RESEARCH ARTICLE

The protective role of natural phytoalexin resveratrol on inflammation, fibrosis and regeneration in cholestatic liver injury

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Liver injuries can trigger a cascade of inflammatory responses and as a result, initiate the process of hepatic regeneration and fibrogenesis. Resveratrol (RSV) has multiple health-promoting benefits. This study evaluated the potential protective effects and mechanism of RSV as related to cholestatic liver injury. RSV was given (4 mg/kg/day, i.p.) for either 3 days or 7 days after bile duct ligation (BDL) injury. RSV significantly reduced serum ALT, AST but not T-bil on Day 3. At this early stage of injury, RSV significantly reduced TNF-α and IL-6 mRNA and decreased the number of Kupffer cells ($CD68^+$) recruited in the injured liver. RSV decreased hepatic fibrosis and reduced collagen Iα1 and TIMP-1 mRNA on Day 7. At the later stages of injury, RSV increased the number of $Ki67^+$ hepatocytes indicating that RSV promoted hepatocyte proliferation. Additionally, it resulted in decreased expression of 4-hydroxynonenal and increased expression of the hepatocyte growth factor protein and mRNA in the RSV-treated BDL group. Meanwhile, RSV reduced the mortality rate of BDL mice. In conclusion, RSV attenuated inflammation and reduced Kupffer cells activation. RSV decreased fibrosis and promoted hepatocyte regeneration, which increased the survival of BDL mice. RSV was beneficial for the treatment of cholestatic liver injury.

Received: June 5, 2011
Revised: August 23, 2011
Accepted: August 29, 2011

Keywords:
Hepatocyte / Inflammation / Kupffer cell / Regeneration / Resveratrol

1 Introduction

Liver injury is a common health problem and can be caused by different agents. Severe injury will cause liver failure, resulting in high morbidity and mortality. Liver injury can trigger a cascade of inflammatory responses and may initiate the process of hepatic regeneration to replace the damaged tissue and restore normal function [1, 2]. In the injury, hepatocyte quickly responds to the insult caused directly by a toxin or indirectly by noxious cellular mediators released from different inflammatory cells including neutrophils, lymphocytes and Kupffer cells [3, 4]. If the injury persists, it will activate the hepatic stellate cells and trigger the process of fibrogenesis [5]. The hepatic parenchyma will then be progressively substituted by fibrotic tissues composed of abundant extracellular matrix (ECM) including fibrillar collagen and fibronectin [6]. Regeneration and fibrosis processes are thus considered as wound-healing responses of the liver.

Kupffer cells are considered to play a key role in modulating inflammation in liver injury [7, 8]. Kupffer cells can be

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Abbreviations: ALT, alanine aminotransferase; **AST,** aspartate aminotransferase; **BDL,** bile duct ligation; **ECM,** extracellular matrix; **HGF,** Hepatocyte growth factor; **4-HNE,** 4-hydroxynonenal; **MMP,** matrix metalloprotease; **RSV,** Resveratrol; **T-bil,** total bilirubin; **TGF,** transforming growth factor; **TIMP,** tissue inhibitor of metalloprotease; **TNF,** tumor necrosis factor

stimulated and activated by cytokines, chemokines and toxic metabolic products that damage the hepatocytes [8–10]. Activated Kupffer cells can produce pro-inflammation cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α to activate hepatic stellate cells and promote liver fibrogenesis [8, 11]. Furthermore, IL-6 and TNF-α may also initiate liver regeneration and stimulate hepatocyte proliferation to repopulate the damaged tissue [10, 12]. Hepatic stellate cells can also trigger the hepatocyte growth factor (HGF), which facilitates hepatocytes moving from the G0 phase into the G1 phase of the cell cycle [1]. Therefore, the process of hepatocyte regeneration after liver injury was participated by inflammatory cytokines and well regulated by both Kupffer cell and hepatic stellate cell.

Resveratrol (3,4,5-trihydroxystibene, RSV) is a natural phytoalexin synthesized in a wide variety of plants such as grapes and peanuts [13]. RSV possesses anti-inflammatory, antioxidant and anti-tumor effects [14, 15]. Through these effects, RSV has been reported to exert multiple health-promoting benefits. RSV can protect the retinal pigment epithelial cells against hyperglycemia-induced inflammation [16]. RSV also significantly reduces the amount of pro-inflammation cytokine released as a result of acute small intestinal inflammation [17]. Long-term supplementation of RSV attenuates chronic colonic inflammation in mice and reverses pro-inflammatory cytokine profile and oxidative DNA damage in ageing hybrid mice [18, 19]. RSV has been reported to reduce renal fibrosis by inhibiting collagen and fibronectin mRNA expression [20]. Moreover, RSV attenuates lung injury following trauma-hemorrhage by reducing the intercellular adhesion molecule (ICAM)-1 and IL-6 [21]. These results imply that RSV may be used as a powerful anti-inflammatory agent against different types of injuries. Although RSV has been shown to exert anti-fibrotic effects on rats after chronic CCl_4 administration [22], the roles of RSV in cholestasis-induced liver injury have not been completely revealed.

