

The Protective Effect of Resveratrol on Dimethylnitrosamine-Induced Liver Fibrosis in Rats

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(Received October 1, 2009/Revised January 19, 2010/Accepted January 24, 2010)

Oxidative stress in liver injury is a major pathogenetic factor in progress of liver fibrosis. Resveratrol, a representative antioxidant derived from grapes, has been reported to show widespread pharmacological properties. In this study, we investigated the protective effects of resveratrol on dimethylnitrosamine (DMN)-induced liver fibrosis in rats. Rats were treated with resveratrol daily by oral gavage for seven days after a single intraperitoneal injection of DMN (40 mg/kg). Resveratrol remarkably recovered body and liver weight loss due to DMN-induced liver fibrosis. Liver histology showed that resveratrol alleviated the infiltration of inflammatory cells and fibrosis of liver tissue. Resveratrol decreased the level of malondialdehyde and increased the levels of glutathione peroxidase and superoxide dismutase. Also, resveratrol significantly inhibited the mRNA expression of inflammatory mediators including inducible nitric oxide, tumor necrosis factor- α and interleukin-1 β . In addition, resveratrol showed not only reduced mRNA expression of fibrosis-related genes such as transforming growth factor beta 1, collagen type I, and alpha-smooth muscle actin, but also a significant decrease of hydroxyproline in rats with DMN-induced liver fibrosis. Our results suggest that resveratrol could be used to treat liver injury and fibrosis and be useful in preventing the development of liver fibrosis and cirrhosis.

Key words: Liver injury, Resveratrol, Antioxidant enzyme, TGF- β_1 , Collagen type I, α -SMA

INTRODUCTION

Liver fibrosis is a wound-healing process in injured liver and characterized by the excessive production and deposition of an extracellular matrix (ECM) (Friedman, 2000; Shafritz et al., 2006). As the main components of liver fibrosis, activated hepatic stellate cells (HSCs) produce ECM such as α -SMA and collagen type I (Malinski et al., 1996; Shafritz et al., 2006). And activated HSCs have been implicated in hepatic inflammation through the secretion of cytokines, such as transforming growth factor beta 1 (TGF- β_1) in the liver fibrogenesis process (Pinzani et al., 2001).

Oxidative stress and reactive oxygen species (ROS), which are physiological disturbances in the redox status of biological molecules, have been suggested to be closely associated with various pathological conditions (Caro et al., 2004). ROS induce liver fibrosis, cholestasis, hepatic inflammation, and necrosis of liver cells (Cressman et al., 1996; Tilg et al., 2000). Also, it has been reported that ROS in hepatocellular injury mediates inflammation response by tumor necrosis factor alpha (TNF- α), interleukin-1 β (IL-1 β), and inducible nitric oxide synthase (iNOS) and enhances the proliferation of cultured stellate cells and their collagen synthesis (Casini et al., 1994; Hierholzer et al., 1998; Ramadori et al., 2001). On the contrary, the activation and proliferation of stellate cells are inhibited by antioxidants or glutathione (GSH) precursors such epigallocatechin-3-gallate and N-acetylcysteine (Kim et al., 2001; Chen et al., 2003; Wu et al., 2005).

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Dimethylnitrosamine (DMN) exerts a potent hepatotoxin, carcinogen and mutagen through metabolic activation by CYP2E1 in animal experimental models (George et al., 2001). The formation of ROS has been demonstrated during the metabolism of nitrosamines resulting in oxidative stress and the stimulation of Kuffer cells leading to the generation of O_2^- in mouse livers (Wallace et al., 1981; Wills et al., 2006). Moreover, a single DMN injection can induce necrosis of the hepatocyte, rapid excessive deposition of collagen fibers, and activation of HSCs (Shiba et al., 1998; Yasuda et al., 1999).

