



Research Article

Protective effect of wild ginseng cambial meristematic cells on D-galactosamine-induced hepatotoxicity in rats

Seok-Joo Kim¹, Hyo-Sun Choi¹, Hong-Ik Cho¹, Young-Woo Jin², Eun-Kyong Lee², Jeung Youb Ahn², Sun-Mee Lee^{1,*}¹ School of Pharmacy, Sungkyunkwan University, Suwon, Korea² Plant Stem Cell Institute, Unhwa Corp., Jeonju, Korea

ARTICLE INFO

Article history:

Received 26 February 2015

Received in Revised form

11 April 2015

Accepted 13 April 2015

Available online 30 April 2015

Keywords:

D-galactosamine

heme oxygenase-1

hepatitis

Panax ginseng

wild ginseng cambial meristematic cells

ABSTRACT

Background: *Panax ginseng* has a wide range of biological activities including anti-inflammatory, anti-oxidant, and immunomodulatory functions. Wild ginseng cambial meristematic cells (CMCs) were obtained from *P. ginseng* cambium. This study examined the protective mechanism of wild ginseng CMCs against D-galactosamine (GalN)-induced liver injury. GalN, a well-known hepatotoxicant, causes severe hepatocellular inflammatory damage and clinical features similar to those of human viral hepatitis in experimental animals.

Methods: Hepatotoxicity was induced in rats using GalN (700 mg/kg, i.p.). Wild ginseng CMCs was administered orally once a day for 2 wks, and then 2 h prior to and 6 h after GalN injection.

Results: Wild ginseng CMCs attenuated the increase in serum aminotransferase activity that occurs 24 h after GalN injection. Wild ginseng CMCs also attenuated the GalN-induced increase in serum tumor necrosis factor- α , interleukin-6 level, and hepatic cyclooxygenase-2 protein and mRNA expression. Wild ginseng CMCs augmented the increase in serum interleukin -10 and hepatic heme oxygenase-1 protein and mRNA expression that was induced by GalN, inhibited the increase in the nuclear level of nuclear factor-kappa B, and enhanced the increase in NF-E2-related factor 2.

Conclusion: Our findings suggest that wild ginseng CMCs protects liver against GalN-induced inflammation by suppressing proinflammatory mediators and enhancing production of anti-inflammatory mediators.

Copyright © 2015, The Korean Society of Ginseng, Published by Elsevier. All rights reserved.

1. Introduction

Hepatitis is a serious clinical problem caused by exposure to different agents such as viruses, alcohol, and chemicals, and by autoimmune diseases. Hepatitis may occur with limited or no symptoms and often leads to anorexia, jaundice, and hepatic carcinoma [1]. Although extensive studies into the treatment of liver disease with several oral hepatoprotective agents have been carried out, few beneficial liver drugs are currently available in the clinic. D-Galactosamine (GalN) is a specific hepatotoxicant that depletes the uridine triphosphate pool and thereby inhibits macromolecule synthesis, inducing hepatotoxicity that resembles that of human viral and drug-induced hepatitis [2]. In GalN-induced acute liver

injury, overproduction of reactive oxygen species from hepatocytes, infiltrated leukocytes, and activated Kupffer cells, accompanied by enhanced activity of the proinflammatory cytokine signaling pathway, contributes to liver damage [3]. Heme oxygenase (HO)-1 is an endogenous cytoprotective enzyme induced in response to cellular and environmental stresses. The critical role of HO-1 in the protection against *in vivo* and *in vitro* inflammatory disease models has been reported, and upregulation of HO-1 exerts an anti-inflammatory effect in experimental settings of hepatic ischemia/reperfusion and acute hepatitis [4].

Panax ginseng Meyer (Araliaceae) is a medicinal herb that has been used worldwide for > 5,000 yrs. *P. ginseng* has been widely used for treatment of inflammation, cardiovascular diseases,

* Corresponding author. School of Pharmacy, Sungkyunkwan University, 2066 Seobu-ro, Jangan-gu, Suwon-si, Gyeong gi-do, Korea.
E-mail address: sunmee@skku.edu (S.-M. Lee).

trauma, and bleeding caused by injury. Several investigators have reported the biologic role of *P. ginseng* in experimental models of liver diseases, including fatty liver disease, liver fibrosis, hepatic carcinoma, and chemical-induced liver injury [5]. Its biologic activities are primarily attributable to ginsenosides, which produce an array of pharmacologic responses [6]. Ginsenosides are located in the root, leaf, seed, and flower of *P. ginseng*, and have antitumor and immunomodulatory activity in various organs [7]. However, the yield of active components from natural sources can be highly variable depending on the source plant, location, season of harvest, and the prevailing environmental conditions [8]. In addition, a dramatic depletion of the plant population has occurred as a result of its consumption for medicinal purposes. The isolation of cambial meristematic cells (CMCs) from selected *P. ginseng* species may ensure a stable ginsenoside content and provide a sustainable supply for therapeutic applications [9]. However, there are no reports that demonstrate the biologic activity of natural compounds isolated from wild ginseng CMCs.

The objective of this study is to investigate the cytoprotective effects of wild ginseng CMCs against GalN-induced acute liver injury, particularly the modulation of inflammatory responses.

2. Materials and methods

2.1. Materials/chemicals

The followings are the materials and chemicals we used in this study: a 3-L bioreactor and a 20-L bioreactor (Samsung Science, Seoul, Korea), a 250-L bioreactor (Fermentec Co. Ltd, Cheongwon, Korea), 0.05% trifluoroacetic acid (Daejung Chemicals & Metals, Siheung, Korea), ginsenoside 20(S)-Rg3 and ginsenoside 20(S)-Rh2 (Chromadex, Irvine, CA, USA), mixture of ginsenoside Rk1 and Rg5 (Ambo Institute, Seoul, Korea), acetonitrile (Merck, Darmstadt, Germany), methanol (J.T. Baker, Center Valley, PA, USA), GalN and silymarin (Sigma-Aldrich, St. Louis, MO, USA), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assay kits (IVDLab Co., Uiwang, Korea), tumor necrosis factor (TNF)- α , interleukin (IL)-6 and IL-10 enzyme-linked immunosorbent assay kits (BD Science, San Diego, CA, USA), PRO-PREP and enhanced chemiluminescence detection system (iNtRON Biotechnology, Seongnam, Korea), NE-PER and BCA Protein Assay Kits (Thermo Fisher Scientific Inc., Rockford, IL, USA), polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA), Semi-Dry Trans-Blot Cell (Bio-Rad Laboratories, Hercules, CA, USA), RNAiso Plus and EcoDry cDNA Synthesis Premix (Takara Bio Inc., Shiga, Japan), Thermocycler and SYBR Green detection System (Roche Applied Science, Mannheim, Germany). The following antibodies were used in this study: cyclooxygenase-2 (COX-2; Cayman Chemicals, Ann Arbor, MI, USA), HO-1 (Enzo Life Sciences, Farmingdale, NY, USA), kelch-like ECH-associated protein 1 (Keap1) and lamin b1 (Abcam, Cambridge, MA, USA), nuclear factor-kappa B (NF- κ B)/p65, p-cJun and NF-E2-related factor 2 (Nrf2; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β -actin (Sigma-Aldrich).