The aim of this study was to evaluate potential protective effects and identify possible underlying mechanisms of RSV in cholestatic liver injury.

2 Materials and methods

2.1 Animal model of cholestatic liver injury

Hepatic fibrosis was induced in C57BL/6 mice by bile duct ligation (BDL). BDL was performed by surgical isolation and ligation of the bile duct after laparotomy under ketamine anesthesia (Katalar 10 mg/10 g for mice). The bile duct was double-ligated and transected between two 6-0 ligatures after which the abdomen was closed. The animals were allowed to recover under careful observation and protection. The mice were randomly allocated to receive either sham operation or BDL with or without treatment of RSV. Administration of RSV was given intraperitoneally at dose of 4 mg/kg daily for either 3 days or 7 days starting from the first day of surgery. At the end of treatment, the blood and livers of sham, BDL and BDL+RSV groups ($n = 4$–6 in each group) were collected and processed properly for subsequent studies. In the survival study, the survival rates were recorded by pulling data of three study groups (sham $n = 8$, BDL $n = 14$ and BDL+RSV $n = 30$ in total) from two independent experiments. All animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication no. 86-23, revised 1985) and approved by the Institutional Animal Care and Use Committee (IACUC) of Taipei Veterans General Hospital (VGH97-110). In all experiments, the authors adhered to the American Physiological Society Guiding Principles for the Care and Use of Laboratory Animals.

2.2 Liver biochemistry

Immediately after induction of anesthesia with ketamine (Katalar, 10 mg/10 g), a laparotomy was performed and blood was drawn out from the vena cava for biochemistry study. The serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin (T-bil) were measured by standard laboratory methods in an automatic chemistry analyzer.

2.3 Assessment of liver histology

Consecutive sections of paraffin-embedded liver (5 μm thick) were cut for hematoxylin and eosin staining (H&E) and Masson Trichrome staining to facilitate the evaluation of liver injury, necrosis and fibrosis. Areas of bile infarct and hepatic necrosis were evaluated in five microscopic fields (10× magnification) photo-taken by using a digital camera and computerized image analysis software (Microcam, M&T OPTICS, Taiwan).

2.4 Western blots

Tissue lysates were prepared in a buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA, 1 mM Na orthovanadate, 1 mM Na fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin and 1 μg/mL pepstatin, on ice. Specified amounts of total cellular protein (DC-Bradford protein assay, Bradford, Bio-Rad, Hercules, CA, USA) were subjected to SDS-PAGE gel electrophoresis and then transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk and incubated overnight at 4°C with primary antibodies to 4-hydroxynonenal (4-HNE), HGF and

β-actin. Membranes were washed in phosphate-buffered saline solution containing tween (TBST: 10 mM 0.05%, Tris pH 8, 0.9% sodium chloride, Tween 20 0.05%) and incubated for 1 h at room temperature with secondary antibodies. Immunoreactive bands were visualized by ECL as per manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA) and captured with a digital image system (Chemigenius2 photo-documentation system, Syngene, Cambridge, UK). Intensities of specific bands were measured.

2.5 RNA extraction and quantification by real-time PCR

RNA was entirely extracted from whole liver using TRIZOL reagent (Sigma, USA). The concentration of RNA is determined spectrophotometrically and 1 μg of RNA was used to synthesize cDNA. cDNA was amplified at 95°C for 5 min, 45 cycles of 95°C for 30 s, 60°C for 30 s with and extension at 72°C for 10 min. Real-time polymerase chain reaction (PCR) was performed according to manufacturer's recommendation by using ABI 7900 thermocycler. Amplification reaction was performed using an SYBR Green PCR Master Mix (Roche, USA). The primers used in this study are listed in Table 1. All samples were analyzed in triplicate. GAPDH was used as an internal control.

