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Ramalin, an antioxidant compound derived from Antarctic lichen, prevents progression of liver fibrosis induced by dimethylnitrosamine (DNM) in rats

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ABSTRACT

Hepatic fibrosis is characterized by the excessive accumulation of extracellular matrix (ECM), primarily collagen, within the liver. Because reactive oxygen species (ROS) has been implicated in its pathogenesis, the use of antioxidants as a potential treatment has been broadly explored. Here, we investigated the hepatoprotective properties of ramalin (RM), a compound extracted from the Antarctic lichen *Ramalina terebrata*, against hepatic fibrosis *in vitro* and *in vivo*. RM suppressed hepatic stellate cell (HSC) activation *in vitro* without any significant signs of adverse effects on the cells tested, and the accumulation of ECM was dramatically reduced in the liver tissue. Oral administration of RM in rats noticeably improved the gross appearance of the liver with increased body and liver weight relative to the DMN injected rats, and all of the serum biochemical markers returned to the normal range. RM treatment have ameliorated hepatic fibrosis in rats induced by DMN by repressing α-smooth muscle actin (α-SMA) and upregulating heme oxygenase-1 (HO-1). In addition, RM significantly reduced collagen accumulation, and levels of malondialdehyde (MDA) and hydroxyproline (HP) in the liver tissue of DMN injected rats. The efficacy exerted by RM was through erythroid 2–related factor 2 (Nrf2) mediated antioxidant response proteins such as HO-1 and NAD(P)H quinone dehydrogenase 1 (NQO-1). Our results show the beneficial effect of RM against the progression of hepatic fibrosis.

1. Introduction

Chronic liver disease affects many people worldwide, and its prevalence continues to rise due to numerous causes and contributing factors, such as viral hepatitis B and C infections, excessive alcohol consumption, chronic inflammation, and obesity [1]. However, despite the various processes by which liver disease can progress, the common pathology of chronic injury is hepatic fibrosis, which is characterized by the excessive formation of extracellular matrix (ECM)—primarily collagen—in the liver [2].

Hepatic fibrosis is a response to liver injuries where the liver itself induces tissue scarring to minimize further damage [3]. From a pathophysiological point of view, HSCs are the major inductive cells of hepatic fibrosis. Chronic inflammation transforms HSCs into myofibroblasts that produce ECM, which results in tissue scarring [3]. Continuous and repeated liver scarring usually results in hepatic cirrhosis or hepatic cancer [4]. At the cirrhosis stage, hepatic tissue is structurally deformed, preventing the liver from functioning properly. As the accumulating ECM becomes progressively thicker, the liver is unable to recover normal functionality, and the condition becomes almost impossible to reverse [2–4].

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Anti-fibrotic therapies mainly attempt to remove inflammatory stimuli, to recover cellular dysfunction, and to prevent further deposition of ECM. Oxidative stress is closely linked with the etiology of hepatic fibrosis [3,5,6]. The production of reactive oxygen species (ROS) is a natural byproduct of several essential biochemical reactions, including cytochrome P450 activation and the mitochondrial electron transport chain and is generally tolerated due to the presence of antioxidants [3,5,6]. An abundance of ROS without efficient removal, however, is implicated in the pathogenesis of hepatic fibrosis [5,7,8]. For this reason, there have been continuous attempts to use antioxidants in the treatment of hepatic fibrosis [3,5–10].

Both natural and synthetic antioxidants can protect the body from free radical-induced oxidative stress [11]. However, synthetic antioxidants have shown toxic side effects, such as teratogenesis and carcinogenesis, in primates [12]. However, natural antioxidants are known for their capacity to protect organisms from damage caused by oxidative stress without any negative side effects. Natural antioxidants can be acquired from various sources, including plants, macromicetes, and lichens [13]. Lichen-derived compounds have attracted a great deal of interest and have been screened for biological activities, including antibiotic, antiviral, anti-inflammatory, analgesic, and antioxidant properties [13–16]. Although the antioxidant activities of lichen-derived compounds from tropical or sub-tropical species are known, studies on the antioxidant activities of lichen-derived compounds from regions in Antarctica are scarce.

Preliminary studies have revealed that RM possesses potent antioxidant properties when compared to other commercial and natural antioxidant compounds [17,18]. In this study, we explored the preventative anti-fibrotic effects of RM on DMN-induced hepatic fibrosis in rats. Our data show that oral administration of RM ameliorated hepatic fibrosis in rats induced by DMN through Nrf2 mediated antioxidant response protein pathways.

2. Materials and methods

2.1. Reagents

Dimethylnitrosamine (DMN), hydroxyproline (HP), platelet-derived growth factor (PDGF), Histodenz®, and perchloric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, and trypsin-ethylenediaminetetraacetic acid (EDTA) disodium salt were purchased from Gibco BRL (Paisley, Scotland, U.K.). Ramalin (RM) ($C_{11}H_{16}N_3O_4$, bat. no.; KSL-GPH-024) was provided by the Korea Polar Research Institute (KPRI).