2.2. Plant cell culture

Wild ginseng CMCs were obtained from the cambium of *P. ginseng* Meyer (wild ginseng; native to Kangwon province, Korea, for > 50 yrs; appraised by the Korea Association of Wild Ginseng Appraiser) [10]. Wild ginseng CMCs were gradually scaled up from 250-mL flasks to a 3-L bioreactor, a 20-L bioreactor, and a 250-L bioreactor. The bioreactors were all the airlift type. The working volume of the 250-L bioreactor was 210 L, 84% of the total volume. Wild ginseng CMCs were cultured in two stages: (1) a proliferation stage to obtain biomass in the 250-L bioreactor and (2) a production

stage to obtain secondary metabolites such as ginsenosides. In general, plant growth regulators, such as 2,4-dichlorophenoxyacetic acid or naphthaleneacetic acid, are used to induce cell division. However, to obtain biomass under the proliferation culture condition without using growth regulators, wild ginseng CMCs underwent a cell habituation process for 1 yr without growth regulators [9]. Wild ginseng CMCs obtained through this process were maintained for 37 mo. Culture conditions for the proliferation stage to obtain biomass of wild ginseng CMCs were Murashige and Skoog medium (MS medium) containing 3% sucrose, pH 5.8, cell inoculum of 2.09 g/L [dry cell weight (DCW)], and aeration rate of 0.038 vvm (air volume/media volume/min), 0.052 vvm, and 0.066 vvm for Days 1–5, Days 5–10, and Days 11–13 (Dwyer Instruments, Michigan City, IN, USA), respectively. When proliferation was complete, wild ginseng CMCs were subcultured every 13 days. Culture conditions for the production stage to obtain secondary metabolites from wild ginseng CMCs were 1/2 MS media containing 3% brown sugar, cell inoculum of 3.94 g/L (DCW), and 100 μ M methyl jasmonate. The air flow rate was 0.06 vvm, and the cells were cultured for 7 d. Proliferation and production culture were performed in the same room in the dark at a temperature of $21 \pm 1^\circ\text{C}$. After proliferation and production culture, biomass and major ginsenosides (Rb1, Rb2, Rc, Rd) of wild ginseng CMCs were obtained. The cells were then heat-treated in an extractor at 95°C for 48 h to obtain rare ginsenosides. During the heat treatment, major ginsenosides were converted to Rg3, Rh2, Rg5, and Rk1. The biomass was harvested and freeze-dried.

2.3. HPLC analysis (chromatographic analysis of wild ginseng CMCs)

An Agilent HPLC 1260 DAD system (Agilent Technologies, Santa Clara, CA, USA) and Agilent Zorbax Eclipse plus C18 column (4.6×100 mm, 3.5 μ m, Agilent Technologies) were used for the analysis of rare ginsenosides in wild ginseng CMCs. The detection wavelength was 203 nm, the temperature of the column was 30°C , and the mobile phase was 0.05% trifluoroacetic acid in water and 0.05% trifluoroacetic acid in acetonitrile with a flow rate of 1 mL/min. Standards used for the analysis were ginsenoside 20(S)-Rg3, ginsenoside 20(S)-Rh2, and a mixture of ginsenoside Rk1 and Rg5. Solvents used for the analysis were acetonitrile, methanol, and trifluoroacetic acid. Each standard was weighed and the concentration was adjusted to 0.5 mg/mL using methanol. Freeze-dried wild ginseng CMCs were ground to a fine powder, and 80% of methanol solution was added to 0.5 g of ground wild ginseng CMCs to a volume of 10 mL. This solution was extracted for 2 h under ultrasonic waves, centrifuged, and filtered through a 0.2- μ m syringe filter (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) for HPLC analysis.

2.4. Treatment of animals

Male Sprague–Dawley rats weighing 250–300 g (Orient Bio, Seongnam, Korea) were fasted overnight but given access to water *ad libitum*. All animal experiments were approved by the Animal Care Committee of Sungkyunkwan University School of Pharmacy, Suwon, Korea (SUSP14-04) and performed in accordance with the guidelines of National Institutes of Health (NIH publication No. 86-23, revised 1985). To generate GalN-induced hepatitis model, rats were injected intraperitoneally with 700 mg/kg of GalN dissolved in phosphate-buffered saline. The dose of GalN was selected according to previous reports [11,12]. There was no mortality in each experimental group, and all animals were shown to induce hepatotoxicity after GalN administration. Wild ginseng CMCs were suspended in distilled water and administered orally once a day for 2 wks and then 2 h prior to and 6 h after GalN injection. Animals were randomly assigned among the following seven groups ($n = 10$

per group): (1) vehicle-treated control (control), (2) wild ginseng CMCs (300 mg/kg)-treated control (wild ginseng CMCs + control), (3) vehicle-treated and GalN-treated (vehicle + GalN), (4–6) wild ginseng CMCs (75 mg/kg, 150 mg/kg, and 300 mg/kg)-treated and GalN-treated (wild ginseng CMCs 75 mg/kg, 150 mg/kg, and 300 mg/kg + GalN), and (7) silymarin (positive control, 50 mg/kg)-treated and GalN-treated (silymarin 50 mg/kg + GalN). Silymarin was suspended in 10% Tween 80 saline and administered orally once a day for 2 wks. The animals were euthanized under overdose of ether 24 h after GalN injection, and blood samples were taken from the inferior vena cava. The liver was isolated and used immediately for the preparation of mRNA, which was stored at -75°C for later analysis. A portion of the left lobe of the liver was used for histologic analysis.

2.5. Serum aminotransferase activities

Serum alanine ALT and AST activities were determined with standard spectrophotometric procedures using the ChemiLab ALT and AST assay kits, respectively.

2.6. Histologic analysis

Liver tissues were removed from a portion of the left lobe, fixed immediately in 10% neutral-buffered formalin, embedded in paraffin, and sectioned at 5- μm thickness. Serial sections were stained with hematoxylin and eosin for evaluation of portal inflammation, hepatocellular necrosis, and inflammatory cell infiltration. Sections were examined in a blinded manner under an Olympus CKX 41 microscope (Olympus Optical Co. Ltd., Tokyo, Japan).

2.7. Serum TNF- α , IL-6, and IL-10 levels

Serum levels of TNF- α , IL-6, and IL-10 were quantified at 24 h after GalN injection using commercial mouse ELISA kits according to the method described by Kang et al [13].