Table 1. Primer sequences used in this study

Col Type Iα1	Forward: GAGCGGAGAGTACTGGATCG	
	Reverse: GCTTCTTTTCCTTGGGGTCG	158 kb
Fibronectin	Forward: GTGGCTGCCTTCAACTTCTC	
	Reverse: GTGGGTTGCAAACCTTCAAT	132 kb
TNF-α	Forward: GGTGATCGGTCCCAACAAGGA	
	Reverse: CACGCTGGCTCAGCCACTC	173 kb
IL-6	Forward: TCCAGTTGCCTTCTTGGGAC	
	Reverse: GTGTAATTAAGCCTCCGACTTG	140 kb
TIMP-1	Forward: CCTTGCAAACTGGAGAGTGACA	
	Reverse: GTGGGTTGCAAACCTTCAAT	91 kb
MMP-2	Forward: ACAGCCTGGCATGGGGCAGT	
	Reverse: TTCTCCTCCATCCAGTGGAG	292 kb
TGF-β1	Forward: TTGCCCTCTACAACCAACACAA	
	Reverse: GGCTTGCGACCCACGTAGTA	103 kb
HGF	Forward: CCTGTCAGCGTTGGGATT	
	Reverse: CTCGGATGTTTGGGTCAGTGG	147 kb
GAPDH	Forward: TGCACCACCAACTGCTTAGC	
	Reverse: GGCATGGACTGTGGTCATGAG	87 kb

2.6 Immunohistochemistry study

The liver specimens were preserved in 4% buffered *para*-formaldehyde and dehydrated in a graded alcohol series. For the immunohistochemistry staining, the detection of $CD68^+$ cells (NB100-683, Novus, USA) was performed using the MM detection kit (Biocare, USA) and DAB according to manufacturer's protocol. The tissue was counterstained with Hematoxylin. The number of $CD68^+$ cells was counted by computer software (Image J 1.43, NIH).

2.7 Detection of proliferative hepatocyte

The harvested livers were fixed and embedded in paraffin. Afterwards they were sectioned for subsequent immunohistochemistry study in order to detect the proliferative hepatocytes using peroxidase-coupled mouse monoclonal antibodies against anti-Ki67 (DAKO, USA). Ten pictures of randomly selected areas per animal were photographed under a microscope at 200× magnification. The mean numbers of Ki67-positive cells per area per animal were used for statistical analysis.

2.8 Cell culture study and proliferation assay

Mouse non-transformed hepatocyte cell line, AML12 (ATCC CRL-2254), was grown in 10% FBS in DMEM to near confluence. The hepatocyte was washed with PBS and treated with RSV. At the end of experiment, the proliferation assay was evaluated by MTT assay. The amount of MTT formazan product was determined using a microplate reader at an absorbance of 560 and 670 nm (SpectraMax 250, Molecular device, CA, USA).

2.9 Statistical analysis

The results are expressed as mean ± SEM. Statistical analysis was performed by using an independent Student *t*-test, one-way ANOVA with the Tukey post hoc test as appropriate. The survival curves were created and analyzed using GraphPad Prism 4 software (GraphPad Software, Inc. CA, USA). A *p*-value less than 0.05 was considered as statistically significant.

3 Results

3.1 RSV attenuated cholestatic liver injury induced by BDL

After ligation surgery, mice had significantly higher levels of ALT, AST and T-bil indicating the impairment of liver functions and the presence of cholestasis (Fig. 1A–C).