2.2. Cell culture and cell cytotoxicity assay

Chang liver cells (human hepatocyte-derived cell line) were obtained from the American Type Culture Collection (Manassas, VA, USA). For cytotoxicity assays, EZ-cytox cell viability assay kit (Daeil Lab Service, Korea) was used to measure cell viability. EZ-cytox kit reagents were added to the medium according to manufacturer's protocol. The optical density was measured at 450 nm using a microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

2.3. Preparation of hepatic stellate cells (HSCs)

HSCs were isolated from normal rat livers or injured livers after DMN administration. HSCs were obtained using the perfusion method, as previously described [19]. HSCs were identified via typical light microscopic appearance. Cells were then incubated for 12-h in serum-free DMEM with or without 400 pmol/L PDGF.

2.4. Analysis of gene expression in HSCs and liver tissue via RT-PCR

Total RNA was extracted from HSCs and frozen rat liver tissues using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA). Single-strand cDNA was synthesized from 1 μg total RNA in a total volume of 20 μl using a reverse transcription system (Promega, Madison, WI, USA), according to the manufacturer's instructions. Equal amounts of cDNA were subsequently amplified via PCR in a 25-μl reaction volume containing 1X PCR reaction buffer, 200 μM dNTPs, 0.5 pmol specific primers for each gene and 1 unit Taq DNA polymerase (Takara Biotech, Shiga, Japan). GAPDH was used as the standard protocol for DNA synthesis and PCR amplification. The primer sets were purchased from Thermo Fisher Scientific (Waltham, MA, USA) as follows: HO-1 (Rn01536933_m1), Collagen I (Rn01482925_m1), α-SMA (Rn01759928_g1), β-actin (Rn00667869_m1), GAPDH (Rn99999916_m1).

2.5. Animal experiments

All animal experiments were performed in accordance with the guidelines of the IACUC INHA-University. Male Sprague-Dawley rats (Orient-Bio, Gyeonggi-do, Korea) weighing 200–240 g were housed in conventional cages with free access to water and rodent chow at 25 °C with a 12-h dark/light cycle. All animals received humane care with unlimited access to chow and water.

Thirty-two rats were divided into four groups. Rats in group 1 were designated as the control group. Rats in groups 2 and 4 were given 20 mg/kg RM orally three times per week for 4 weeks. Rats in groups 3 and 4 received an intraperitoneal injection of DMN in 1-ml doses (diluted 1:100 with 0.15 M sterile NaCl) per 100 g body weight. DMN injections were given on three consecutive days of each week for 4 weeks. At the end of the fourth week, all of the rats were sacrificed under ketamine anesthesia and their livers were excised and weighed. Blood samples were collected via cardiac puncture and centrifuged at 3000 rpm for 15-min at 4 °C. The liver specimens were immediately fixed in 10% neutral buffered formaldehyde for histochemical studies.

2.6. Blood biochemistry

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein, alkaline phosphatase (ALP), total bilirubin (T-bilirubin), direct bilirubin (D-bilirubin), and albumin activities were analyzed by Samkwang Medical Laboratories (Seoul, Korea).

2.7. MDA, SOD, GPx and HP measurements

Malondialdehyde (MDA) levels were determined using a Bioxytech MDA-586 Assay Kit (Oxis Research, Oregon, USA). Superoxide dismutase (SOD) levels in homogenized liver tissue and homogenized glutathione peroxidase (GPx) levels were measured using commercial kits (Cayman Chemical, Ann Arbor, MI, USA). Hydroxyproline (HP) content in the liver was measured using a modified version of previously described methods [20].

2.8. Histological study and immunohistochemical staining

Liver specimens were fixed in 10% buffered formaldehyde, embedded in paraffin, and sectioned. After deparaffinization and dehydration, paraffin blocks were stained with hematoxylin-eosin (H&E) and Masson's trichrome (MT) before observation using a light microscope (Leica Microsystems, Wetzlar, Germany). Immunohistochemical staining was performed using formalin-fixed and deparaffinized tissue sections. The primary antibodies used were

anti-α-SMA, HO-1, NQO-1 and Nrf2 (Santa Cruz Biotechnology, CA, USA). Alexa Fluor® 555-conjugated secondary antibodies were used for fluorescence microscopy (Carl Zeiss, Oberkochen, Germany).

2.9. Western blot analysis

Protein extracts were obtained by homogenizing frozen tissues in radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific) containing protease inhibitors (Thermo Scientific). Protein concentrations were determined using a bicinchoninic acid-based method [21]. Primary antibodies were mouse monoclonal anti-α-SMA, mouse monoclonal anti-β-actin, rabbit polyclonal anti-Nrf2, anti-type I procollagen, anti-NQO-1 (Santa Cruz Biotechnology, CA, USA), and mouse monoclonal anti-HO-1 (ENZO Life Science, Farmingdale, NY, USA). Secondary antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA).

2.10. Statistical analysis

All statistical analyses (one-way analysis of variance (ANOVA)) were performed using Graph Pad Prism (version7.04). All the data are expressed as the mean ± standard deviation (SD).