2.8. Preparation of protein extracts and Western blot immunoassay

Fresh liver tissue was isolated and homogenized in PRO- for whole protein samples and in NE-PER for extraction of nuclear and cytosolic protein samples, according to the manufacturer's instructions. Protein concentrations were determined using the BCA Protein Assay kit. Whole protein samples (20 μg) were used to determine the content of COX-2 and HO-1, cytosolic protein samples (20 μg) were used to determine the content of Keap1, and nuclear protein samples (10 μg) were used to determine the content of nuclear NF- κB /p65, phospho-cJun (p-cJun), and Nrf2. Protein samples were separated by sodium dodecyl sulfate/polyacrylamide

gel electrophoresis and transferred to polyvinylidene difluoride membranes using the Semi-Dry Trans-Blot Cell. After the transfer, the membranes were washed with 0.1% Tween 20 in Tris-buffered saline and blocked for 1 h at room temperature with 5% (w/v) skim milk powder in Tris-buffered saline. Blots were incubated overnight at 4°C with primary antibodies against COX-2 (1:4,000), HO-1 (1:4,000), Keap1 (1:4,000), NF- κB /p65 (1:4,000), p-cJun (1:4,000), and Nrf2 (1:2,000). On the following day, the blots were incubated with the appropriate secondary antibodies and detected using an enhanced chemiluminescence detection system according to the manufacturer's instructions. Immunoreactive bands were evaluated densitometrically with ImageQuant TL software (GE Healthcare, Piscataway, NJ, USA). Signals were normalized to that achieved with antibodies to β -actin (1:4,000) for whole and cytosolic proteins, or lamin B1 (1:10,000) for nuclear proteins.

2.9. Total RNA extraction and real time-polymerase chain reaction

For mRNA analysis, total RNA was extracted from liver tissue of rats in the different treatment groups using RNAiso Plus, and cDNA was synthesized using a reverse transcription reaction. The cDNA was amplified using real-time polymerase chain reaction with a thermocycler and the SYBR Green detection system. The amplification cycling conditions were as follows: 45 cycles of 30 s at 94°C , 30 s at 56°C , and 30 s at 72°C for TNF- α and HO-1; 32 cycles of 30 s at 94°C , 45 s at 58°C , 30 s at 72°C for IL-6; 45 cycles of 30 s at 94°C , 30 s at 64°C , and 30 s at 72°C for IL-10; 45 cycles of 30 s at 94°C , 30 s at 65°C , and 30 s at 72°C for COX-2; and 45 cycles of 30 s at 94°C , 30 s at 62°C , and 30 s at 72°C for β -actin. The gene-specific primers used are listed in Table 1. mRNA expression levels were normalized to the expression level of β -actin and analyzed with respect to the average of all delta C_t values in each sample using the cycle threshold (C_t) method. All samples were run in duplicate to ensure amplification integrity.

2.10. Statistical analysis

All results are presented as mean \pm standard deviation. The overall significance of the data was analyzed with one-way analysis of the variance. Differences between the groups were considered significant at $p < 0.05$ with the appropriate Bonferroni correction made for multiple comparisons.

3. Results

3.1. Qualitative and quantitative analysis of wild ginseng CMCs

Wild ginseng CMCs were ultrasonically extracted and analyzed through HPLC. The analyzed chromatogram is as shown in Fig. 1.

Table 1
Real-time RT-PCR primers used in study and the amplified product length

Gene	Accession number	Primer sequences (5'–3')	Product length
TNF- α	NM_012675	Sense: AGA ACA GCA ACT CCA GAA CAC CCT Antisense: ATC TCG GAT CAT GCT TTC CGT GCT	148
IL-6	NM_012589	Sense: ATA TAC CAC TTC ACA AGT CCG Anti-sense: GGC AAA TTT CCT GGT TAT ATC C	151
IL-10	NM_012854	Sense: GAC CAG CTG GAC AAC ATA CT Antisense: GAG GGT CTT CAG CTT CTC TC	143
COX-2	NM_017232	Sense: TCC AGT ATC AGA ACC GCA TTG CCT Antisense: AGC AAG TCC GTG TTC AAG GAG GAT	149
HO-1	NM_012580	Sense: AAC AAG CAG AAC CCA GTC TAT GC Antisense: GGC CCA CGC ATA TAC CCG CTA CCT	212
β -Actin	NM_031144	Sense: CTA CAA TGA GCT CGG TGT GGC Antisense: CAG GTC CAG ACG CAG GAT GGC	229

COX, cyclooxygenase; HO, heme oxygenase; IL, interleukin; RT-PCR, reverse transcription-polymerase chain reaction; TNF- α , tumor necrosis factor alpha

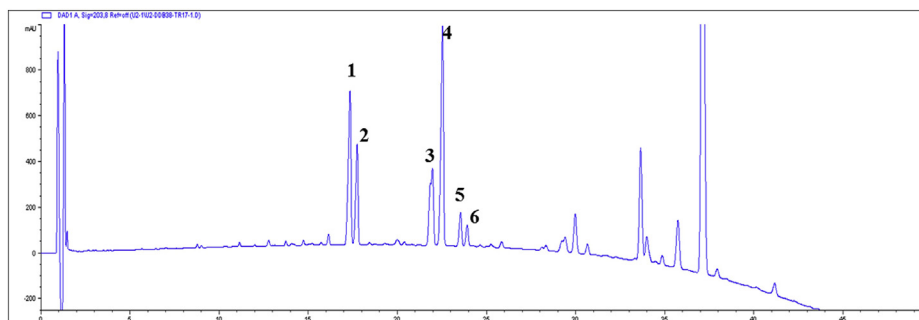


Fig. 1. HPLC chromatogram of ginsenosides detected from the wild ginseng CMCs. Numbers on the HPLC chromatogram indicate: (1) ginsenoside 20(S)-Rg3, (2) ginsenoside 20(R)-Rg3, (3) ginsenoside Rk1, (4) ginsenoside Rg5, (5) ginsenoside 20(S)-Rh2, and (6) ginsenoside 20(R)-Rh2. CMC, cambial meristematic cells. CMCs, cambial meristematic cells.

Major ginsenosides Rb1, Rb2, Rc, and Rd that were detected at the end of production culture stage were not detected after heat treatment, confirming that all these major ginsenosides have transferred to rare ginsenosides. Rare ginsenosides contained in wild ginseng CMCs were isolated and identified, and each compound was confirmed through electrospray ionization-mass spectrometry and nuclear magnetic resonance. In the analysis condition, compounds that were detected at 17.1 min, 17.7 min, 21.8 min, 22.3 min, 23.4 min, and 24.0 min were 20(S)-Rg3, 20(R)-Rg3, Rk1, Rg5, 20(S)-Rh2, and 20(R)-Rh2, respectively. The contents of Rg3, Rk1, Rg5, and Rh2 were confirmed through their standard compounds as shown in Table 2. To calculate the contents of Rk1 and Rg5, areas of the individual peaks of Rk1 and Rg5 were added. Rg3 and Rh2 were detected as isomers of the S form and R form, and their contents were calculated by summing the area of the individual peaks. Contents of rare ginsenosides, such as Rg3, Rh2, and Rk1 + Rg3 in dry cell of wild ginseng CMCs were 25.5 mg/g, 13.0 mg/g, and 29.0 mg/g, respectively. Rare ginsenosides are reported to be contained in red ginseng in a small amount, but wild ginseng CMCs produced high contents of rare ginsenosides. In particular, 20(S)-Rh2, 20(R)-Rh2, Rk2, Rh3, and 20(S)-PPD, which are rarely detected or are not contained in red ginseng, were highly produced in wild ginseng CMCs.