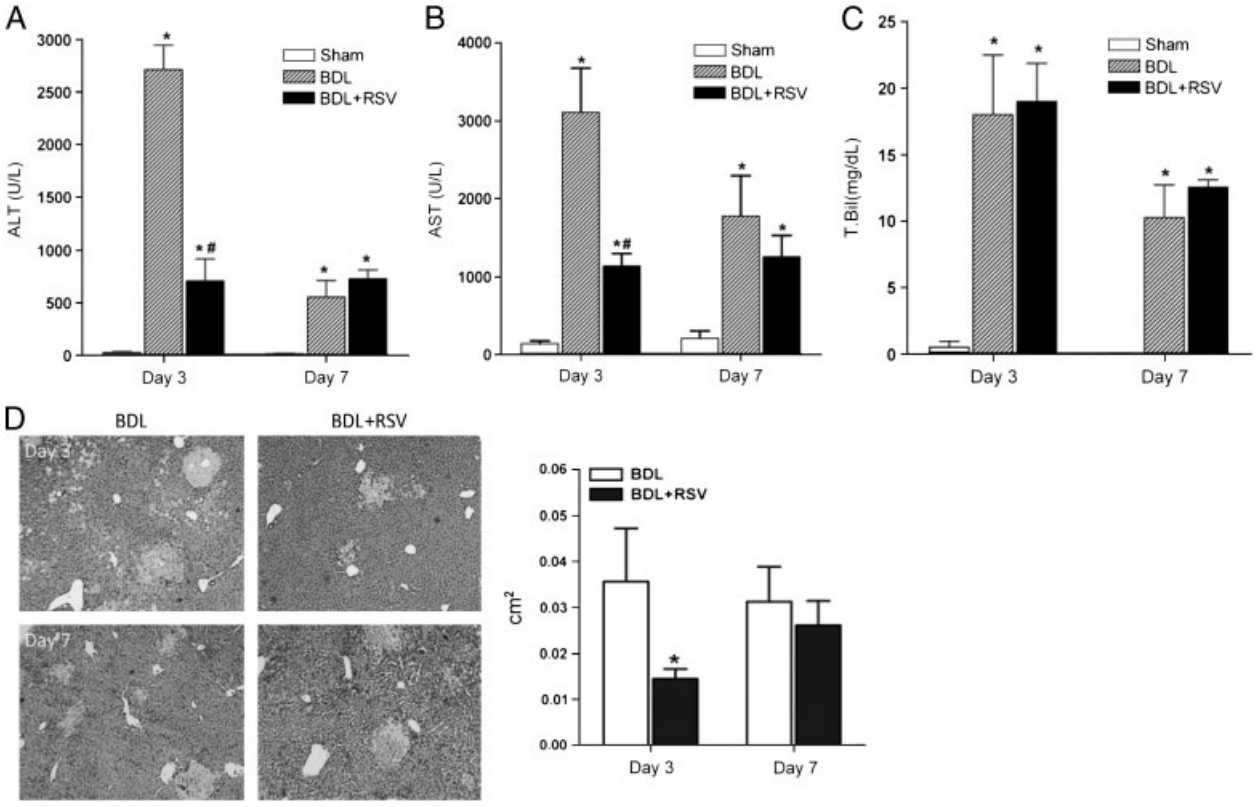

Figure 1. The biochemistry and liver histology in bile duct ligated (BDL) mice treated with resveratrol (RSV). (A–C) RSV treatment reduced ALT, AST in BDL mice on Day 3. RSV treatment did not alter the level of total Bilirubin. (D) RSV decreased the liver necrosis area on Day 3 (H&E staining, 40 ×). (*$p<0.05$ versus sham group, $^{\sharp}p<0.05$ versus BDL group).

RSV-treated BDL mice ($n=6$) had significantly lower serum ALT and AST on Day 3 as compared with the vehicle-treated BDL mice ($n=6$) ($p<0.05$) which showed levels similar to the sham group ($n=6$, $p>0.05$). On Day 7, the serum ALT and AST in BDL mice was significantly higher than those of the sham-operated mice ($n=6$, $p<0.05$). However, no difference in serum ALT and AST was found between vehicle-treated and RSV-treated BDL mice ($n=4$, respectively, $p>0.05$). RSV treatment did not alter the level of T-bil in BDL mice. In all mice with BDL, increases in bile infarcts within the liver were observed histologically (Fig. 1D). The areas of necrosis and infarction were reduced by RSV on Day 3 but no difference was noted on Day 7 between RSV-treated ($n=4$) and the vehicle-treated BDL mice ($n=4$) ($p>0.05$).

3.2 RSV decreased pro-inflammatory cytokines, recruitment of Kupffer cells and oxidative stress

Liver injuries initiated inflammatory reactions and rapidly triggered the release of pro-inflammatory cytokines at the early stages of injury; on account of these actions, we evaluated the anti-inflammatory effect of RSV on injured livers. It was found that RSV significantly reduced TNF-α and IL-6 mRNA expressions in BDL mice (Fig. 2A). We also counted $CD68^+$ cells in different groups. Those counts indicated that the BDL group mice had a higher accumulation of $CD68^+$ cells than the sham group and RSV treatment significantly reduced the number of $CD68^+$ cells in the BDL group ($p<0.05$) (Fig. 2B). Since oxidative stress may contribute to inflammatory liver injury, we further evaluated the effect of RSV on 4-HNE, which is a marker of oxidative stress. RSV significantly attenuated the hepatic expression of 4-HNE in the BDL group ($p<0.05$) (Fig. 2C).