Resveratrol (3,4,5-trihydroxystilbene) is natural phytoalexin that is synthesized in various plant species including grapes as a reaction against environmental stress or fungal infection (Rivera et al., 2008). The efficacy of resveratrol in biological systems is comprehensive as a chemopreventive agent (Wolter et al., 2004), an efficient anti-inflammation factor (Donnelly et al., 2004), and an antioxidant agent (Cai et al., 2003). Several studies have reported the possible role and protective effects of resveratrol against some kinds of oxidant damage, as a hydrogen-electron donor from its hydroxyl groups (Lopez-Velez et al., 2003). The consequences are a capacity to scavenge ROS, a protective effect against DNA damage and lipid peroxidation in the cell membrane (Leonard et al., 2003).

In this study, we investigated whether resveratrol was capable of preventing DMN-induced liver fibrosis and its molecular mechanism. We report that resveratrol can show functional and morphologic improvement by increasing anti-oxidant enzymes and decreasing the expression of TGF- β_1 , collagen type I and α -SMA as well as inflammatory mediators in rats with DMN-induced liver fibrosis.

MATERIALS AND METHODS

Chemicals

Resveratrol, dimethylnitrosamine (DMN) and carboxymethylcellulose (CMC) were purchased from Sigma Aldrich, and thiobarbituric acid (TBA) was obtained from Alfa Aesar (Johnson Matthey). All other chemicals were analytical grade.

Animals

All animal care and experiments were performed in accordance with the Guide for Animal Experiments edited by the Korean Academy of Medical Sciences. The animals were housed in an air-conditioned room at 25°C with a 12 h dark/light cycle. All animals received humane care during the study with unlimited access to food and water. Male Sprague-Dawley rats

were obtained from the Orient-Bio laboratory Animal Research Center Co., Ltd. Eighteen rats (six weeks old) were divided into three groups of six each (Con, DMN, and DMN + Res groups). The DMN + Res group was treated with resveratrol suspended in a 0.7% CMC at a dose of 10 mg/kg by oral administration for seven days daily after intraperitoneal injections of 40 mg/kg DMN. The DMN group was treated with DMN and equivalent volumes of the 0.7% CMC solution. The control group was treated with only vehicles, saline and 0.7% CMC solution equivalent to the treatment given to the resveratrol group. The day after the final administration, all rats were sacrificed under ketamine anesthesia.

Biochemical indicators of liver function

Serum aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total bilirubin (T-Bilirubin), and direct bilirubin (D-Bilirubin) levels were measured at Inha University Hospital.

Liver histopathology

For histopathological analysis, liver slices were cut in equal parts, fixed in 10% formaldehyde, and embedded in paraffin. The 5 μ m thick sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome (MT) before observation under a light microscope.

Determination of MDA, SOD and GPx

The liver tissues were washed with PBS to remove blood and homogenized in 50 mM Tris buffer (pH 7.4) for 2 min. Tissue homogenates were used for measurement of malondialdehyde (MDA). For glutathione peroxidase (GPx) assay, tissue homogenates were centrifuged at 13,000 rpm for 30 min and then the supernatant was examined. For superoxide dismutase (SOD) assay, tissue homogenates were mixed with the same volume of ethanol/chloroform (5:3) and centrifuged at 5,000 rpm for 20 min and then transparent upper layer was analyzed. MDA assay was performed according to the thiobarbituric acid method (Niehaus et al., 1968). SOD and GPx levels were measured using a commercial assay kit (Cayman).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the liver sample with Trizol reagent (Invitrogen) following the manufacturer's protocol. An aliquot of the total RNA was reverse transcribed and amplified using *MMuLV* reverse transcriptase and *Taq* DNA polymerase (Promega), respectively. The primer pairs for iNOS, TNF- α , IL-

β , collagen type I, α -SMA and TGF- β_1 were used following the designed primer. The PCR products were electrophoresed on a 1.5% agarose gel, and the bands were quantitated using densitometry. PCR primer sequences and fragment size are shown in Table I.