3. Results

3.1. Ramalin (RM) suppresses PDGF-induced proliferation of HSCs and prevented HSC activation

As shown in Fig. 1A, RM is a potent antioxidant extracted from the Antarctic lichen *Ramalina terebrata*, which contains two hydroxyl moieties in the phenolic and the carboxylic acid functional groups that may donate hydrogen atoms to scavenge free radicals. To explore the beneficial effects of RM as a potent therapeutic agent, the cytotoxicity was measured in both rat and human hepatocytes (Fig. 1B). The data showed no substantial cytotoxicity for HSCs within the range of concentration (<10 μg/mL) tested in this study. Consequently, effects of RM on primary rat hepatic stellate cells (HSCs) were evaluated (Fig. 1C and D). After being plated, primary rat HSCs were grown in the presence and absence of 400pM of PDGF, which is one of the most potent mitogens available, to activate the HSCs (data not shown). RM effectively inhibited both non-PDGF- and PDGF-induced HSC proliferation by ~50% (Fig. 1C). In the activated HSCs, mRNA expression of α-SMA, the most reliable marker for HSC activation, became apparent in the PDGF-induced cells. However, mRNA expression of α-SMA reverted to control levels in the cells treated with RM alone or with RM and PDGF. Moreover, treatment with RM alone or RM with PDGF induced the mRNA expression of heme oxygenase 1 (HO-1), which is a marker for antioxidant pathway activation (Fig. 1D). These data suggest that RM suppresses PDGF-induced proliferation of HSCs and prevents HSC activation by repressing α-SMA and upregulating HO-1.

3.2. Ramalin (RM) ameliorares DMN-induced hepatic fibrosis in vivo

As shown in Fig. 2A, RM was orally administered three times per week for 4 weeks (white arrows) while DMN was given *i.p.* for 3 consecutive days per week for 4 weeks (black arrows). DMN injection induced severe liver damage in rats, resembling human hepatic fibrosis, as indicated by a severe reduction in body and liver weight (Table 1). The serum biochemical markers also suggested liver injury with elevated serum levels of ALP, AST, ALT, and total bilirubin with reduced serum albumin levels (Table 2). The efficacy of RM was assessed by administering RM orally during the same time period as the DMN injections (Fig. 2A, Tables 1 and 2). The administration of RM dramatically increased both body and liver weight that had been lost after treatment with DMN (Fig. 2B, Table 1), and all of the serum biochemical markers returned to normal ranges (Table 2). The oral administration of RM noticeably improved the gross appearance of the liver compared to the liver injured by DMN injections (Fig. 2C, DMN *vs.* DMN + RM). The histological analysis revealed that the liver treated with RM alone showed no pathological changes compared to the control liver tissue and had a normal lobular architecture with central veins and radiating hepatic cords (Fig. 2D, H&E, Con *vs.* RM). In contrast, DMN-induced periportal deposition and centrilobular fibers in the liver and caused continuous fibrotic septa between the central and portal veins. DMN also caused an increase in necrotic hepatocytes and the incorporation of degenerated hepatocytes into pseudolobules (Fig. 2D, H&E, DMN). However, rats treated with RM and DMN showed a significant suppression of fibril deposition and a marked amelioration of all pathological symptoms seen in rats with DMN-induced hepatic fibrosis (Fig. 2D, H&E, DMN + RM). Additionally, Masson's trichrome (MT) staining showed that RM significantly reduced collagen accumulation in the liver damaged by DMN (Fig. 2D, MT panel). Taken, together these data demonstrate that RM ameliorates hepatic fibrosis *in vivo*.

3.3. Effects of ramalin (RM) on hepatic fibrosis in DMN-induced rats

The effects of RM on the expressions of collagen I and α-SMA in liver tissue were evaluated by RT-PCR, Western blot and immunofluorescence analyses (Fig. 3). The results clearly showed that the mRNA and protein expressions of collagen I and α-SMA were elevated after DMN injection alone in the rat liver while the oral administration of RM blocked the elevation of those liver fibrosis markers (Fig. 3A, B and 3C). Levels of malondialdehyde (MDA), a marker of hepatic oxidative stress, and hydroxyproline (HP), a marker for collagen in hepatic fibrosis, were also measured in the liver tissue to show the efficacy of RM in DMN-induced hepatic fibrosis. Treatment with RM significantly reduced the DMN-elevated amount of MDA and HP in the liver tissue (Fig. 3D), suggesting that DMN-induced hepatic oxidative stress was relieved by RM and that RM also attenuated hepatic collagen deposition in the liver caused by DMN.