3.2. Serum aminotransferase activities

Serum ALT and AST activities in the control were 37.1 ± 17.6 U/L and 75.6 ± 30.3 U/L, respectively. At 24 h after GalN injection, serum ALT and AST activities significantly increased 4.8- and 6.8-fold, respectively, compared with the control. These increases were attenuated by treatment with wild ginseng CMCs at 75 mg/kg, 150 mg/kg, and 300 mg/kg and silymarin at 50 mg/kg. Wild ginseng CMCs alone (300 mg/kg) did not affect serum aminotransferase activities (Fig. 2A). Wild ginseng CMCs at 300 mg/kg was selected as the optimally effective dose for evaluating the molecular mechanisms by which wild ginseng CMCs attenuate GalN-induced hepatic injury.

3.3. Histologic analysis

The histologic features of livers from control animals indicated normal liver lobular architecture and cell structure (Fig. 2B).

Table 2
Analysis of ginsenosides content (mg/g, DCW) of wild ginseng CMCs

Rare ginsenosides	Rg3	Rk1 + Rg5	Rh2	Total content
Content (mg/g, DCW)	25.5	29.0	13.0	67.5

CMCs, cambial meristematic cells; DCW, dry cell weight

However, livers exposed to GalN showed obvious broad hemorrhagic necrosis, extensive areas of portal inflammation, and a moderate increase in inflammatory cell infiltration. These pathologic alterations were ameliorated in animals that were treated with wild ginseng CMCs.

3.4. Cytokine levels

The serum level and hepatic mRNA expression of the proinflammatory cytokine TNF- α in the GalN-treated group were approximately 3.1- and 8.0-fold higher than that in the control group, respectively (Fig. 3A). Similarly, the serum level and hepatic mRNA expression of IL-6 in the GalN-treated group were approximately 13.5- and 12.2-fold higher than that in the control group, respectively. These changes were attenuated by wild ginseng CMCs (Fig. 3B). As shown in Fig. 3C, the serum level and hepatic mRNA expression of the anti-inflammatory cytokine IL-10 increased 4.5- and 3.1-fold at 24 h after GalN injection, respectively. These changes in IL-10 expression were augmented by wild ginseng CMCs.

3.5. Hepatic COX-2 and HO-1 protein and mRNA expression

The levels of hepatic COX-2 protein and mRNA expression in rats in the GalN group were approximately 8.2- and 4.1-fold higher than that in the control group, respectively. The increased expression of COX-2 mRNA and protein expression was attenuated by wild ginseng CMCs (Fig. 4A). Fig. 4B shows that levels of hepatic HO-1 protein and mRNA expression increased 1.8- and 2.0-fold after GalN injection compared with those of the control. These increases were augmented by wild ginseng CMCs.

3.6. Nuclear translocation of NF- κ B, p-cJun, and Keap1/Nrf2

Nuclear levels of NF- κ B/p65, p-cJun, and Nrf2, and the cytosolic level of Keap1 were measured using immunoblot analysis (Fig. 5). At 24 h after GalN injection, the nuclear level of NF- κ B/p65 increased 4.5-fold compared with that of the control, whereas the cytosolic level of NF- κ B/p65 decreased compared with that of the control. In the GalN-treated group, the nuclear protein level of p-cJun increased 2.3-fold compared with the control. Wild ginseng CMCs attenuated these changes in the levels of nuclear and cytosolic NF- κ B/p65 but did not affect the level of nuclear p-cJun (Fig. 5A, B). At 24 hours after GalN injection, the nuclear level of Nrf2 increased 2.7-fold over the control, whereas the cytosolic level of Keap1 decreased. Wild ginseng CMCs augmented the increase in the nuclear level of Nrf2 and attenuated the decrease in the cytosolic level of Keap1 (Fig. 5C, D).