Immunohistochemistry

Frozen tissue sections were washed with PBS. After being blocked with normal goat serum (Vector Laboratories) for 1 h, the sections were incubated for 1 h at room temperature in a dilution of 1:100 of collagen type I antibody (Santa Cruz Biotechnology). The sections were visualized by an avidin-biotin peroxidase complex solution using an ABC kit (Vector Laboratories). The sections were washed in PBS, developed with a diaminobenzidine tetrahydrochloride substrate for 15 min and counterstained with hematoxylin.

Hydroxyproline assay

Hepatic hydroxyproline content was measured using a modified version of the previous methods (Jamall et al., 1981; Seifert et al., 1995). Briefly, the liver samples were placed in 1.0 mL of 6 M HCl at 100°C in a vacuum oven for 24 h. The samples were hydrolyzed into individual amino acids. The 6 M HCl was evaporated in a vacuum oven at 50°C for 36-48 h and dried pellets were resuspended in 2.0 mL of 1.0 mM HCl and vortexed for 1 min. 100 μ L of chloramine T solution was added and incubated at room temperature for 10 min after vortexing for 30 sec. Then 1.3 mL of Ehrlich's reagent was added and placed in a water bath at 55°C for 20-25 min after vortexing for 30 sec. The standard and samples were measured by a spectrophotometer at 558 nm. Trans-4-hydroxy-L-proline was used as a

standard.

Statistical analysis

Data were expressed as mean \pm S.D. Statistical analysis was performed using ANOVA and an unpaired Students t-test. A *p*-value of 0.05 or less was considered statistically significant. Statistical calculations were performed using SPSS software for Windows operating system (Version 10.0; SPSS).

RESULTS

Body and liver weights

Treatment with DMN caused a significant decrease in both body and liver weight compared with the control group (Fig. 1). The body weight of DMN group was only 84% of the control, whereas that of DMN + Res group was 91% of the control. Also, the final liver weight in the DMN group was 79% of the control. However, the liver weights in the DMN + Res group were 90% of the control (*p* < 0.05). This result showed that resveratrol administration prevented the loss of liver weight induced by DMN. In addition, the DMN group caused 5% decrease in the relative liver weight, which is the average of the liver weight at the final body weight multiplied by 100, compared to the control (*p* < 0.05, 4.26 ± 0.17 vs. 4.04 ± 0.08). The relative liver weight was recovered to normal level by co-treatment with resveratrol (*p* < 0.05, 4.27 ± 0.09). Resveratrol caused a recovery of body and liver weights, as well as relative liver weight in rats with DMN-induced liver injury.

Serum parameters

As shown in Table II, DMN increased ALP, T-Biliru-

Table I. PCR primers and size of expected PCR product

Gene	Primer sequence (5'- 3')	Product length (bp)
iNOS	Sense: CAG AGC CTC TAG ACC TCA ACA AA Antisense: GCT GAA CTT CCA ATC GTT GTA CT	479
TNF- α	Sense: ACA AGG AGG AGA AGT TCC CAA AT Antisense: GAC TTT CTC CTG GTA TGA AAT GG	332
IL-1 β	Sense: AAA TAG CAG CTT TCG ACA GTG AG Antisense: GAT TTT GTC GTT GCT TGT CTC TC	488
TGF- β_1	Sense: CGG CAG CTG TAC ATT GAC TT Antisense: TCA GCT GCA CTT GCA GGA GC	288
Collagen type I	Sense: TGC CGT GAC CTC AAG ATG TG Antisense: CAC AAG CGT GCT GTA GGT GA	462
α -SMA	Sense: ACT GGG ACG ACA TGG AAA AG Antisense: CAT CTC CAG AGT CCA GCA CA	240
GAPDH	Sense: CCA CTG GCG TCT TCA CCA C Antisense: CCT GCT TCA CCA CCT TCT TG	501