3.4. Effects of ramalin (RM) are linked to the Nrf2 mediated antioxidant response element (Nrf2/ARE) pathway

A major protective mechanism against oxidative or electrophilic stress is the activation of the Nrf2-mediated oxidative stress response signaling pathway that controls the expression of genes whose protein products are involved in the removal of intracellular ROS. To investigate that RM protects the liver from DMN-induced oxidative stress, the total superoxide dismutase (SOD) levels in the liver tissues were measured, as shown in Fig. 4 A. The total SOD level decreased four-fold in the liver injured by DMN compared to the control tissue, but the reduced SOD level was significantly recovered with the administration of RM. We then explored whether RM mediated protection against DMN-induced hepatic fibrosis involves the activation of Nrf2. As shown in Fig. 4B and C, DMN downregulated the protein expressions of Nrf2, NQO-1, and HO-1. However, subsequent RM administration significantly increased the levels of Nrf2 and Nrf2 mediated antioxidant response proteins such as HO-1 and NQO-1. The results support the notion that RM exerts its potential protective mechanism of action through the Nrf2-mediated pathway.

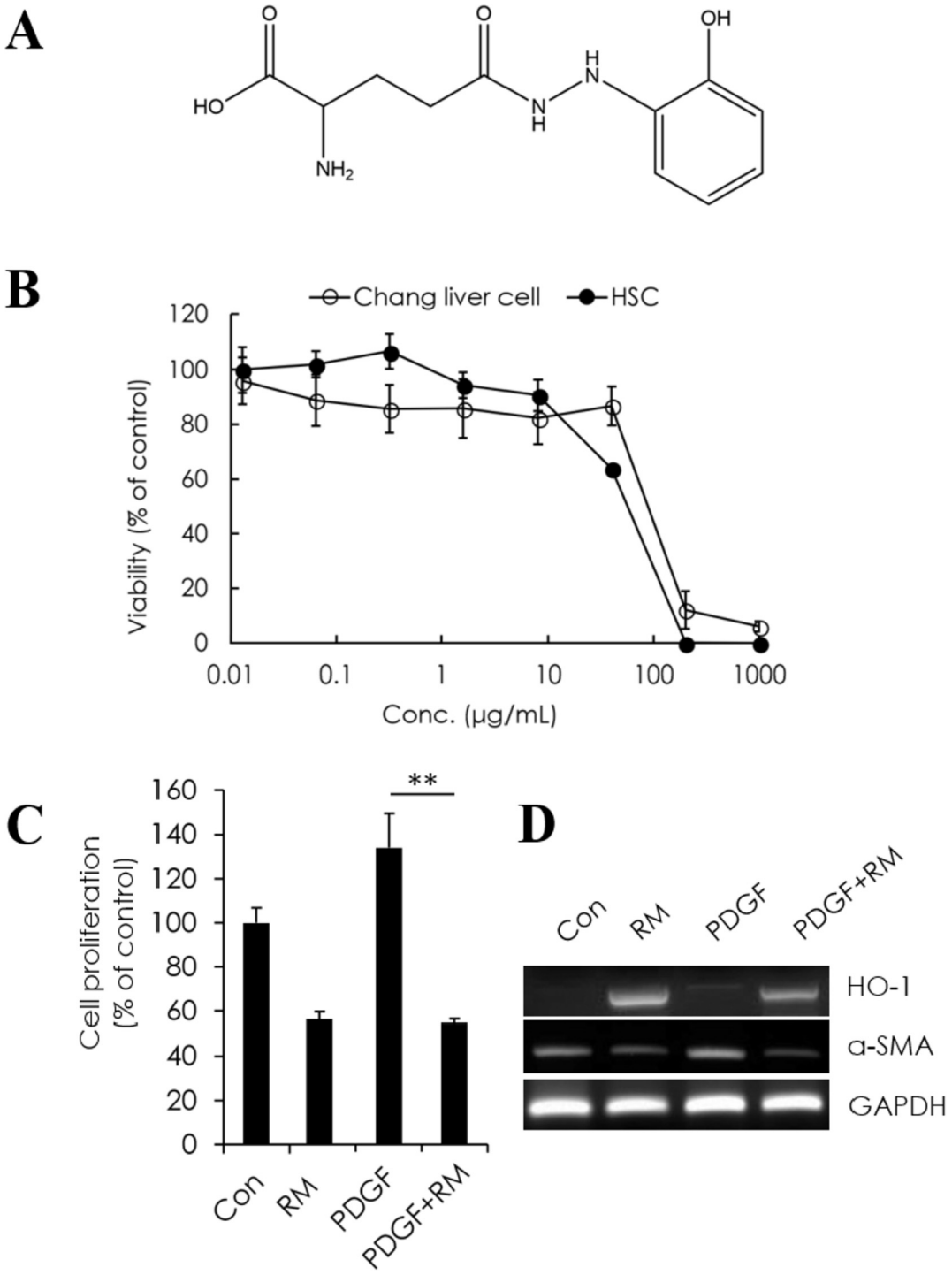

Fig. 1. Ramalin (RM) suppresses PDGF-induced proliferation of HSCs and prevents HSC activation.
(A) Chemical structure of ramalin (RM) [γ-glutamyl-N′-(2-hydroxyphenyl)hydrazide]. (B) Cytotoxicity assay was performed to evaluate the treatment dosage of RM against human hepatocytes (Chang liver cells) and isolated rat HSCs. (C and D) Isolated HSCs were incubated in the presence or absence of PDGF (400 pmol/L) with or without RM (10 μg/mL). Cell proliferation (C) was measured via WST-1 assay and the mRNA expression levels of α-SMA and HO-1 (D) were examined by RT-PCR. Data are presented as the mean ± SD of 3 independent experiments (**P < 0.01).