3.3 RSV reduced hepatic fibrosis

The injury initiated inflammatory reaction would subsequently lead to liver fibrosis at a later stage. The histological study revealed that the degree of liver fibrosis in BDL mice was reduced by RSV treatment (Fig. 3A). In addition, RSV treatment significantly reduced the mRNA expression of collagen Iα1 in BDL mice on Day 7 (Fig. 3B). The mRNA expression of fibronectin was also reduced. The deposition and degradation of the matrix were regulated by a dynamic balance between

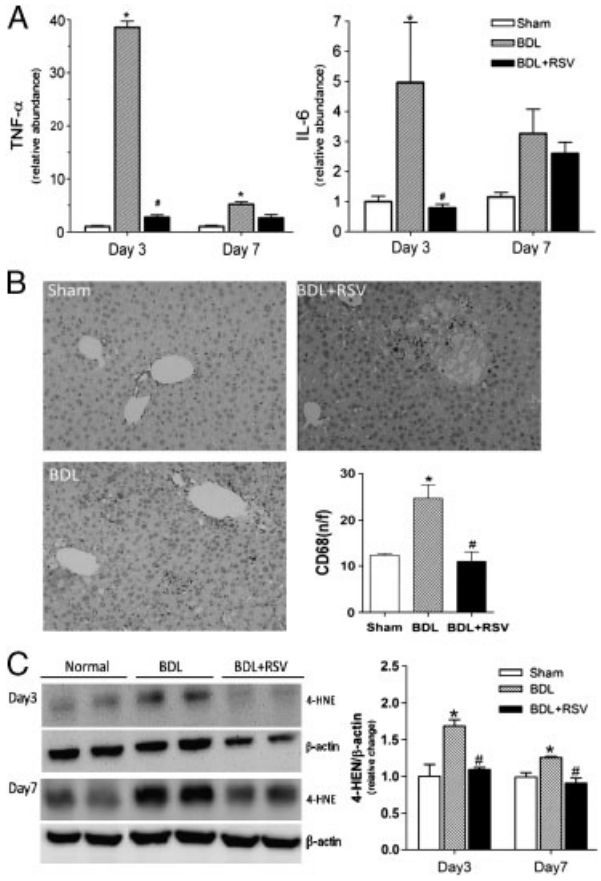

Figure 2. The effect of resveratrol (RSV) on pro-inflammatory cytokines, Kupffer cells accumulation and oxidative stress in bile duct ligation (BDL) mice on Day 3. (A) RSV reduced TNF-α and IL-6 mRNA expression on Day 3. (B) Representative photomicrographs of CD68 staining for Kupffer cells. Compared with sham mice, increased Kupffer cell numbers were detected in BDL mice. RSV treatment reduced the accumulation of Kupffer cells. (C) Representative Western blots of 4-HNE protein. RSV treatment significantly lowered the hepatic expression of 4-HNE in BDL mice (*$p<0.05$ versus sham group, ♯ $p<0.05$ versus BDL group).

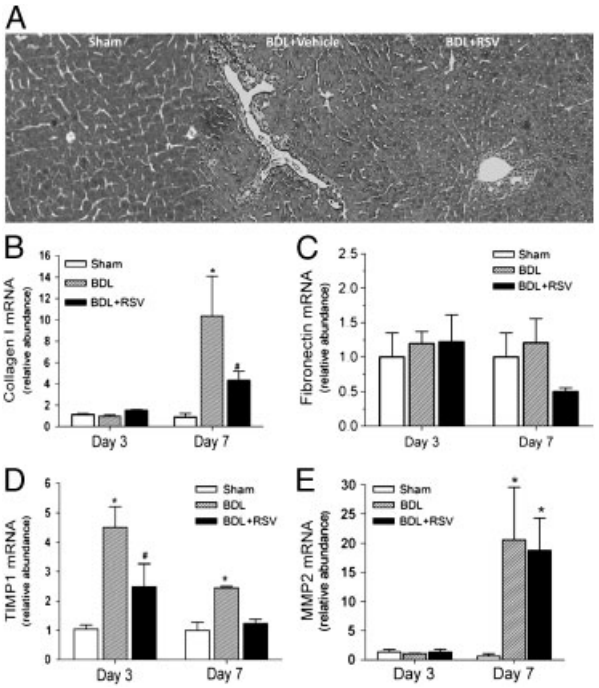

Figure 3. The effect of resveratrol (RSV) on hepatic fibrogenesis. (A) RSV treatment significantly reduced the degree of liver fibrosis on Day 7 after BDL (Masson Trichrome staining, 40 ×). (B) RSV treatment significantly reduced the collagen Ia1 mRNA expression in BDL mice. RSV also attenuated the increase in the TIMP-1 mRNA. MMP-2 mRNA increased significantly but was not altered by RSV treatment. (*$p<0.05$ versus sham group, ♯$p<0.05$ versus BDL group).

tissue inhibitor of metalloprotease (TIMP) and matrix metalloprotease (MMP). The expression of TIMP-1 and MMP-2 were then evaluated. Compared to sham-operated mice, BDL mice had higher levels of TIMP-1 mRNA. RSV significantly reduced the expression of TIMP-1 mRNA (Fig. 3D). The mRNA expression of MMP-2 increased on Day 7 and remained at high levels despite RSV treatment (Fig. 3D).