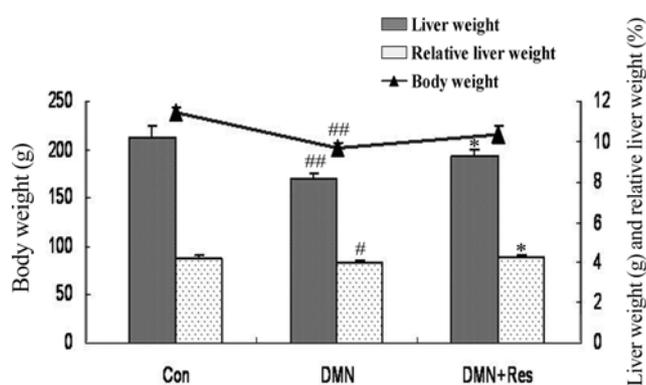


Fig. 1. Effect of resveratrol on the body and liver weights in DMN-induced liver fibrosis rats. The relative liver weight is the average of the liver weight divided by the final body weight multiplied by 100. Values are expressed as the mean \pm S.D. ($n = 6$). # $p < 0.05$ and ## $p < 0.01$ vs. control, and * $p < 0.05$ vs. DMN group, respectively.

bin, and D-Bilirubin. However, the administration of resveratrol significantly lowered ALP, T-Bilirubin, and D-Bilirubin levels which represent liver damage by about 60% ($p < 0.05$). There were no significant differences although resveratrol showed a tendency to decrease ALT and AST level.

Liver histopathology

The results of both H&E and Masson's trichrome (MT) staining showed a similar pattern. The control group showed normal architecture, whereas the DMN group exhibited massive and severe hepatocyte necrosis, as well as infiltration of inflammatory cells (Fig. 2). In the DMN + Res group, hepatocytes were relatively well preserved. Also, serial sections were stained with MT for collagen. The DMN group showed increased collagen and displayed bundles of collagen fibers surrounding the lobules. However, collagen expression of DMN + Res group was nearly absent.

Hepatic level of MDA, SOD, and GPx

Oxidative stress was assayed through the levels of MDA, SOD, and GPx in liver tissue homogenates. MDA, a product of lipid peroxidation was increased in liver injury induced by DMN. However, the level of liver MDA in the DMN + Res group was decreased compared to the DMN group (Fig. 3A). The liver SOD level of the DMN group was significantly lower than those of the control ($p < 0.05$), whereas the SOD level of the DMN + Res group was 2.5-fold higher than the DMN group ($p < 0.01$, Fig. 3B). Furthermore, the liver GPx level in the DMN + Res showed a 3.5-fold increase compared to the DMN group ($p < 0.01$, Fig. 3C).

The mRNA expression of iNOS, TNF- α , and IL-1 β

We examined the effect of resveratrol on mRNA expressions of inflammatory related gene in rats with DMN-induced liver fibrosis (Fig. 4). As shown in Fig. 4, iNOS, TNF- α , and IL-1 β mRNA expression level were about 2-fold increased by DMN treatment compared with control ($p < 0.01$). However, resveratrol decreased 1.5 or 2-fold those of iNOS, TNF- α , and IL-1 β in DMN + Res group ($p < 0.05$).

The mRNA expression of TGF- β_1 , collagen type I, and α -SMA

The mRNA expression levels of TGF- β_1 , collagen type I, and α -SMA were amplified by RT-PCR. As shown in Fig. 5, the mRNA expression level of TGF- β_1 in the DMN group was higher than the control group. However, the mRNA expression level of TGF- β_1 in the DMN + Res group was significantly lower than that of the DMN group ($p < 0.05$). Like the TGF- β_1 , the mRNA expression level of collagen type I in the DMN + Res group also showed a significant difference compared to the DMN group ($p < 0.05$). In addition, the mRNA expression level of α -SMA in the DMN + Res group was

