease the increase induced by I/R with respect to the I/R group. GSP could evidently decrease the aminotransferase level induced by I/R. We examined whether GSP treatment could alter the liver pathology after I/R. After reperfusion, the pathologic features of I/R liver tissue displayed severe lobular distortion with widespread necrosis, apparent edema, hemorrhage, and neutrophil infiltration in the I/R group(Figure 1E). However, the GSP treatment considerably relieved the aforementioned pathologic changes, even not displayed.Amild architectural damage characterized by interstitial edema and less neutrophil infiltration was observed (Figure 1F). From the Suzuki score (Figure 1C), a much higher score appeared in the I/R group,whereas GSP treatment presented a decreasing grade. We speculated that GSP treatment effectively alleviated the pathologic changes induced by I/R.

***GSP treatment increasesanti-inflammatory cytokines but decreases pro-inflammatory cytokines***

To detect the effect of GSP on the involvement of inflammatory cytokines in liver I/R, we detected the serum concentration (by ELISA) and the mRNA expression level (by RT-PCR) of TGF-β1, IL-10, TNF-α, and IL-6. Compared with the I/R group, the serum levels of TGF-β1 and IL-10 were significantly increased in the GSP group (Figure 2A and B). GSP treatment considerably reduced the secretion of TNF-α and IL-6 (Figure 2C and D). To further analyze whether the increase in serum cytokines was caused by intrahepatic production, we detected the mRNA expression of hepatic cytokines by real-time PCR. As shown inRT-PCR detection, a similar trend of mRNA change was observed in RT-PCR. Thus, GSP treatment could reverse the decreased anti-inflammatory cytokines including TGF-β1 (Figure 2E) and IL-10 (Figure 2F) and the increased pro-inflammatory cytokinesincluding IL-6 (Figure 2G) and TNF-α (Figure 2H) induced by the I/R.

***GSP treatment increases SOD level and decreases MDA concentration***

SOD (Figure 3A) and MDA (Figure 3B) are two indices of oxidative stress. The SOD decreased in the I/R group compared with the sham group, but the SOD level in the GSP group significantly increased compared with the I/R group. However, the MDA concentration of the I/R group increased higher than the GSP group. This result reveals that GSP could effectively decrease the injury of ROS (SOD, MDA) induced by ER stress.

***GSP relieves the apoptotic level induced by ER stress***

The result of TUNEL staining is shown in Figure 4. TUNEL-positive cells were barely observed in the GSP group (Figure 4C), but were abundantly observed in the I/R group (Figure 4B). It (Figure 4D) shows the decreased apoptotic cells in the GSP group compared with the I/R group. Protein procaspase-12 (Figure 5D), one of the apoptosis-regulated proteins, increased abundantly in the I/R group compared with the GSP group as detected by Western blot. We speculated that GSP treatment could exert its anti-apoptosis effect based on our findings.

***GSP treatment relieves the expression of protein GRP78, IRE1, and ATF-4 and downregulatesIRE1–NF-κb, ATF-4-CHOP signaling pathways***

GRP78, IRE1, and ATF-4 are marked proteins when severe ER stress occurs. As shown in Figure 5, the expression of ER stress protein GRP78 (Figure 5A) 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 I/R group. However, the proteins IRE1 (Figure 5B) and ATF-4 (Figure 5E) 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 relieve the injury by moderately increasing the GRP78 expression and decreasing the IRE1 and ATF-4 expressions.

NF-κb is a classical factor to the generation of inflammatory response. NF-κb can be regulated by the IRE1-NF-κb signaling pathway[[2](#_ENREF_2)]. Apparently, the NF-κb expressed higher levels when the expression of IRE1 increased. Figure 5 shows that much higher expression of IRE1 (Figure 5B) and NF-κb (Figure 5C) were observed in the I/R group compared with the sham group. However, the increase induced by I/R could be much reversed by GSP treatment. We speculated that the relieved inflammatory response in the GSP group may be connected with the attenuated ER stress by inhibiting IRE1 expression.

Previousstudies reported that PERK-elF2-ATF4-CHOP[[2](#_ENREF_2),[23](#_ENREF_23)] is one of the signaling pathways of ER stress. Thus, ATF-4 expression in the liver tissues was analyzed by Western blot.The high expression level of ATF4 (Figure 5E) markedly increased the CHOP (Figure 5F) expression, whereas the rats treated with GSP showed a low level of ATF4 and CHOP compared with the I/R group. This result indicates that GSP could markedly relieve theapoptosis level by this signal pathway to protect the liver againstI/R.

