Food and Chemical Toxicology 56 (2013) 231–239

Contents lists available at SciVerse ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

# Platycodi Radix attenuates dimethylnitrosamine-induced liver fibrosis in rats by inducing Nrf2-mediated antioxidant enzymes

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ARTICLE INFO

*Article history:*
Received 17 October 2012
Accepted 14 February 2013
Available online 26 February 2013

*Keywords:*
Platycodi Radix
Liver fibrosis
Antioxidant enzymes
Nrf2
COX-2

ABSTRACT

The purpose of this study was to investigate the anti-fibrotic effects of the aqueous extract of the Platycodi Radix root (Changkil: CK) on dimethylnitrosamine (DMN)-induced liver fibrosis in rats. DMN treatment for 4 weeks led to marked liver fibrosis as assessed by serum biochemistry, histopathological examination, and hepatic lipid peroxidation and collagen content. CK significantly inhibited DMN-induced increases in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities, fibrosis score, and hepatic malondialdehyde and collagen content. CK also inhibited DMN-induced reductions in rat body and liver weights. Reverse transcription polymerase chain reaction (RT-PCR) and western blot analyses revealed that CK inhibited DMN-induced increases in matrix metalloproteinase-13 (MMP-13), tissue inhibitor of metalloproteinase-1 (TIMP-1), and tumor necrosis factor-α (TNF-α) mRNA, and collagen type I and α-smooth muscle actin protein. DMN-induced cyclooxygenase-2 (COX-2) expression and nuclear factor-kappa B (NF-κB) activation was reduced by CK treatment. Furthermore, CK induced activation of nuclear erythroid 2-related factor 2 (Nrf2)-mediated antioxidant enzymes such as γ-glutamylcysteine synthetase (γ-GCS), heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1), and glutathione-S-transferase (GST) in HepG2 cells. These results demonstrated that CK attenuates DMN-induced liver fibrosis through the activation of Nrf2-mediated antioxidant enzymes.



## 1. Introduction

Liver fibrosis is a wound healing response to a variety of chronic stimuli, including alcohol intake, viral infection, drugs, and metabolic disease (Begriche et al., 2011). During the development of liver fibrogenesis, quiescent hepatic stellate cells proliferate and undergo a phenotypic transformation to myofibroblast-like cells (Friedman, 2003). Activated hepatic stellate cells produce excessive extracellular matrix (ECM), and fibrosis-associated factors are the major pathogenic cell type in fibrogenesis (Pan et al., 2012). Liver fibrosis is associated with a number of pathological and biochemical changes that lead to structural and metabolic abnormalities (George and Chandrakasan, 2000). The progression of liver injury leads to fibrosis, a condition characterized by distortion of normal architecture, septae and nodule formation, altered blood flow, portal hypertension, hepatocellular carcinoma, and ultimately, liver failure (Han et al., 2004).

Dimethylnitrosamine (DMN) treatment is widely used in experimental model for hepatic fibrosis. DMN is a potent hepatotoxin, carcinogen, and mutagen that belongs to a family of N-nitrosamine compounds. Repeated DMN exposure causes chronic liver injury with necrosis, fibrosis, and nodular regeneration through metabolic activation of CYP2E1 in experimental animals (Guengerich et al., 1991). Activation of CYP2E1 by DMN in the liver stimulates Kupffer cells, leading to the generation of reactive oxygen species (ROS) that can damage liver cells (Teufelhofer et al., 2005). The injured liver cells release a number of cytokines; these cause inflammation and contribute to the pathogenesis of various acute and chronic liver injuries, such as acetaminophen (APAP) overdose, alcohol-induced liver injury, toxin exposure, and viral hepatitis. More specifically, DMN-induced liver fibrosis reproduces most of the features of human liver fibrosis, such as ascites, nodular regeneration, overproduction of ECM including collagen, and histopathological changes (Bataller and Brenner, 2005). Thus this is a