Table II. Effect of resveratrol on serum biomarkers

Parameter	Group		
	Control	DMN	DMN + Res
ALT (U/L)	44.6 \pm 2.2	62.0 \pm 19.8	55.0 \pm 7.7
AST (U/L)	145.7 \pm 31.7	196.5 \pm 69.7	193.6 \pm 47.3
Alkaline phosphatase (U/L)	1109.5 \pm 85.3	1255.7 \pm 37.1 [#]	1011.4 \pm 85.7 [*]
T. Bilirubin (mg/dl)	< 0.001	0.195 \pm 0.041 [#]	0.074 \pm 0.019 [*]
D. Bilirubin (mg/dl)	0.006 \pm 0.003	0.152 \pm 0.022 [#]	0.060 \pm 0.050 [*]

Effects of resveratrol on serum parameters in rat with DMN-induced liver fibrosis. Values are expressed as mean \pm S.D. ($n = 6$).

[#] $p < 0.05$ vs. control and ^{*} $p < 0.05$ vs. DMN group. Control, treated only vehicle group; DMN, DMN-induced liver fibrosis group; DMN+Res, DMN plus resveratrol treated group; ALT, alanine aminotransferase; AST, aspartate aminotransferase; T-Bilirubin, total bilirubin; D-Bilirubin, direct bilirubin.

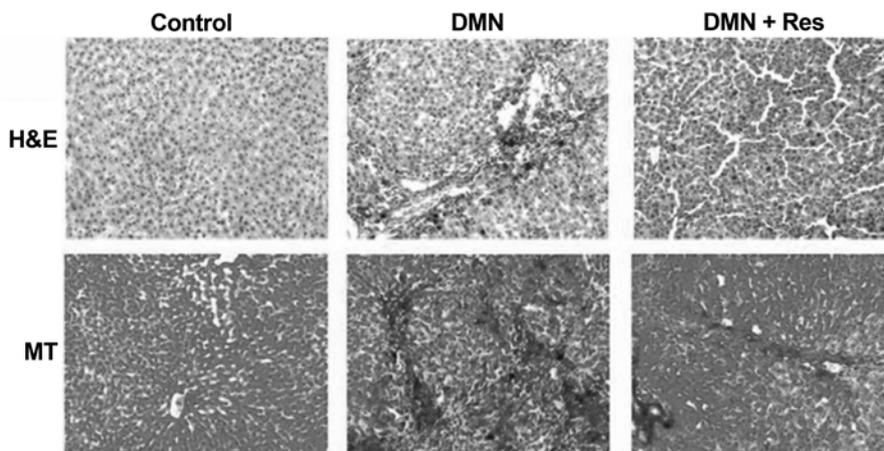


Fig. 2. Histological analysis of liver sections. Liver tissues were stained with H&E and MT ($\times 200$).