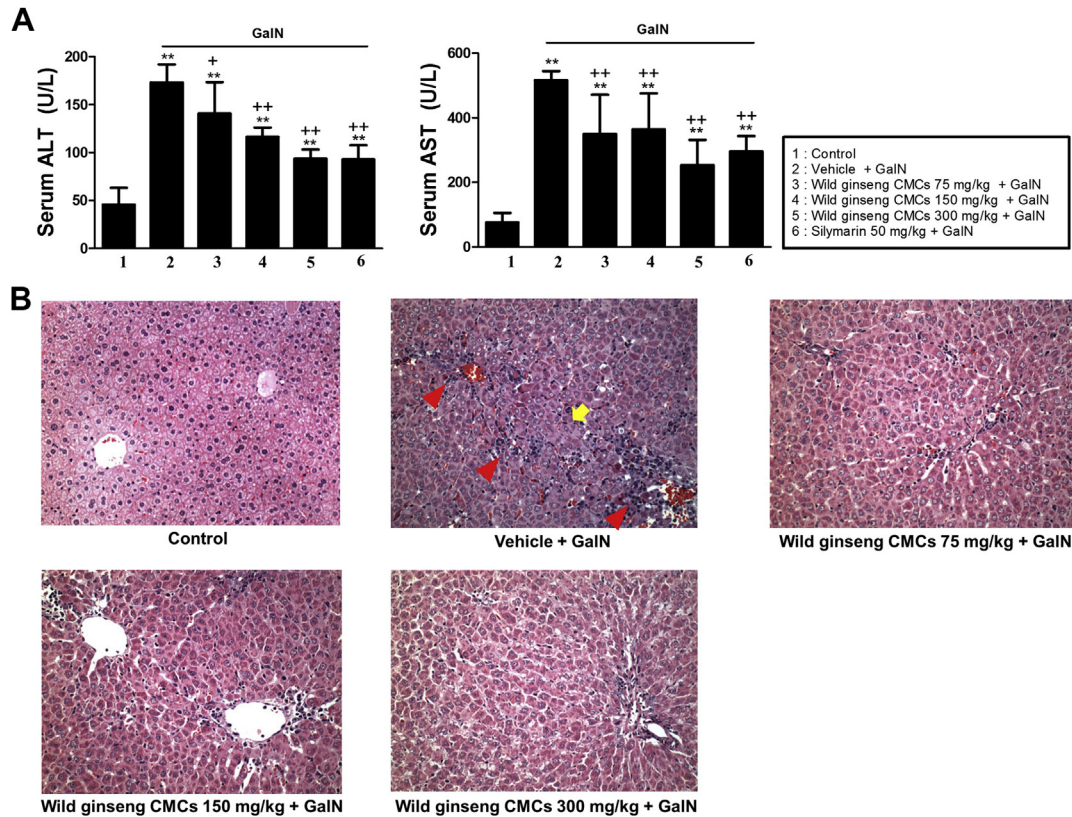


Fig. 2. Effects of wild ginseng CMCs on serum ALT, AST levels (A) and histological changes (B) in the rat liver after GalN (700 mg/kg) injection (original magnification $\times 400$). All values are means \pm SD of 10 rats per group. Rats received oral injection of vehicle or wild ginseng CMCs (75, 150, and 300 mg/kg) once daily for 2 wks and 2 h prior to and 6 h after GalN (700 mg/kg) treatment. $**p < 0.05$, significantly different from control group. $^1p < 0.05$, $^1p < 0.01$, significantly different from GalN group. Histological features of liver sections stained with H&E at 24 h after GalN injection. Typical images were chosen from each experimental group (original magnification, $\times 400$). Arrow and arrowhead indicate hemorrhagic necrosis and inflammatory cell infiltration, respectively. ALT, alanine aminotransferase; AST, aspartate aminotransferase; CMCs, cambial meristematic cells; H&E, hematoxylin and eosin; GalN, D-galactosamine; SD, standard deviation.

4. Discussion

P. ginseng is one of the most abundantly used herbal medicines in Korea, China, and Japan. Many studies have reported that *P. ginseng* possesses antidiabetic, antihepatotoxic, antifatigue, antistress, and blood pressure regulatory effects [14]. Wild ginseng, however, is rarely available, and the price is very high owing to its rarity. In addition, extensive efforts on quality control are required because the contents of ginsenosides vary depending on the ginseng species and cultivation environments [15]. To overcome these problems, plant cell culture emerged as an attractive alternative. Various tissue culture techniques are being used to produce large amounts of wild ginseng with increased yield of ginsenosides in a relatively shorter time [16]. However, it is often not commercially viable because of the difficulties associated with culturing dedifferentiated plant cells on an industrial scale and stable material production. To bypass the dedifferentiation step, some of us established a technique of isolation and culture of innately undifferentiated CMCs. The isolation of CMCs from selected *P. ginseng* species may ensure a stable method to provide high contents of ginsenosides and sustainable supply for therapeutic applications [10].

GalN is a highly specific hepatotoxic agent that induces liver injury closely resembling viral hepatitis when given to rats. Administration of GalN disrupts the permeability of the plasma membrane, causing leakage of enzymes from the cell. In our study, a significant elevation of serum ALT and AST was detected after GalN injection, and this increase was attenuated by treatment with wild ginseng CMCs. Normal lobular architecture and cell structure

were observed in the control group, whereas increased hepatocellular necrosis, portal inflammation, and inflammatory cell infiltration were observed in the liver section obtained from GalN-treated animals. These pathologic changes were attenuated by treatment with wild ginseng CMCs, suggesting that wild ginseng CMCs may have a potential clinical application in the treatment of liver disease.

The liver is an inflammatory organ, and inflammatory processes contribute to a number of pathologic events. Kupffer cells, the resident macrophages of the liver, reside in a strategic position within the liver sinusoids. They interact with hepatocytes, leukocytes, and various mediators from the gut and therefore represent the first line of defense against microorganisms crossing the gut mucosal barrier and entering the portal circulation. Moreover, activated Kupffer cells produce a number of signaling molecules that promote inflammatory reactions and cause cell dysfunction and enhanced expression of adhesion molecules in endothelial cells. Stachlewitz et al [17] demonstrated the integral role of Kupffer cells in inflammatory cell interactions after GalN injection. Furthermore, the interaction of neutrophils with endothelial cells and subsequent neutrophil infiltration are observed in GalN-induced hepatic injury [18]. TNF- α is one of the key proinflammatory cytokines involved in the responses that ultimately result in liver failure [19]. TNF- α participates in necrotic, apoptotic, and inflammatory pathways in GalN-induced hepatotoxicity. IL-6, promptly and transiently produced in response to infections and tissue injuries, contributes to host defense through the stimulation of acute phase responses, hematopoiesis, and immune reactions. IL-

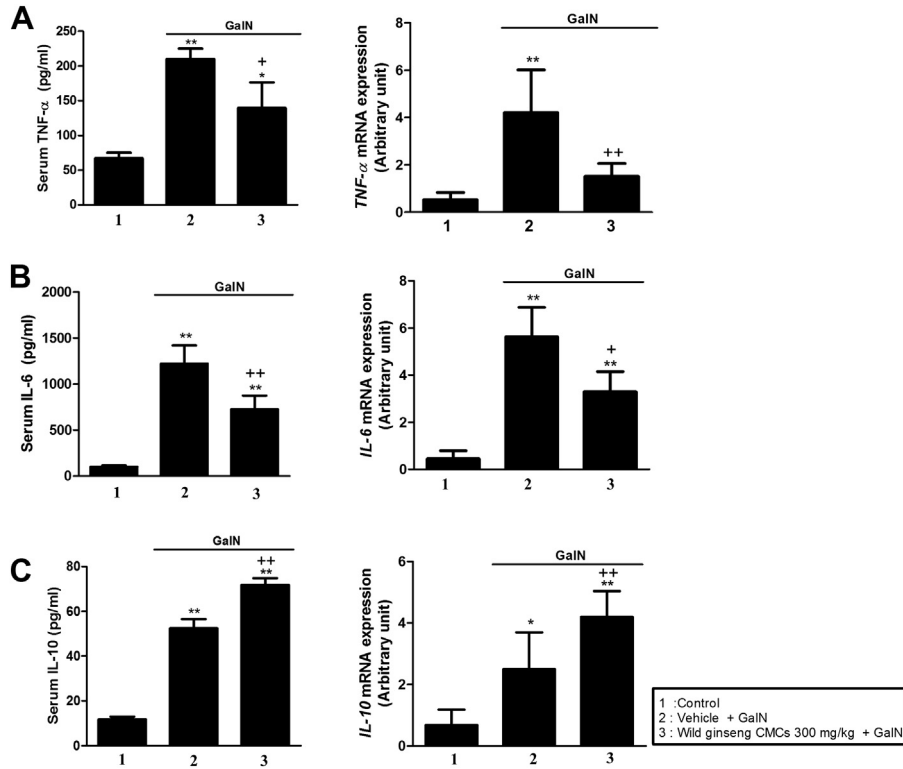


Fig. 3. Effects of wild ginseng CMCs (300 mg/kg) on levels of serum TNF- α and hepatic mRNA expression (A), and serum IL-10 and hepatic mRNA expression (B) after GalN (700 mg/kg) treatment. All values are means \pm SD of 10 rats per group. * $p < 0.05$, ** $p < 0.01$, significantly different from control group. † $p < 0.05$, †† $p < 0.01$, significantly different from GalN group. CMCs, cambial meristematic cells; GalN, D-galactosamine; IL, interleukin; TNF- α , tumor necrosis factor alpha; SD, standard deviation

6 produced by Kupffer cells in response to liver damage also plays a critical role in the acute phase response in the liver [20]. In our study, we found that the serum level and hepatic gene expression of TNF- α and IL-6 increased after GalN injection, and that these increases were attenuated by wild ginseng CMCs.