3.4 RSV enhanced hepatocyte proliferation

To explore the cause of liver function improvement, we investigated the effect of RSV on hepatocyte proliferation. Figure 4A shows the effect of RSV on the proliferation of hepatocytes. On Day 3, at the early stages of injury, there were few proliferative hepatocytes in all groups. In contrast, RSV significantly increased the number of the $Ki67^+$ hepatocytes, indicating that RSV treatment promoted hepatocytes proliferation at a later stage of injury. Because HGF and transforming growth factor (TGF)-β1 could regulate the proliferation of hepatocytes, their expression was evaluated. There were increased HGF protein and mRNA expression in the BDL group on Day 7 after RSV treatment (Fig. 4B and C). The mRNA expression of TGF-β1 was significantly higher in the BDL group. RSV treatment did not significantly lower the expression of TGF-β1 (Fig. 4C). In cell culture study, RSV at concentrations of 2, 5 and 10 μM significantly increased the proliferative response of hepatocyte (Fig. 4D).

3.5 RSV improved the survival of BDL mice

Finally, we evaluated whether the treatment of RSV had any effect on the survival of BDL mice. The mortality rates of the sham-operated, BDL and BDL mice with RSV treatment were observed daily for one week ($N = 8$, 14 and 30, respectively). It was found that RSV-treated mice had significantly lower mortality rates one week after BDL surgery as compared with those without RSV treatment ($p<0.05$, Fig. 5).

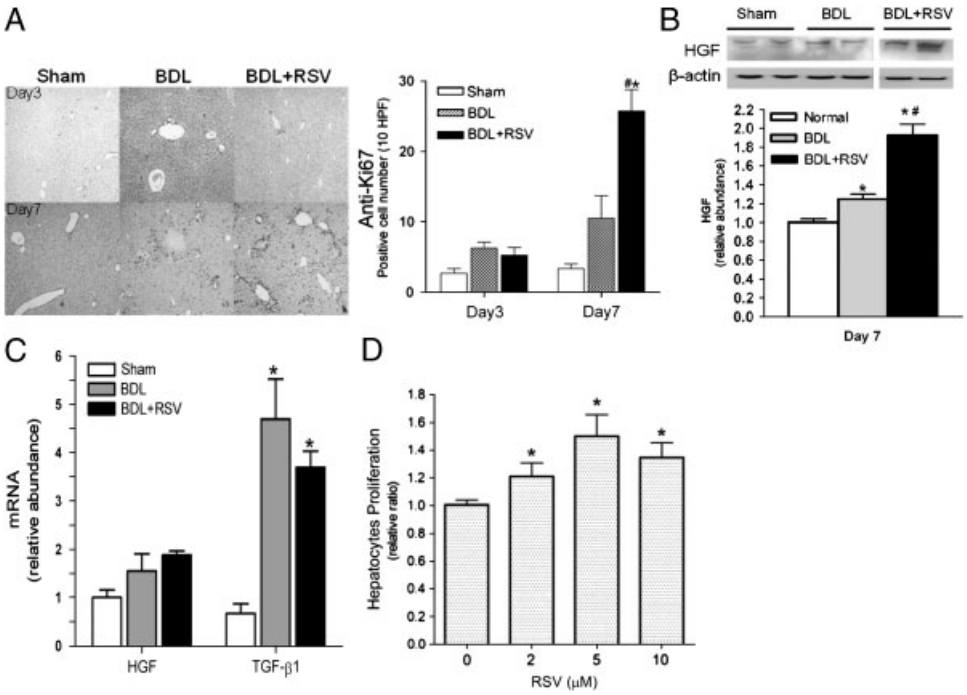

Figure 4. The effect of resveratrol (RSV) on the proliferation of hepatocytes and the expression of hepatocyte growth factor (HGF) and TGF-β1. (A) The proliferation of hepatocytes was detected by anti-Ki67 immunohistochemical staining. RSV significantly increased the numbers of proliferative hepatocytes on Day 7. (B) HGF protein expression was increased in BDL group after RSV treatment. (C) HGF mRNA expression was increased in RSV-treated BDL group on Day 7. The mRNA expression of TGF-β1 was increased in the BDL group. RSV treatment did not significantly lower the expression of TGF-β1. (*$p<0.05$ versus sham group). (D) In vitro cell culture study revealed that RSV (2, 5 and 10 μM) increased the proliferation of hepatocyte (*$p<0.05$ versus RSV = 0 μM).