**DISCUSSION**

This study revealedthat GSP protected the liver against I/R injury owing to its anti-inflammatory, anti-oxidate, and anti-apoptotic effects by alleviating ER stress.

The increased ALT (Figure 1A) and AST (Figure 1B) coupled with the pathological changes in the I/R (Fig. 1E) group than the sham group (Figure 1D) revealed the severe damage induced by I/R. Reduced ALT (Figure 1A) and AST (Figure 1B) levels and the minor pathologic changes in the GSP group (Figure 1F) compared with the I/R group (Figure 1E) confirmed the protective effect of GSP on I/R. Meanwhile, the high level of ER stress proteins [GRP78 (Figure 5A), IRE1 (Figure 5B), and ATF-4 (Figure 5E)] indicated that ER stress was successfully induced in the animal models in the IR group compared with the sham group. GSP treatment could effectively decrease 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 injury is unknown.

The inhibition of inflammation to alleviate I/R injury was investigated. We observed that the IL-6 (Figure 2C and G) and TNF-α (Figures 2D and 4H) decreased in the GSP group, but the TGF-β1 (Figure 2A and E) and IL-10 (Figure 2B and F) were inversed. TGF-β1 and IL-10 inhibited the production of pro-inflammatory cytokines, such as IL-1, IL-6, IL-8, and TNF-α[[24](#_ENREF_24)]. Thus, we speculated that GSP relievedthe inflammation response by decreasing pro-inflammatory 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-kb[[25](#_ENREF_25)]. The ROS-NF-κb signaling way is one of down-streaming ways for ER stress. Antioxidants reduce NF-κb activation[[26](#_ENREF_26)]; in the present study, the GSP treatment decreased MDA (Fig. 3B) and NF-κb (Figure 5C) and increased SOD (Figure 3A). We may conclude that GSP inhibited inflammation through the ROS-NF-κb signaling way. However, we also observed that NF-κb could be relieved by GSP through the IRE1-p-IRE1-IKK-TRAF2-NF-κb signaling way. IRE1-mediated tumor necrosis factor receptor-associated factor2 (TRAF2) can promote NF-κb-mediated inflammation[[8](#_ENREF_8)]. In our experiment, the IRE1 (Figure 5B) and NF-κb (Figure 5C) were significantly decreased as detected by western blot. In summary, GSP relieved the inflammation induced by ER stress in the I/R rats.

The anti-apoptosis role of GSP in I/R rats by relieving ER stress was explored. In the GSP group, the anti-apoptotic effect of GSP was affirmed by decreasing procaspase-12 expression (Figure 5D) and protectingapoptosis (Figure 4C) compared with the I/R group (Figure 4B). The mechanism involved includes the following: first, GSP can affect caspase-12-dependent cell apoptosis induced by ER stress. Caspase-12, which is regularly in the presence of inactive form of procaspase-12, is linked with the ER membrane[[27](#_ENREF_27)]. When ER stress occurs, the procaspase-12 is activated after its dissociation with ER. With its activation, the downstream apoptotic pathways areinitiated[[27](#_ENREF_27)]. Caspase-12-deficient mice are reported to be resistant to ERstress-induced apoptosis[[28](#_ENREF_28)], which supports our finding. Our results demonstrated that the level of procaspase-12 was considerably downregulatedby GSP, which inevitably alleviated the pathway. Secondly, the proapoptotic transcription factor CHOP, downstream of the PERK-eIF2-ATF4 pathway in UPR[[29](#_ENREF_29)], is vital in ER stress-induced apoptosis[[30](#_ENREF_30)]. P-eIF2plays a key role in cell death signal pathway and results in a significantly increased activation of ATF4 and CHOP[[31](#_ENREF_31)] when ER stress is initiated. The transcription factor ATF4 induces the CHOP transcription through the branch signal mediated by p-eIF2[[32](#_ENREF_32)].Our result demonstrated that the expression level of ATF-4 and CHOP was significantly decreased in the GSP group compared with the I/R group. Therefore, we may deduce that downregulatingthe ATF4-CHOP signal way may be one of the protective mechanisms. Moreover, the higher increase of protein GRP78 in the GSP group than that in the I/R groupindicated that GSP may effectively relieve ER stress by increasing GRP78. The effect of upregulated GRP78 increasing proper protein folding is confirmed by the experiment that tunicamycin treatment easily induces ER stress in the GRP78-depleted cells[[26](#_ENREF_26),[33](#_ENREF_33)]. Up regulating GRP78 reduced the expression of CHOP and apoptosis during the ER stress[[30](#_ENREF_30)]. Nevertheless, liver I/R pathological development processinvolvesa variety of complicated mechanisms, but primary causehas not yet been completely clarified. In this research, we propose that the relationship betweenthe effects of GSP (including anti-apoptosis, anti-inflammation, and anti-oxidant) and the attenuation of ER stress should be further explored. In addition, further research is important to identify an effective drug within the ER stress pathway that hastens the fight against liver and other systemic diseases.