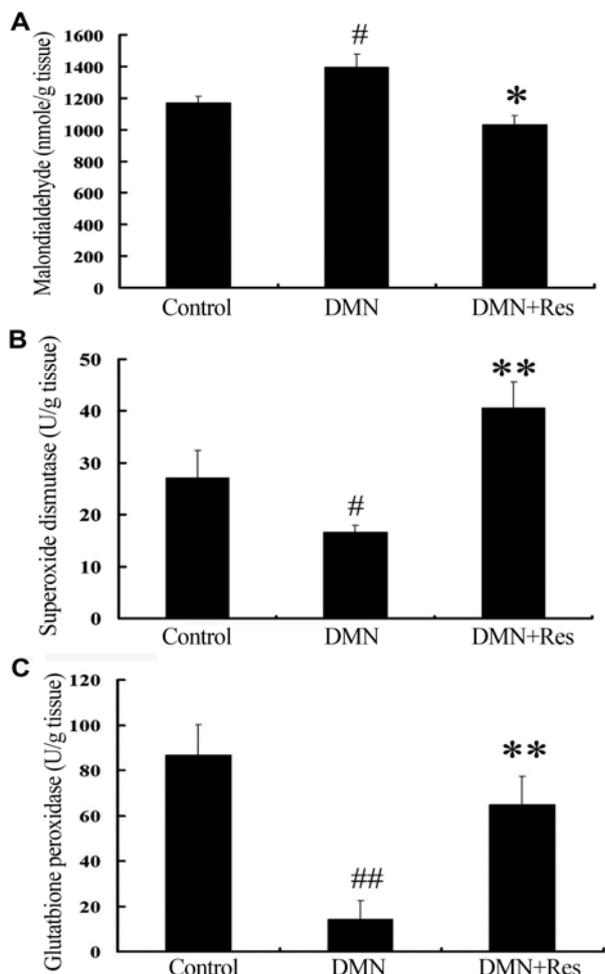


Fig. 3. Hepatic levels of (A) malondialdehyde (MDA, nmole/g tissue), (B) superoxide dismutase (SOD, U/g tissue), and (C) glutathione peroxidase (GPx, U/g tissue) after resveratrol treatment in rats with DMN-induced liver fibrosis. Each value represents the mean \pm S.D. in at least three separate experiments. [#] $p < 0.05$ and ^{##} $p < 0.01$ vs. control, and ^{*} $p < 0.05$ and ^{**} $p < 0.01$ vs. DMN group, respectively.

decreased by 40% compared to the DMN group ($p < 0.05$).

Collagen accumulation

Collagen accumulation was confirmed by immunohistochemistry and hydroxyprolin analysis. In the control group, collagen type I regions were weakly detected around the portal area and the central vein (Fig. 6A). Also, collagen type I expression in the DMN group was increased in periportal fibrotic band areas and central veins, and was diffused at the fibrous septa, whereas collagen type I expression of the DMN + Res group was obviously decreased compared to the DMN group. In addition, we measured hydroxyproline content using hydroxyproline assay, which is a directive method to determine collagen amount in tissue. As shown in Fig. 6B, the hydroxyproline content of the DMN + Res group was decreased significantly compared to the DMN group ($p < 0.05$).

DISCUSSION

Liver injury mediates several pathological functions, including the proliferation of HSC, secretion of proinflammatory cytokines, and increased deposition of ECM proteins, finally leading to liver fibrosis. In this study, we utilized DMN to induce hepatic injury and fibrogenic stimulation and investigated that resveratrol exerted protective effects on DMN-induced hepatic fibrosis in rats. Our study showed that resveratrol recovered the fibrosis of liver tissue through antioxidative and anti-inflammatory effect.

The ROS has the capability to initiate the lipid peroxidation of membrane and affects the antioxidant system (Nordmann, 1994). Additionally, it has been suggested that ROS formation could represent the link between different forms of liver injuries and