COX-2 plays a key role in the pathogenesis of inflammation and its expression is markedly upregulated by inflammatory stimuli leading to increased synthesis of prostanoids, which are potent lipid inflammatory mediators, in inflamed tissues. A previous report demonstrated that genetic overexpression of COX-2

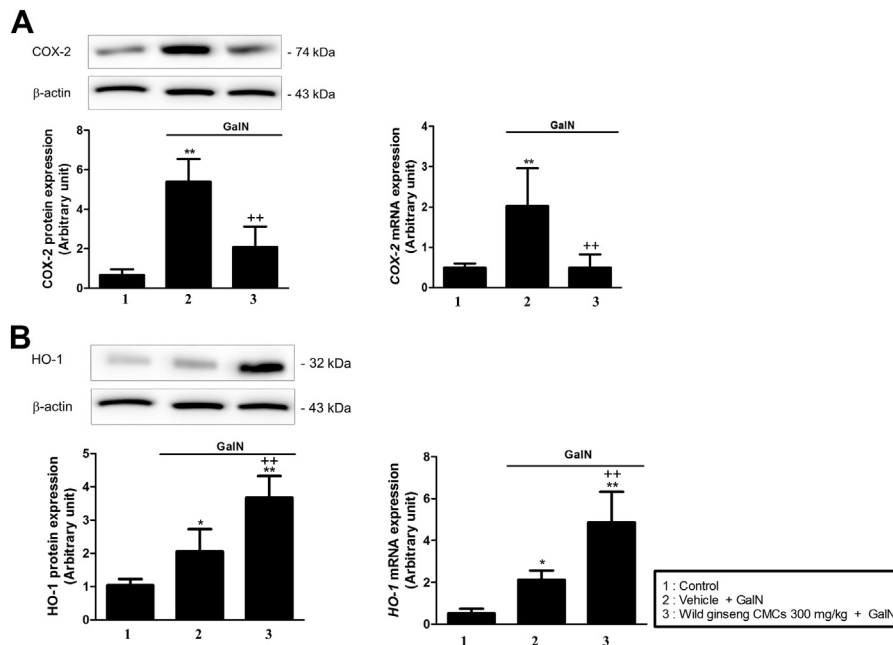


Fig. 4. Effects of wild ginseng CMCs (300 mg/kg) on levels of hepatic COX-2 protein and mRNA expression (A), and hepatic HO-1 protein and mRNA expression (B) after GalN (700 mg/kg) treatment. All values are means \pm SD of 10 rats per group. * $p < 0.05$, ** $p < 0.01$, significantly different from control group. † $p < 0.05$, †† $p < 0.01$, significantly different from GalN group. CMCs, cambial meristematic cells; COX-2, cyclooxygenase-2; GalN, D-galactosamine; HO-1, heme oxygenase-1; SD, standard deviation.

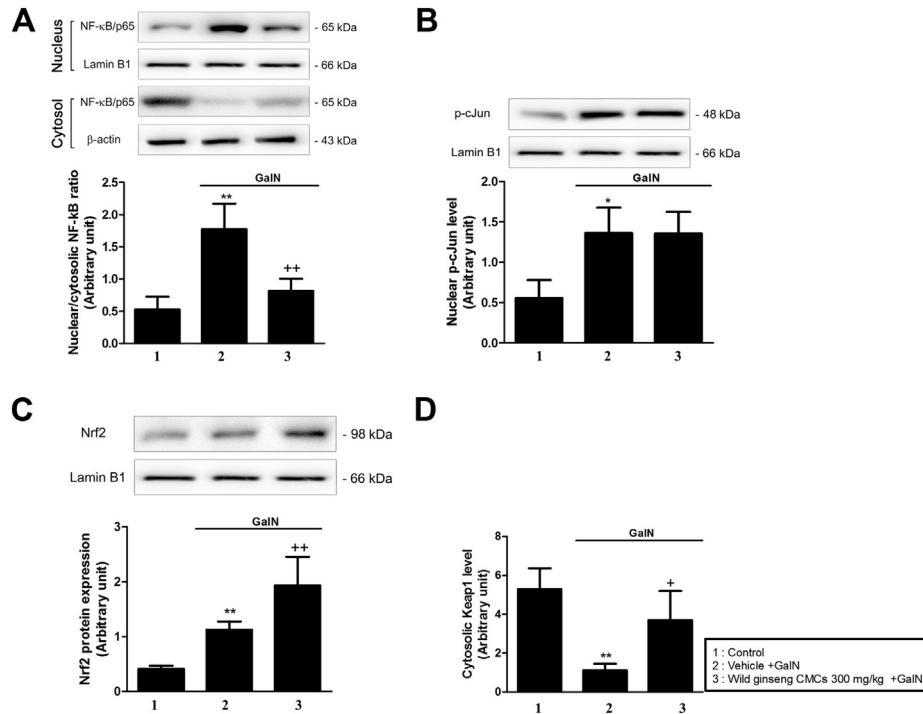


Fig. 5. Effects of wild ginseng CMCs (300 mg/kg) on hepatic levels of nuclear NF-κB (A), p-c-Jun (B), and Nrf2 (C), and cytosolic Keap1 (D) protein expression. All values are means \pm SD of 10 rats per group. * $p < 0.05$, ** $p < 0.01$, significantly different from control group. † $p < 0.05$, †† $p < 0.01$, significantly different from GalN group. CMCs, cambial meristematic cells; GalN, D-galactosamine; Keap1, kelch-like ECH-associated protein 1; NF-κB, nuclear factor-kappa B; Nrf2, NF-E2-related factor 2; TNF, tumor necrosis factor.

accelerates endotoxin-induced inflammation and subsequent liver damage [21]. Modulation of COX-2 expression is an effective strategy for regulating inflammation in an animal model of GalN-induced hepatic failure. We demonstrated increased mRNA and protein expression of hepatic COX-2 in GalN-treated animals, which was ameliorated by wild ginseng CMCs. These results indicate that wild ginseng CMCs suppress GalN-induced production of proinflammatory mediators at the transcriptional level.