In conclusion, our study proved that GSP possesses anti-oxidant, anti-inflammatory, and anti-apoptosis effects by relieving ER stress to achieve the defensive effect on liver I/R. This may be one of protective mechanisms about the botanical ingredients to protect the liver against I/R. This finding may serve as a guide to prevent the damage induced by I/R and possess important clinical significance.

**COMMENTS**

***Background***

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

***Research frontiers***

Previous study proved that GSP possess anti-bacterial, anti-viral, anti-carcinogenic, anti-mutagenic, anti-inflammatory, anti-allergic effects. Also, it reported that GSP relived the I/R injury owing to its botanical effects. But the relative mechanism needs to be explored.

***Innovations and breakthroughs***

The authors explored the mechanism that grape seed proanthocyanidin (GSP) protected liver from ischemia/reperfusion (I/R) injury. And it may also be a new finding of the occurance of I/R injury. This finding may serve as a guide to prevent the damage induced by I/R.

***Applications***

This study proved that GSP possesses anti-oxidant, anti-inflammatory, and anti-apoptosis effects by relieving ER stress to achieve the defensive effect on liver I/R. This finding may serve as a guide to prevent the damage induced by I/R and possess important clinical significance.

***Terminology***

The ER regulates protein folding, calcium storage, and the bio-synthesis 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 anti-bacterial, anti-viral, anti-carcinogenic, anti-mutagenic, anti-inflammatory, anti-allergic, and vasodilatory effects

***Peer-review***

This is a good descriptive study in which the authors suggested that GSP can protect rat liver injury of ischemia/reperfusion by anti-inflammatory, anti-oxidate, anti-apoptotic effects and attenuating liver ER stress. The authors present the Grape Seed Proanthocyanidin as a potent reagent.

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| **Table 1 Primer sequences**

|  |  |
| --- | --- |
| Gene | Direction and sequence |
| IL-6 | F: 5′-CCCTGCGTTTCTCTGCAAAC-3′R: 3′-TTTCAGGGTGGAAGGCAGAC-5′ |
| TNF-a | F: 5′-CATCCGTTCTCTACCCAGCC-3′R: 3′-AATTCTGAGCCCGGAGTTGG-5′ |
| IL-10 | F: 5′-CCTCTGGATACAGCTGCGAC-3′R: 3′-GTAGATGCCGGGTGGTTCAA-5′ |
| TGF-β1 | F: 5′-AGGGCTACCATGCCAACTTC-3′R: 3′-CCACGTAGTAGACGATGGGC-5′ |

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|  |

**Table 2 Suzuki scores for the assessment of liver damage after liver I/R**

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

**Figure 1 Effects of grape seed proanthocyanidin on serum aminotransferase levels after reperfusion were detected.** The levels of ALT (A) and AST (B) significantly increased in the I/R group compared with the sham group. GSP administration could significantly reduce the ALT and AST levels (*n* = 8, ALT: 56.12 ± 1.41 *vs* 2494 ± 49.47, b*P* < 0.01; 2494 ± 49.47 *vs* 887 ± 79.07, d*P* < 0.01; AST: 104.3 ± 4.21 *vs* 3020 ± 174.5, b*P* < 0.01; 3020 ± 174.5 *vs* 1803 ± 94.67, c*P* < 0.05). Histopathologic analysis of livers harvested 6 h after reperfusion. Histopathologic changes (× 200): sham group (D) showed normal liver structure; severe lobular distortion, apparent edema, hemorrhage, neutrophil infiltration, and cell apoptosis were observed in the I/R group (E); GSP administration (F) could significantly improve the condition. The Suzuki score is shown in Figure 1C; the I/R group obtained a higher score than the GSP group. (*n* = 8, 2.17 ± 0.48 *vs* 11.33 ± 0.67, b*P* < 0.01; 11.33 ± 0.67 *vs* 7 ± 0.77, d*P* < 0.01).