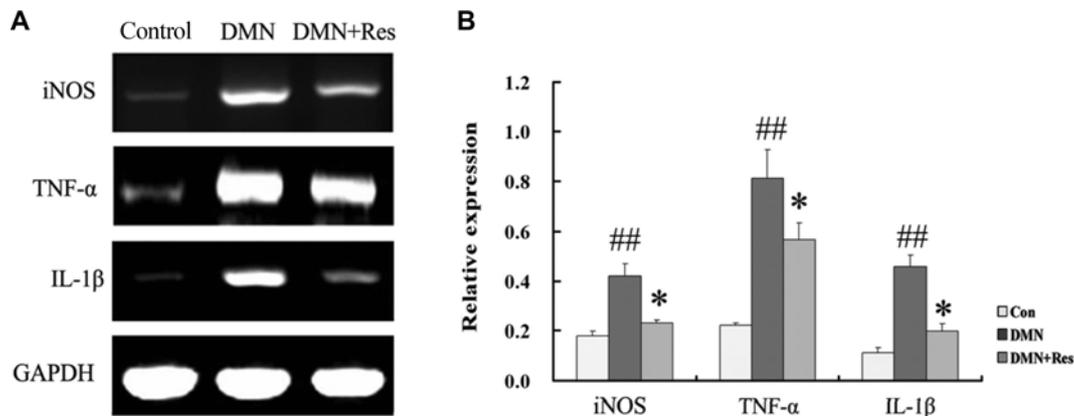


Fig. 4. The mRNA expressions levels of inducible nitric oxide (iNOS), tumor necrosis factor-alpha (TNF- α) and interleukin-1beta (IL-1 β) by RT-PCR. (A) Total RNA was prepared and RT-PCR was performed. The PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. (B) Quantification of mRNA expression of iNOS, TNF- α and IL-1 β was performed by densitometric analysis. Values were expressed as mean \pm S.D. of iNOS/GAPDH, TNF- α /GAPDH and IL-1 β /GAPDH for at least three separate experiments. [#] $p < 0.05$ vs. control and ^{*} $p < 0.05$ vs. DMN group, respectively.

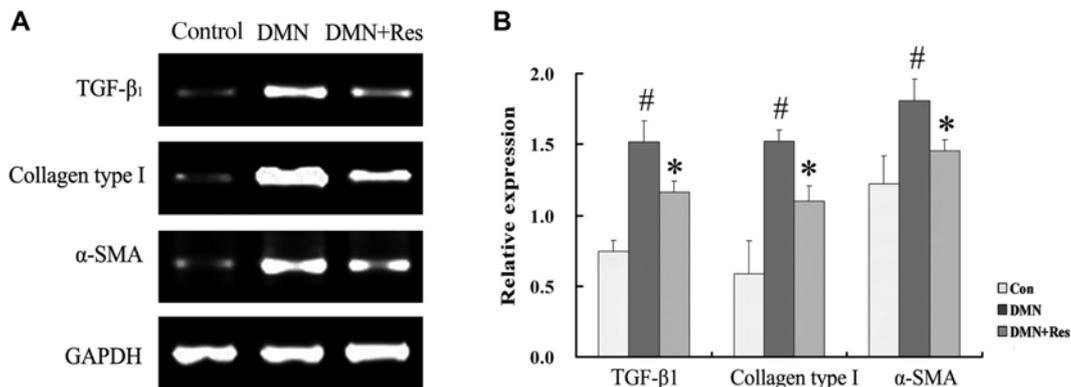


Fig. 5. The mRNA levels of transforming growth factor 1 (TGF- β_1), collagen type I, and alpha-smooth muscle actin (α -SMA) after resveratrol treatment by RT-PCR. (A) Total RNA was prepared and RT-PCR was performed. The PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. (B) Quantification of TGF- β_1 , collagen type I, and α -SMA mRNA expression was performed by densitometric analysis. Values were expressed as the mean \pm S.D. of TGF- β_1 /GAPDH, collagen type I/GAPDH, and α -SMA/GAPDH for at least three separate experiments. [#] $p < 0.05$ vs. control and ^{*} $p < 0.05$ vs. DMN group, respectively.

hepatic fibrosis both *in vitro* and *in vivo* (Nordmann, 1994; Rushmore et al., 2002; Ohara et al., 2008). Thus, antioxidant treatments in animals and humans could be beneficial in preventing or reducing some complications of ROS (Rushmore et al., 2002). In addition, resveratrol has been reported to enhance antioxidant enzymes in CCl₄-induced liver fibrosis and to reduce ethanol-induced oxidative tissue damage in rats (Kasdallah-Grissa et al., 2007; Bujanda et al., 2008; Chavez et al., 2008). Indeed, our study showed that resveratrol increased SOD and GPx with concomitant decrease of MDA production in rats with DMN-induced liver fibrosis. Recently, as another mechanism, Shang et al. reported that resveratrol also improves alcoholic and non-alcoholic fatty liver disease by activating AMP activated protein kinase

(AMPK) (Shang et al., 2008; Ajmo et al., 2008).