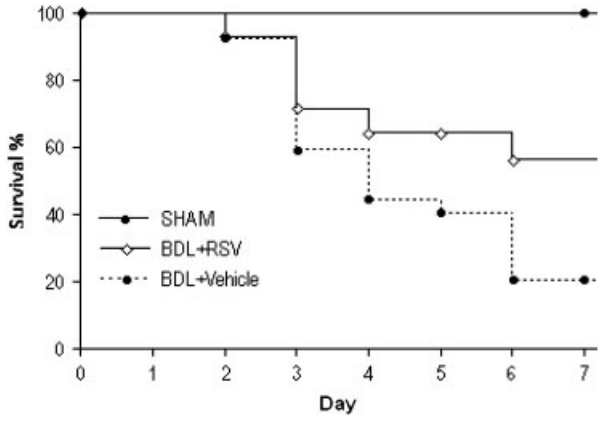

Figure 5. The survival of BDL mice after resveratrol (RSV) treatment. RSV treatment significantly increased the one-wk survival rate of BDL mice ($p<0.05$).

4 Discussion

RSV is a natural substance that can be extracted from grapes [13]. In the recent years, investigators have demonstrated the beneficial effects of RSV in coronary disease and cancer [23, 24]. Other investigators reported that RSV protected liver and gastric tissue against the oxidative stress in cholestatic rats [25–27]. Although the anti-inflammation and anti-fibrotic effects of RSV are well known, their effects on liver parenchyma and non-parenchyma cells in cholestasis-induced liver injury in mice has not been completely evaluated. In this study, we found that RSV significantly reduces cholestatic liver injury, pro-inflammation cytokines release and Kupffer cell accumulation. RSV also attenuated the degree of liver fibrosis and promoted hepatocyte proliferation. Moreover, RSV increased the survival rate of BDL mice. Therefore, these results demonstrated that RSV could be a potential protective agent for cholestatic liver injury.

In our study, we found that RSV did not have any effect on the level of total bilirubin in BDL mice. Previous study showed that administration of RSV 10 mg/kg in BDL rats for 28 days reduced total bilirubin from 2.7 mg/dL to 2.3 mg/dL and inhibited bile ductular proliferation [25]. Thus, the absence of decrease in bilirubin in our study could be due to different rodent models, lower dose and a shorter duration of RSV usage.

Kupffer cells are resident tissue macrophage located within the lumen of liver sinusoids. They are activated and involved in inflammation as a result of many different types of liver injury [28, 29]. Regarding acute inflammatory responses as related to liver injuries, previous studies suggest that macrophages are activated early and respond by vigorously releasing chemokines and pro-inflammatory cytokines [30]. The cytokines and other toxic metabolic

products produced from Kupffer cells may directly or indirectly contribute to inflammatory liver injury [10, 31]. Other studies indicate that Kupffer cells are important for liver injuries and that the depletion of Kuppfer cells can reduce hepatic inflammation and hepatocyte apoptosis [32]. In addition, previous studies demonstrated that RSV at dosage of 4 mg/kg exerted immune modulation effects in mice [33, 34]. Our results showed that at the same dose, RSV treatment significantly reduced the accumulation of $CD68^+$ Kupffer cell in BDL mice. TNF-α and IL-6 are two important pro-inflammatory cytokines produced by Kupffer cells. TNF-α was found to induce the synthesis of IL-6 and other cytokines [35]. RSV has been reported to reduce endotoxinin-induced TNF-α production in rat Kupffer cells [36]. Furthermore, study has demonstrated that RSV pleiotropic effects appeared early in the event of liver damage. RSV abrogated the activation of nuclear transcriptional factor NF-KB and subsequently inhibits the increased expression inflammatory genes [37]. In this study, we found that RSV treatment significantly lower the mRNA expression of TNF-α and IL-6 in BDL mice especially in the early days of cholestatic liver injury similar to the previous study [25]. The inhibitory effects of RSV on Kupffer cells and pro-inflammation cytokines were beneficial to the cholestatic liver injury in its early stage and contributed to the improvement of liver biochemistry. Another important biological effect of RSV is its antioxidant activity. RSV has been shown to increase antioxidant enzyme activity including superoxide dismutase, catalase, glutathione peroxidase and decrease malondialdehyde and nitric oxide (NO) in BDL rats [25, 27]. In our study, we found that RSV reduced the expression of 4-HNE in BDL mice. The 4-HNE is a major reactive aldehyde produced from reactive oxygen species (ROS)- and NO-induced lipid peroxidation of membrane polyunsaturated fatty acid. It has been recognized as a marker of oxidative stress and a modulator of signal transduction pathway in many inflammatory diseases [38–40]. Since it is well established that there is a link between oxidative stress and inflammation, the contribution of the antioxidative properties of RSV to its anti-inflammatory effects should be reminded.