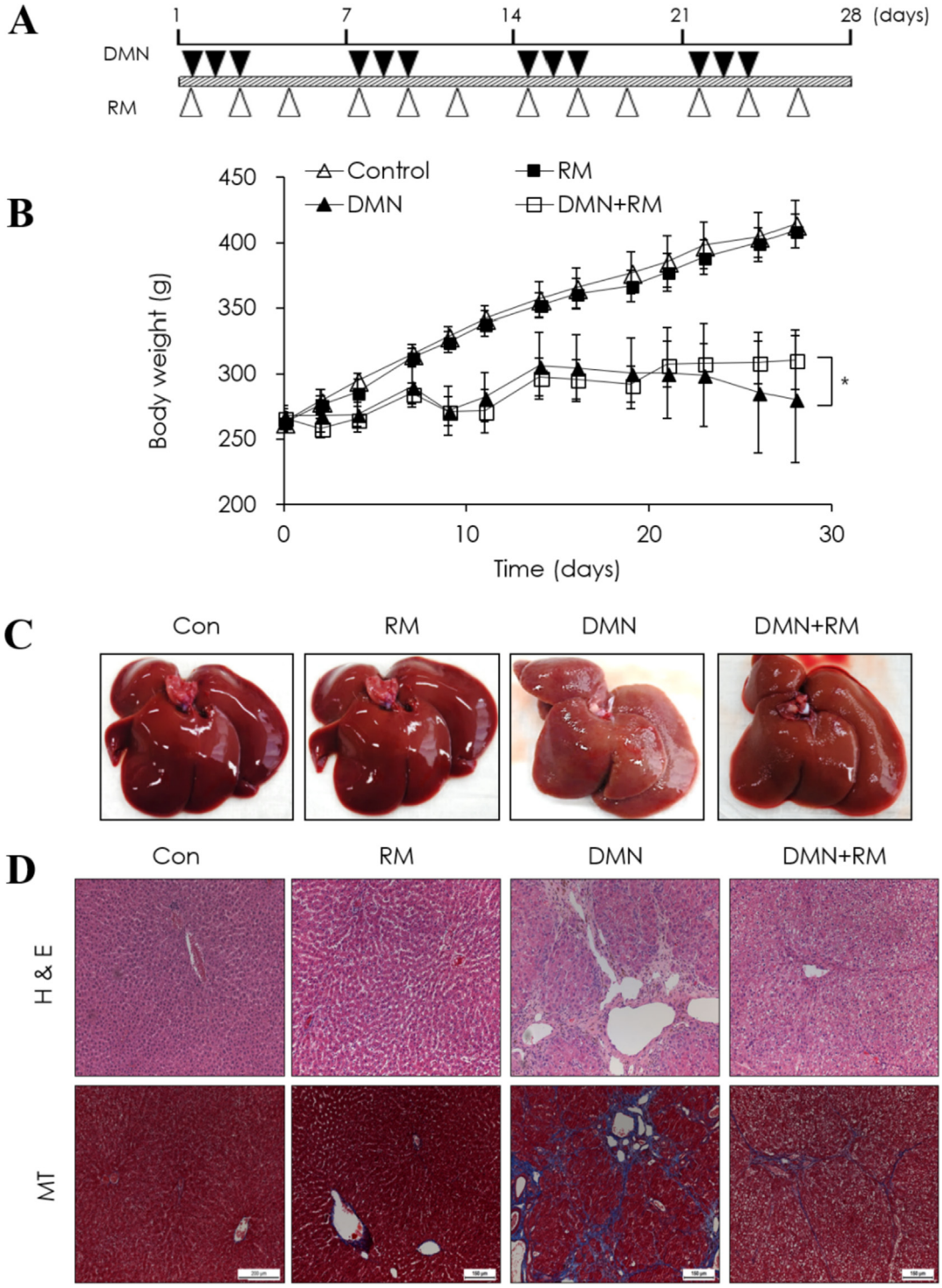

Fig. 2. Ramalin (RM) ameliorates hepatic fibrosis *in vivo*.
(A) Schematic illustration of *in vivo* experiments: RM was orally administered three times per week for 4 weeks (white arrows) while DMN was given *i.p.* for 3 consecutive days per week for 4 weeks (black arrows). (B) Body weight of rats after 30 days of each treatment were measured. (C) Morphological observation of the whole liver recovered from rats after each treatment. (D) Histopathological examination of rat liver tissue. Rat liver sections were stained with hematoxylin-eosin (H&E) and Masson's trichrome (MT).

Table 1
Effects of RM on body and liver weights in DMN-induced rat model of hepatic fibrosis.