ROS induces inflammation by up-regulating the expression of inflammatory mediators (Jaeschke et al., 2000; Nove et al., 2008). Among those, inflammatory response is mediated by the action of proinflammatory cytokines such as TNF- α and IL-1 β (Weber et al., 2003). Also, iNOS has been known to involve inflammatory actions by producing large amounts of nitric oxide (NO) (Hierholzer et al., 1998). Several studies have already reported that resveratrol showed anti-inflammatory effect *in vitro* and *in vivo* and inhibits the release of inflammatory cytokines (Birrell et al., 2005; Ma et al., 2005; Bujanda et al., 2006; Bishta et al., 2009). Nevertheless, anti-inflammatory effect of resveratrol in DMN-induced liver fibrosis has not been reported yet. In this study, we observed that

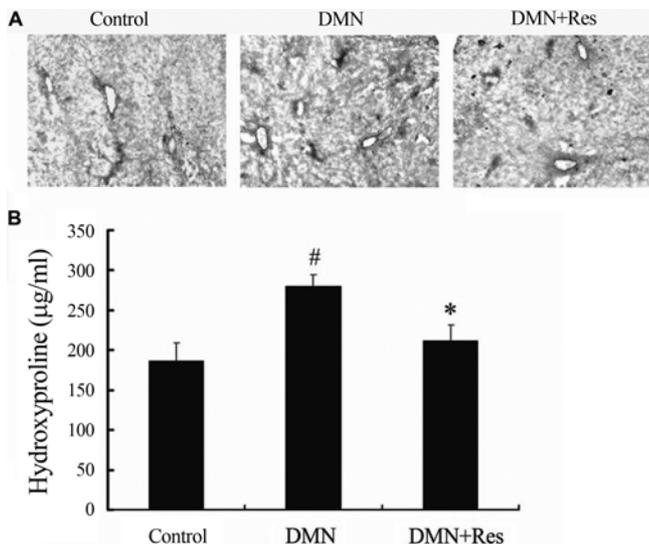


Fig. 6. Expression of collagen type I and the hydroxyproline level after resveratrol treatment in rats with DMN-induced liver fibrosis. (A) Expression of collagen type I after resveratrol treatment in rats with DMN-induced liver fibrosis was measured by immunohistochemistry. (B) Hydroxyproline level in liver tissues. Values were presented as the mean \pm S.D. of at least three separate experiments. [#] $p < 0.05$ vs. control and ^{*} $p < 0.05$ vs. DMN group, respectively.

resveratrol inhibited the expression of inflammation mediators such as iNOS, TNF- α , and IL-1 β . These results indicated that resveratrol was able to improve DMN-induced liver fibrosis by suppressing inflammatory response in rats.

Liver fibrosis is an important factor of cirrhosis and is usually initiated by damage to the hepatocyte, causing the recruitment of inflammatory cells and platelets, activation of HSC, and subsequent release of cytokines. The activated HSCs go through a phenotypic trans-differentiation to contract myofibroblasts (MFB) expressing α -SMA (Ezquerro et al., 2003). In liver fibrogenesis TGF- β_1 is also a mediator which promotes HSC to transit into myofibroblasts, and stimulates the synthesis of ECM such as collagen type II and inhibits its degradation (Breitkopf et al., 2005). Godichaud et al. and Sohara et al. reported that resveratrol can prevent the activation of myofibroblast-like cell growth, the expression of phenotypic markers and α -SMA, and the synthesis of collagen type I in primary cultured HSC cells (Godichaud et al., 2000; Sohara et al., 2002; Bennett et al., 2003). Our study revealed a pivotal role of resveratrol in reducing fibrogenesis progression, as showing that resveratrol decreased the expression of TGF- β_1 , collagen type I, and α -SMA.

In conclusion, the present study demonstrated that resveratrol exhibited a hepatoprotective and anti-fi-

brotic effects through reducing inflammatory mediators and increasing antioxidant enzymes in liver damage induced by DMN injection. Thus, we suggest that resveratrol may be useful in preventing the development of hepatic fibrosis.

ACKNOWLEDGEMENTS

This work was supported by an Inha University Research Grant.

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