The activation of Kupffer cells due to an injury can also promote the activation of hepatic stellate cells to facilitate fibrogenesis [41]. Activated hepatic stellate cells are the key effector cells during hepatic fibrogenesis and are the main ECM-production cells in the injured liver [42, 43]. Hepatic fibrogenesis is characterized by increased deposition of ECM including collagen and fibronectin in the extracellular space [44]. Our results show that RSV can significantly decrease the expression of type I collagen and fibronectin mRNA in BDL mice. The liver histological studies using the Masson Trichrome stain revealed that collagen fiber accumulation in RSV-treated BDL mice were decreased around portal tracts, the major accumulation site of the activated hepatic stellate cells. These results indicated that RSV can reduce the fibrogenic activity of activated hepatic stellate cells. The deposition of ECM is regulated by a dynamic balance between MMP and TIMP [45]. MMP is responsible for the degradation of extracellular matrix proteins. The up-regulated expression of TIMP-1 can inhibit MMP activity and subsequently lead to the accumulation of matrix protein. In addition, 4-HNE has been found to up-regulate collagen and TIMP-1 expression in activated hepatic stellate cells [46]. In the current study, we found that RSV treatment significantly reduced the expression of 4-HNE and TIMP-1. Therefore, the attenuation of liver fibrosis by RSV is most likely due to its inhibitory effect on 4-HNE and TIMP-1 expression allowing greater matrix-digesting activity of MMP-2.

After an injury, the liver will start the process of regeneration to repopulate hepatocytes and other nonparenchymal cells. Ara et al. reported that administration of RSV in BDL rats inhibited the biliary ductular proliferation and lymphocytic inflammation around portal area [25], but the response of hepatocyte was not evaluated in their study. Anti-Ki67 antibody can recognize the cells that are in G1, S, G2 and M phases [47]. Our results show that RSV-treated BDL mice have more $Ki67^+$ hepatocytes in the livers. This result indicated that there were more hepatocyte leaves their resting state (G0) and moves into the replicative phase. Other studies have demonstrated that RSV has the ability to accumulate cells in S phase [48]. Several cytokines have been found to participate in liver regeneration. IL-6 and TNF-α are two important cytokines that can enhance gene transcription and trigger hepatocytes to leave their quiescent state. IL-6 can also protect hepatocytes against TGF-β-induced apoptosis [49]. In our study, RSV reduced the expression of IL-6 and TNF-α more at earlier stages (Day 3) rather than later stages (Day 7) of cholestatic liver injury. To investigate possible factors, which trigger hepatocytes proliferation, we evaluated the expression of HGF. We found increased expression of HGF protein and mRNA in proliferative stages after RSV treatment. Previous studies showed that HGF not only promotes hepatocyte proliferation, but also attenuates liver fibrosis by suppressing TGF-β1 expression and type I collagen deposition [50]. In this study, collagen I mRNA of the RSV treatment group was significantly reduced in the proliferative stage. Thus, it is possible that HGF was involved in the promotion of hepatocyte proliferation and reduction of fibrosis by RSV treatment. Moreover, we found that RSV treatment increased the proliferative response of hepatocyte in culture condition. The mechanism of RSV promoting hepatocyte regeneration is not directly related to TNF-α and IL-6, though it could be due to the combined effects of HGF and RSV itself. Our results show that RSV can stimulate hepatocytes to repopulate the damaged tissue and restore the proper functions.

In summary, we found that RSV attenuates the release of pro-inflammatory cytokine and reduces the activation of Kupffer cells. RSV not only decreased the degree of fibrosis via modulating TIMP, but also promoted the hepatocyte

regeneration to repair the damaged tissue and increase the survival rate. RSV is a multi-functional agent, which could have potential benefits in the therapy of cholestatic liver injury.

The authors gratefully acknowledge Dr. Shih-Hwa Chiou for his excellent technical assistance. This work was supported by grants from National Science Council (NSC97-2314-B-075-027-MY2) and Taipei Veterans General Hospital (VGH 98C1-016, VGH99-C1-056), Taiwan.

The authors have declared no conflict of interest.

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