	Liver wt(g)	Relative liver wt(%)	Body wt(g)
Con	14.6 ± 3.5	3.5 ± 0.9	414.1 ± 26.4
RM	14.8 ± 2.2	3.6 ± 0.4	409.4 ± 18.7
DMN	8.9 ± 2.4*	2.9 ± 0.7*	280.8 ± 69.1*
DMN + RM	14.6 ± 1.0#	4.8 ± 0.5#	310.8 ± 32.2#

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 ± S.D.(n = 10). *p < 0.05 vs. control, and #p < 0.05 vs. DMN group, respectively.

Table 2
Effects of RM on serum parameters in DMN-induced rat model of hepatic fibrosis.

	Con	RM	DMN	DMN + RM
Albumin(g/dL)	2.31 ± 0.10	2.55 ± 0.19	1.82 ± 0.05*	2.30 ± 0.06#
Total protein(g/dL)	5.56 ± 0.18	5.98 ± 0.36	4.59 ± 0.06	5.26 ± 0.12
T-bilirubin(mg/dL)	0.00 ± 0.00	0.00 ± 0.00	1.08 ± 0.97*	0.00 ± 0.00#
D-bilirubin(mg/dL)	0.00 ± 0.00	0.00 ± 0.00	0.73 ± 0.61*	0.00 ± 0.00#
ALP(IU/L)	177.33 ± 33.26	205.00 ± 36.06	610.00 ± 45.13*	256.67 ± 2.52#
AST(IU/L)	114.50 ± 18.71	166.33 ± 5.51	197.67 ± 74.78*	99.67 ± 14.19#
ALT(IU/L)	37.67 ± 4.63	41.33 ± 5.51	167.00 ± 3.61*	63.33 ± 8.15#

*< 0.05 *vs.* the level in group Control.
< 0.05 *vs.* the level in group DMN.

4. Discussion

In this study, our data showed that RM suppresses PDGF-induced proliferation of HSCs and prevented HSC activation *in vitro*. Previous studies have reported that activation of HSCs is a major phenotypic process of hepatic fibrosis in which normal hepatocytes are transformed into α-SMA and collagen type I-expressing myofibroblasts. The incubation of freshly isolated primary HSCs from normal rat livers with the PDGFactivated HSCs while RM treatment successfully attenuated this activation of HSCs *in vitro*.

We also demonstrated that RM ameliorated DMN-induced hepatic fibrosis *in vivo*. DMN, a potent hepatotoxin, causes severe liver damage resembling human hepatic fibrosis pathology in animal. DMN-induced hepatic fibrosis in rats reduced liver and body weights while elevating serum biochemical markers such as AST, ALT, ALP, and bilirubin while decreasing serum albumin levels. All of these changes in biochemical markers are homologous to clinical diagnostic tests for hepatic fibrosis in humans. Furthermore, histological analysis revealed that DMN induced severe hepatic fibrosis pathologies such as hepatic fibril deposition and collagen accumulation in the liver. The elevation of MDA and HP levels indicate that DMN caused the hepatic fibrosis phenotype via oxidative stress and collagen deposition. A major implication of our findings is that the oral administration of RM (20 mg/kg) completely alleviated the all of these serum biochemical and histological changes as well as the hepatic oxidative stress markers of the DMN-injured liver while showing no adverse effects on the animal.

Insufficient ROS removal is implicated in the pathogenesis of hepatic fibrosis [5,7,8]. Natural antioxidants extracted from various organisms in different regions throughout the world have shown therapeutic potential against cellular oxidative stress in tissues such as the liver [7,8,16,19,22,23]. In the present study, we examined the antioxidant properties of RM as an underlying hepatoprotective mechanism. RM attenuated MDA production while increasing the levels of antioxidant enzymes such as SOD, NQO1, and HO-1, suggesting involvement of Nrf2/antioxidant response element (ARE) pathways. Furthermore, we observed that RM administration reversed severe hepatic necrosis and increased SOD levels along with a concomitant decrease in MDA production in a DMN-induced liver injury rat model. This study is the first to report that RM increases antioxidant enzyme levels through the Nrf2/ARE pathway.

However, the mechanism for induction of antioxidants by RM is unclear. Nrf2 is an important transcription factor that regulates ARE-driven NQO1, HO-1, SOD, and GST gene expression. One possible mechanism is that the cytosolic repressor Keap 1 sequesters Nrf2 in the cytoplasm by linking it to the cytoskeleton where it is more likely to degrade, allowing it to have a critical role in the maintenance of the cellular redox balance [7,8,10,22–24]. Oxidative stress enables Nrf2 to escape the Keap1-mediated proteasomal degradation, leading to Nrf2 stabilization, subsequent nuclear translocation, and binding to ARE [7,8,10,22–24]. Nrf2-null mice are extremely susceptible to chemical oxidative and electrophilic stress, contributing to increased hepatotoxicity by acetaminophen [8,23–25] and ethanol [8,23,24]. Several dietary phytochemicals such as triterpenoids, isothiocyanates, flavonoids, and curcuminoids, have antioxidant effects similar to RM and induce Nrf2/ARE-mediated gene expression by increasing Nrf2 protein levels or by suppressing its turnover by ubiquitination [8,10,23,24]. Moreover, most of the genes that code for antioxidant enzymes have an ARE sequence in their promoter region. In our study, Nrf2 expression in DMN-induced livers was lower than in the control group, while RM induced Nrf2 activation.