Toll-like receptors (TLRs) represent a family of pattern recognition receptors that are essential for infectious and inflammatory disease states. In particular, hepatic TLR2 and TLR4 mRNA and protein expression were increased in patients with hepatitis and cirrhosis [22,23]. Recent investigations demonstrated that GalN-induced hepatotoxicity in rat involved the increase of expression of *TLR2* and *TLR4* mRNA [24]. Accumulating data have shown the involvement of transcription factors such as NF-κB and AP-1 in the amplification of inflammatory response in animal models of GalN-induced hepatotoxicity. NF-κB is a ubiquitous transcription factor, and the functional importance of NF-κB in inflammation is based on its ability to regulate the transcription of multiple inflammatory genes, including *TNF-α* and *COX-2* [25]. A significant decrease in hepatic NF-κB activation was noted in TLR4 knockout mice compared to wild type mice following the induction of fulminant hepatic failure by GalN/lipopolysaccharide (LPS). AP-1 is a group of dimeric transcription factors consisting of cJun, cFos, activating transcription factor, and musculoaponeurotic fibrosarcoma, and the combination of proteins in the AP-1 complex affects its DNA binding activity. The *TNF-α* gene contains a cJun binding site in its promoter. Mitogen-activated protein kinases phosphorylation by stimulation of macrophage TLR4 activates the cJun, which subsequently promotes synthesis of proinflammatory mediators [26]. In our study, elevated nuclear NF-κB/p65 and p-cJun protein levels were observed in GalN-treated animals. Wild ginseng CMCs reduced the increased nuclear level of NF-κB/p65 protein, but did not affect the level of nuclear p-cJun protein. These results suggest

that wild ginseng CMCs inhibit the transactivation of NF-κB. Unfortunately, we did not find a way to induce TLR2 and TLR4 mRNA and protein expression in GalN-treated rats; however, this could be a result of the differences in the injury models used by our group (700 mg/kg GalN) and other groups (800 mg/kg GalN or 800 mg/kg GalN + 40 μg/kg LPS) [27]. Further investigations are needed to elucidate the role of TLRs in GalN-induced hepatotoxicity.

A proper balance between pro- and anti-inflammatory responses is essential for maintaining an adequate immune response in GalN-induced liver injury, and the upregulation of anti-inflammatory mediators by therapeutic agent(s) may be helpful in hepatoprotection. IL-10 is an anti-inflammatory cytokine that was initially identified as a product of T helper cells (Th2) and has since been reported to be produced by Kupffer cells, implying a role in the regulation of local immune responses in the liver sinusoids [28]. IL-10 can suppress serum TNF-α, interferon-γ, and IL-1 levels and reduce the mortality induced by GalN/LPS [29]. In the present study, wild ginseng CMCs augmented the GalN-induced increase in serum IL-10 protein level and its mRNA expression.

HO-1 has been implicated in the cellular adaptive response against stress conditions. The primary function of HO-1 is to catabolize the oxidative degradation of heme into carbon monoxide (CO), free iron, and biliverdin, and the CO produced by its activity exerts powerful anti-inflammatory and anti-apoptotic effects. There is accumulating evidence for a critical role of HO-1 signaling in the protection against several inflammatory diseases [30]. Previous reports demonstrated that pharmacologic induction of HO-1 has an anti-inflammatory effect in sterile inflammatory settings of hepatic ischemia/reperfusion, and that genetic overexpression by adenovirus gene transfer of HO-1 (Ad-HO-1) confers cytoprotection [31]. Nrf2 is a transcription factor that promotes the transcription of a series of cytoprotective genes in response to cellular stress. Under basal conditions, Nrf2 is sequestered by Keap1 in the cytosol. In response to stress, Nrf2 is released from Keap1 and translocates into the nucleus, where it induces an array of cytoprotective genes

as an adaptive response. Alam and Cook [32] demonstrated that, upon activation, Nrf2 enters the nucleus where it binds to the ARE in the HO-1 promoter region to trigger gene expression. During acute liver injury, and independent of the toxicants that caused it, Nrf2 plays a protective role by inducing antioxidant enzymes such as NAD(P)H quinone oxidoreductase 1 (Nqo1), glutathione, and many proteins involved in the repair of oxidized proteins, and by attenuating the expression of inflammatory genes including IL-1 β , IL-6, and TNF- α [33]. Increased translocation of HO-1 and Nrf2 to protect the liver has been documented in GalN-induced acute liver injury [34]. In our study, rats treated with GalN showed a decrease in cytosolic levels of Keap1, and an increase in the nuclear level of Nrf2 and hepatic mRNA expression of HO-1. Treatment with wild ginseng CMCs augmented the increase in nuclear Nrf2 level and hepatic HO-1 mRNA expression, and attenuated the decrease in cytosolic Keap1 levels. These results suggest that wild ginseng CMCs may reduce the inflammatory response that occurs after GalN injection via the enhancement of Nrf2 nuclear translocation and subsequent induction of HO-1.

In summary, our findings suggest that wild ginseng CMCs protect against GalN-induced acute liver injury. The mechanism of action of wild ginseng CMCs appear to involve its ability to inhibit overexpression of proinflammatory mediators and nuclear translocation of NF- κ B, and to induce the Nrf2/HO-1 anti-inflammatory pathway. Thus, we propose that wild ginseng CMCs are a potential therapeutic medication for the prevention of acute liver injury in clinical settings. Moreover, our findings may provide important information for novel therapeutic approaches using natural plant products from cultured multipotent cells.

Conflicts of interest

The authors declare they have no conflict of interests.

Acknowledgments

This research was financially supported by the Ministry of Trade, Industry & Energy (MOTIE), Korea Institute for Advancement of Technology (KIAT), and Honam Institute for Regional Program Evaluation through the Leading Industry Development for Economic Region.