**Figure 2 Effects of grape seed proanthocyanidin on inflammatory factors including TGF-β1, IL-10, IL-6, and TNF-α after reperfusion were detected by ELISA and RT-PCR.** ELISA reports showed thatthe serum concentrations of TGF-β1 (A) and IL-10 (B) greatly decreased; however, the concentration of TNF-α (C) and IL-6 (D) levels significantly increased in the I/R group, which were inversed by GSP administration. RT-PCR showed that the mRNA expression of TGF-β1 (E) and IL-10 (F) greatly decreased in the I/R group, but the mRNA expression of TNF-α (G) and IL-6 (H) were markedly increased in the I/R group. An opposite result was observed in the GSP group. Both the ELISA and RT-PCR analyses showed the same trend. (*n* = 8, ELISA: TGF-β1, 425.3 ± 9.01 *vs* 365.6 ± 15.82, b*P* < 0.01; 365.6 ± 15.82 *vs* 891.8 ± 41.82, d*P* < 0.01; IL-10, 37.63 ± 5.58 *vs* 13.41 ± 1.99; a*P* < 0.05; 13.41 ± 1.99 *vs* 48.38 ± 2.53, f*P* < 0.01; IL-6, 54.88 ± 2.43 *vs* 834 ± 87.89; b*P* < 0.01, 834 ± 87.89 *vs* 79.97 ± 14.12; c*P* < 0.05; TNF-α, 19.49 ± 5.53 *vs* 46.33 ± 2.13, a*P* < 0.05, 46.33 ± 2.13 *vs* 6.04 ± 2.24; d*P* < 0.01; RT-PCR: TGF-β1, 0.7017 ± 0.0489 *vs* 0.1037 ± 0.0280, b*P* < 0.001; 0.1037 ± 0.0280 *vs* 1.123 ± 0.0845, d*P* < 0.01; IL-10, 1.247 ± 0.1422 *vs* 0.6453 ± 0.0979, h*P* < 0.01, 0.6453 ± 0.0979 *vs* 5.6060 ± 0.2541, c*P* < 0.05, IL-6, 0.6798 ± 0.1545 *vs* 4.514 ± 0.4803, b*P* < 0.01; 4.514 ± 0.4803 *vs* 0.7864 ± 0.1838, d*P* < 0.05; TNF-α, 1.207 ± 0.2726 *vs* 4.9440 ± 0.7268,b*P* < 0.01; 4.9440 ± 0.7268 *vs* 2.1819 ± 0.5045, c*P* < 0.05).



**Figure 3 Effects of grape seed proanthocyanidin on the superoxide dismutas (A) and methane dicarboxylic aldehyde (B) levels were detected.** An obviously increased level of superoxide dismutase (SOD) in the GSP group was observedwith a reverse result about the methane dicarboxylic aldehyde (MDA) level. The higher SOD and lower MDA levels demonstrate the anti-oxidant effect of GSP compared with the pure I/R damage (*n* = 8, MDA: 2.88 ± 0.15 *vs* 5.45 ± 0.4, b*P* < 0.01; 5.45 ± 0.4 *vs* 2.96 ± 0.46; d*P* < 0.01 SOD: 131.4 ± 4.91 *vs* 73.13 ± 2.46, b*P* < 0.01, 73.13 ± 2.46 *vs* 103.7 ± 3.52, d*P* < 0.01).

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**Figure 4 Effects of grape seed proanthocyanidin on the hepatocytic apoptosis level was detected by tissue TUNEL staining**. We selected three fields under a microscope (× 400) and counted 100 apoptotic cells per field (A). More apoptotic cells appeared in the IR group (B) compared with the GSP group (C). Wespeculated that GSP treatment could relieve the apoptosis level induced by I/R. After statistical analysis, a graph (D) was drawn to display the apoptotic level intuitively. (*n* = 8, 3.77 ± 0.72 *vs* 38.88 ± 4.36, b*P* < 0.01; 38.88 ± 4.36 *vs* 12.62 ± 1.90, d*P* < 0.01).

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**Figure 5 Effects of grape seed proanthocyanidin on the expression levels of the ER stress proteins GRP78, IRE1, and ATF-4 in the liver tissue by Western blot analysis.** The expression of GRP78 (A) increased in the ischemia/reperfusion (I/R) group, but the increase was much lower than the GSP group. The expression of liver IRE1 (B) and ATF-4 (E) both significantly increased in the I/R group, and the increase was markedly attenuated by GSP treatment. These results demonstrated that ER stress was successfully induced in the animal models, and GSP could relieve the ER stress. Effects of GSP on the inflammatory relative protein NF-κB (C) and hepatocytic apoptosis relative protein procaspase-12 (D) were detected. Apparently, GSP treatment could effectively decrease the two proteins’ expression compared with the I/R group.The expression of ATF-4-CHOP signaling pathway relative protein CHOP (F) in the liver tissue was increased in the I/R group, and the increase was largely inversed by GSP treatment.