There is an increasing medical need for new therapeutic options in the treatment of hepatic fibrosis. RM has shown promise as a non-toxic candidate by significantly reducing the activation of HSCs. Furthermore, RM is able to alter the cellular phenotype of hepatic fibrosis, which involves the accumulation of ECM in the liver, by reducing levels of MDS and HP, and collagen expression. Although further studies are needed to determine the exact mechanism by which RM produces these effects, this study has demonstrated that RM can attenuate hepatic fibrosis progression in DMN-induced rats, potentially via the Nrf2/ARE pathway.

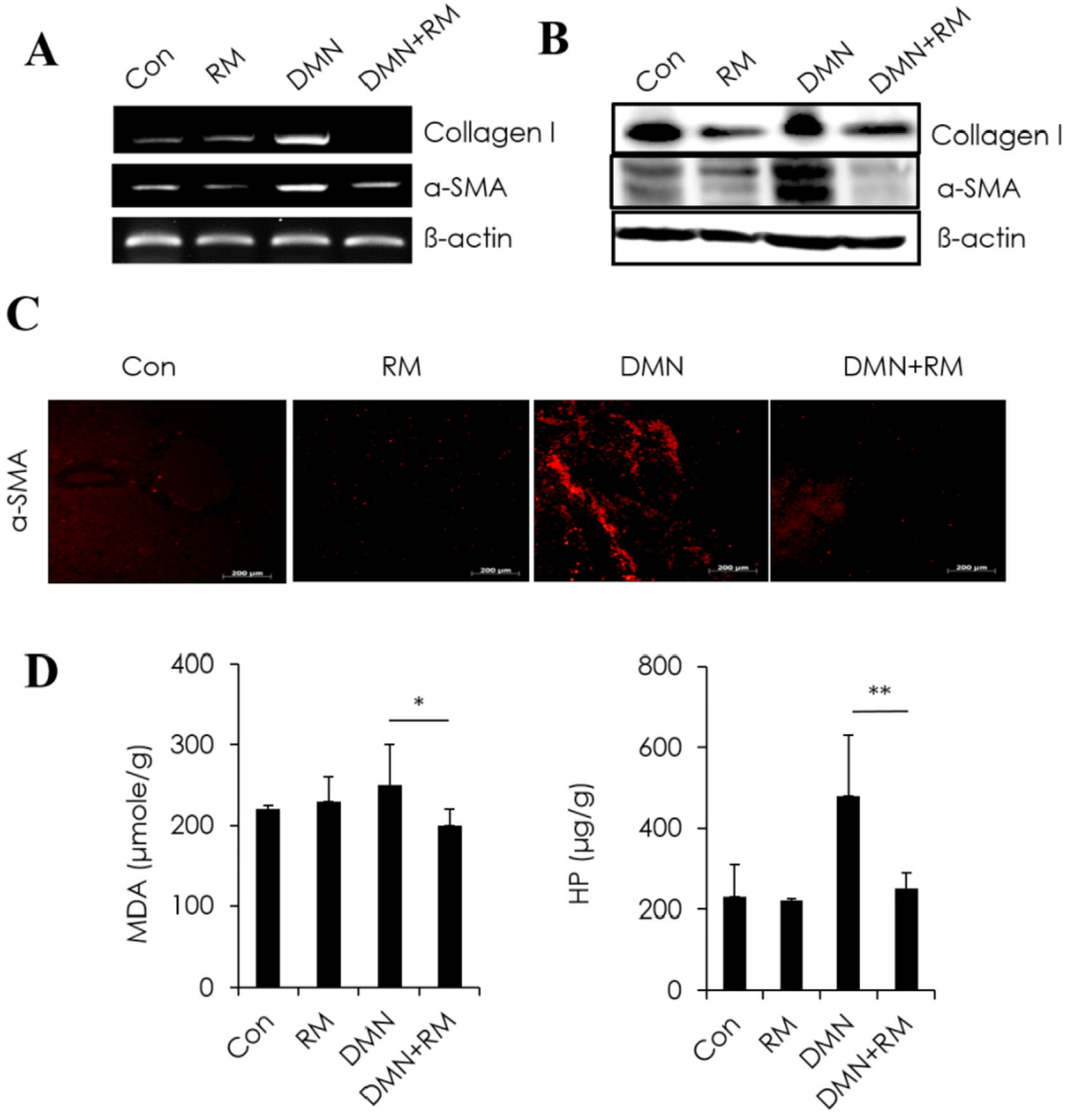

Fig. 3. Effects of ramalin (RM) on hepatic fibrosis in DMN-induced rats.
(A) The mRNA expression levels of collagen I and α-SMA were examined by RT-PCR. (B) Protein expression of collagen type I and α-SMA were determined via Western blot analysis. (C) Confocal microscopy of fluorescent immunohistochemical staining of the myofibroblastic myogenic marker; α-SMA (Red). (D) Hepatic level of MDA and HP were measured after each treatment. Each value represents the mean ± S.E.M. of at least three separate experiments. $^{*}P < 0.05$ and $^{**}P<0.01$, compared to the DMN-induced liver injury group. Con, control; RM, RM alone-treated group; DMN, DMN-induced liver injury group; DMN + RM, DMN plus RM treated group.