References

- Bernal W, Auzinger G, Dhawan A, Wendon J. Acute liver failure. *Lancet* 2010;376:190–201.
- Decker K, Keppler D. Galactosamine hepatitis: key role of the nucleotide deficiency period in the pathogenesis of cell injury and cell death. *Rev Physiol Biochem Pharmacol* 1974;71:77–106.
- Ghosh M, Das J, Sil PC. D(+) galactosamine induced oxidative and nitrosative stress-mediated renal damage in rats via NF-kappaB and inducible nitric oxide synthase (iNOS) pathways is ameliorated by a polyphenol xanthone, mangiferin. *Free Radic Res* 2012;46:116–32.
- Immenschuh S, Baumgart-Vogt E, Mueller S. Heme oxygenase-1 and iron in liver inflammation: a complex alliance. *Curr Drug Targets* 2010;11:1541–50.
- Shukla R, Kumar M. Role of *Panax ginseng* as an antioxidant after cadmium-induced hepatic injuries. *Food Chem Toxicol* 2009;47:769–73.
- Attele AS, Wu JA, Yuan CS. Ginseng pharmacology: multiple constituents and multiple actions. *Biochem Pharmacol* 1999;58:1685–93.
- Hofseth LJ, Wargovich MJ. Inflammation, cancer, and targets of ginseng. *J Nutr* 2007;137:183S–5S.
- Yun UW, Yan Z, Amir R, Hong S, Jin YW, Lee EK, Loake GJ. Plant natural products: history, limitations and the potential of cambial meristematic cells. *Biotechnol Genet Eng Rev* 2012;28:47–59.
- Asano S, Otobe K. Production of phytochemicals by using habituated and long-term cultured cells. *Plant Biotechnol J* 2011;28:51–62.
- Lee EK, Jin YW, Park JH, Yoo YM, Hong SM, Amir R, Yan Z, Kwon E, Elfick A, Tomlinson S, et al. Cultured cambial meristematic cells as a source of plant natural products. *Nat Biotechnol* 2010;28:1213–7.
- Choi JH, Kang JW, Kim DW, Sung YK, Lee SM. Protective effects of mg-CUD against D-galactosamine-induced hepatotoxicity in rats. *Eur J Pharmacol* 2011;657:138–43.
- Kang JW, Kim SJ, Kim HY, Cho SH, Kim KN, Lee SG, Lee SM. Protective effects of HV-P411 complex against D-galactosamine-induced hepatotoxicity in rats. *Am J Chin Med* 2012;40:467–80.
- Kang TJ, Moon JS, Lee SY, Yim DS. Polyacetylene compound from *Cirsium japonicum* var *ussuriense* inhibits the LPS-induced inflammatory reaction via suppression of NF-kB activity in RAW 264.7 Cells. *Biomol Ther* 2011;19:97–101.
- Yun TK. Brief introduction of *Panax ginseng* C.A. Meyer. *J Korean Med Sci* 2001;16:3–5.
- Ahn IO, Lee SS, Lee JH, Lee MJ, Jo BG. Comparison of ginsenoside contents and pattern similarity between root parts of new cultivars in *Panax ginseng* C.A. Meyer. *J Ginseng Res* 2008;32:15–8.
- Kim YS, Hahn EJ, Murthy HN, Paek KY. Adventitious root growth and ginsenoside accumulation in *Panax ginseng* cultures as affected by methyl jasmonate. *Biotechnol Lett* 2004;26:1619–22.
- Stachlewitz RF, Seabra V, Bradford B, Bradham CA, Rusyn I, Germolec D, Thurman RG. Glycine and uridine prevent D-galactosamine hepatotoxicity in the rat: role of Kupffer cells. *Hepatology* 1999;29:737–45.
- Komatsu Y, Shiratori Y, Kawase T, Hashimoto N, Han K, Shiina S, Matsumura M, Niwa Y, Kato N, Tada M. Role of polymorphonuclear leukocytes in galactosamine hepatitis: mechanism of adherence to hepatic endothelial cells. *Hepatology* 1994;20:1548–56.
- Shito M, Balis UJ, Tompkins RG, Yarmush ML, Toner M. A fulminant hepatic failure model in the rat: involvement of interleukin-1beta and tumor necrosis factor-alpha. *Dig Dis Sci* 2001;46:1700–8.
- Wanner GA, Ertel W, Muller P, Hofer Y, Leiderer R, Menger MD, Messmer K. Liver ischemia and reperfusion induces a systemic inflammatory response through Kupffer cell activation. *Shock* 1996;5:34–40.
- Han C, Li G, Lim K, DeFrances MC, Gandhi CR, Wu T. Transgenic expression of cyclooxygenase-2 in hepatocytes accelerates endotoxin-induced acute liver failure. *J Immunol* 2008;181:8027–35.
- Berzsenyi MD, Roberts SK, Preiss S, Woollard DJ, Beard MR, Skinner NA, Bowden DS, Visvanathan K. Hepatic TLR2 & TLR4 expression correlates with hepatic inflammation and TNF- α in HCV & HCV/HIV infection. *J Viral Hepat* 2011;18:852–60.
- Soares JB, Pimentel-Nunes P, Afonso L, Rolanda C, Lopes P, Roncon-Albuquerque Jr R, Goncalves N, Boal-Carvalho I, Pardal F, Lopes S, et al. Increased hepatic expression of TLR2 and TLR4 in the hepatic inflammation-fibrosis-carcinoma sequence. *Innate Immun* 2012;18:700–8.
- Mahmoud MF, Hamdan DI, Wink M, El-Shazly AM. Hepatoprotective effect of limonin, a natural limonoid from the seed of *Citrus aurantium* var. *bigaradia*, on D-galactosamine-induced liver injury in rats. *Naunyn Schmiedebergs Arch Pharmacol* 2014;387:251–61.
- Poligone B, Baldwin AS. Positive and negative regulation of NF-kappaB by COX-2: roles of different prostaglandins. *J Biol Chem* 2001;276:38658–64.
- Kawai T, Akira S. TLR signaling. *Cell Death Differ* 2006;13:816–25.
- Kang JW, Kim DW, Choi JS, Kim YS, Lee SM. Scoparone attenuates D-galactosamine/lipopolysaccharide-induced fulminant hepatic failure through inhibition of toll-like receptor 4 signaling in mice. *Food Chem Toxicol* 2013;57:132–9.
- Knolle P, Schlaak J, Uhrig A, Kempf P, Meyer zum Buschenfelde KH, Gerken G. Human Kupffer cells secrete IL-10 in response to lipopolysaccharide (LPS) challenge. *J Hepatol* 1995;22:226–9.
- Nagaki M, Tanaka M, Sugiyama A, Ohnishi H, Moriwaki H. Interleukin-10 inhibits hepatic injury and tumor necrosis factor-alpha and interferon-gamma mRNA expression induced by staphylococcal enterotoxin B or lipopolysaccharide in galactosamine-sensitized mice. *J Hepatol* 1999;31:815–24.
- Paine A, Eiz-Vesper B, Blasczyk R, Immenschuh S. Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential. *Biochem Pharmacol* 2010;80:1895–903.
- Sun L, Shi T, Qiao H, Jiang X, Jiang H, Krissansen GW, Sun X. Hepatic overexpression of heme oxygenase-1 improves liver allograft survival by expanding T regulatory cells. *J Surg Res* 2011;166:187–94.
- Alam J, Cook JL. Transcriptional regulation of the heme oxygenase-1 gene via the stress response element pathway. *Curr Pharm Des* 2003;9:2499–511.
- Liu J, Wu KC, Lu YF, Ekuase E, Klaassen CD. Nrf2 protection against liver injury produced by various hepatotoxicants. *Oxid Med Cell Longev* 2013;2013:305861.
- Das J, Ghosh J, Roy A, Sil PC. Mangiferin exerts hepatoprotective activity against D-galactosamine induced acute toxicity and oxidative/nitrosative stress via Nrf2-NFkappaB pathways. *Toxicol Appl Pharmacol* 2012;260:35–47.