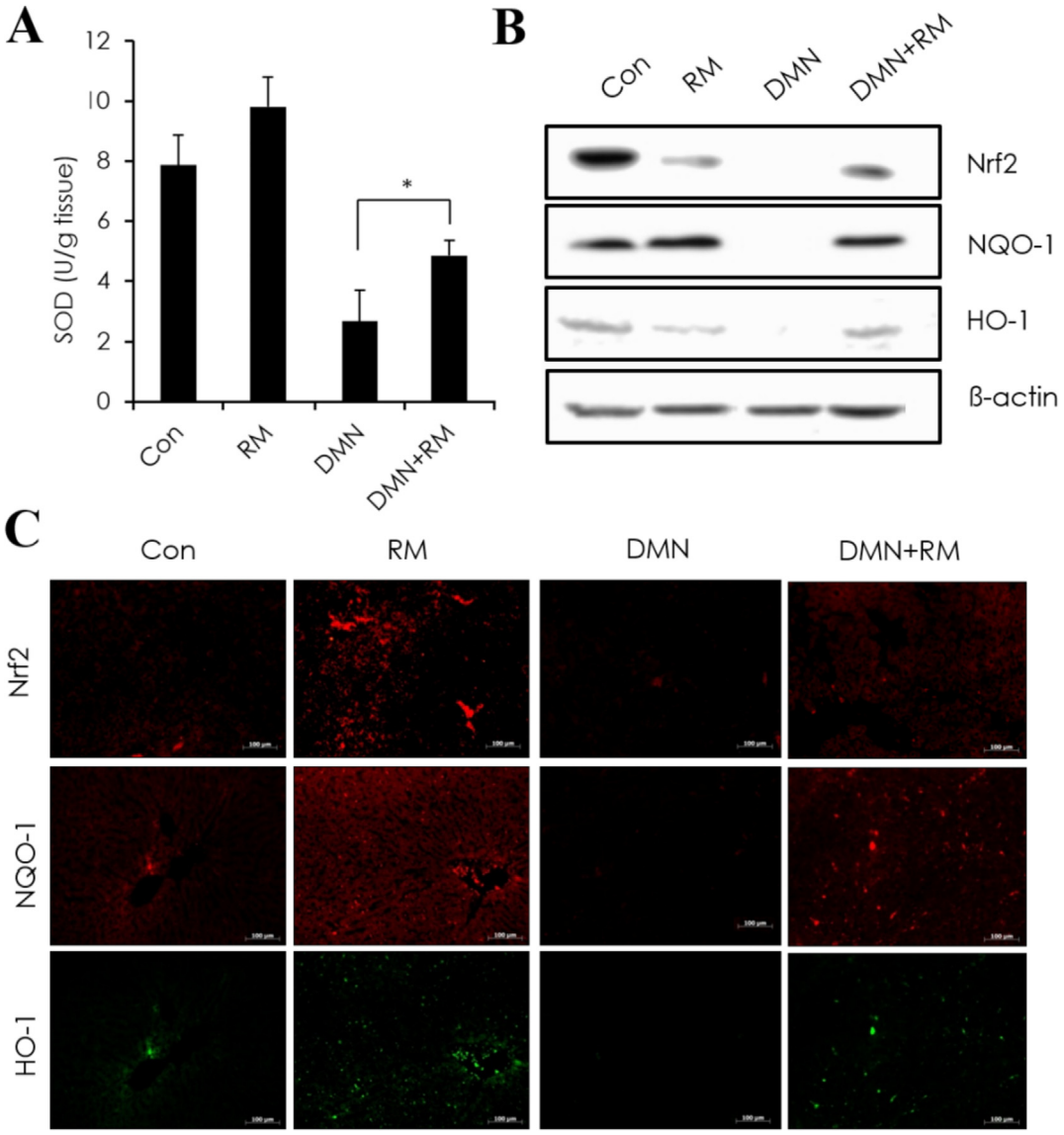

Fig. 4. Induction of antioxidant enzymes by ramalin (RM) through Nrf2 expression.
(A) SOD levels in liver tissues were measured after each treatment. (B) Protein expression of Nrf2, NQO1 and HO-1 were evaluated with or without RM treatment in rats with DMN-induced liver injury by Western blot analysis. (C) Confocal microscopy of fluorescent immunohistochemical staining of Nrf2 (Red), NQO1(Red), and HO-1(Green) with or without RM treatment in rats with DMN-induced liver injury. Each value represents the mean ± S.E.M. of at least three separate experiments. $^{*}P < 0.05$ compared to the DMN-induced liver injury group. Con, control; RM, RM alone-treated group; DMN, DMN-induced liver injury group; DMN + RM, DMN plus RM treated group.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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