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http://dx.doi.org/10.1016/j.fct.2013.02.033

valuable animal model for studying the mechanisms of hepatic fibrosis, and may facilitate the rapid screening of anti-fibrotic agents.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a key transcription factor that protects a variety of tissues and cells against ROS through antioxidant response element (ARE)-mediated induction of diverse antioxidant and phase II detoxification enzymes, such as heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1), and glutathione S-transferase (GST) (Nguyen et al., 2009; Itoh et al., 1997). Upon stimulation, Nrf2 is translocated from the cytosol to the nucleus, where it subsequently binds to the ARE, resulting in a cytoprotective response characterized by upregulation of antioxidant enzymes and decreased sensitivity to oxidative stress damage (Dhakshinamoorthy and Jaiswal, 2001; Jaiswal, 2004).

Platycodi Radix, an aqueous extract of the *Platycodon grandiflorum* A.DC. root (family Campanulaceae), is used as a food and in traditional oriental medicine to treat chronic adult diseases (e.g., bronchitis, asthma, pulmonary tuberculosis, hyperlipidemia, and hypercholesterolemia) and inflammatory diseases (Lee, 1973). Changkil (CK) is an aqueous extract of the Platycodi Radix root, which is cultivated from plants at least 21 years old. Our previous studies reported that CK has anti-oxidant effect (Lee and Jeong, 2002), anti-metastatic activity (Lee et al., 2006), hepatoprotective effects (Lee et al., 2001; Lee and Jeong, 2002; Lee et al., 2004a,b), anti-inflammatory effect (Choi et al., 2009), anti-atopic dermatitis effect (Choi et al., 2012), and stimulates the immune system (Choi et al., 2001). Nonetheless, the effect of CK as an anti-fibrotic agent remains unclear. The objective of this study was to determine the inhibitory effect of CK on DMN-induced liver fibrosis in a rat model.

## 2. Materials and methods

### 2.1. CK preparation

CK, the aqueous extract of a 21-year-old Platycodi Radix root, was supplied by Jangsaeng Doraji Co., Ltd. (Jinju, Korea) and prepared as described previously (Lee and Jeong, 2002). Briefly, 90 °C distilled water was added to the powered root (5 mL/g), and the temperature was maintained for 10 h. The mixture was allowed to cool to room temperature, filtered, and lyophilized. The yield of lyophilized residue corresponded to 33.5% (33.5 g of residue per each 100 g of original dry root). The pale yellow extract was dissolved directly in sterilized saline. CK composition was previously published (Kim et al., 1995); CK consisted of saponin (~2.5%), inulin (~60%) and oligosaccharide (~25%).

### 2.2. Cell culture

Human hepatocarcinoma (HepG2) cells obtained from American Type Culture Collection (ATCC; Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; GibcoBRL, Grand Island, NY, USA), streptomycin (100 μg/mL), and penicillin (100 U/mL), at 37 °C in a humidified chamber with 5% $CO_2$.

### 2.3. Animals and DMN-induced liver injury

Five-week-old male Sprague–Dawley rats were obtained from Samtako (Osan, Korea). The animals were allowed free access to Purina rodent chow (Seoul, Korea) and tap water, and were maintained under specific pathogen-free conditions. Animals were acclimated to the temperature (22 ± 2 °C) and humidity (55 ± 5%) of controlled rooms with a 12-h light/dark cycle for at least 1 week prior to experimentation. All animal experiments were performed according to the rules and regulations of the Animal Ethics Committee at Chosun University. The rats were divided into three groups. To induce hepatic fibrosis, we treated rats with DMN (Sigma Chemical Co., St. Louis, MO, USA) dissolved in sterile saline (10 mg/kg body weight) via intraperitoneal injection three times per week for 4 weeks. CK was dissolved in saline. Rats were intragastrically (i.g.) administered 200 mg/kg of CK six times per week for 4 weeks 1 h before DMN applying each time. The control and DMN-treated groups were administered saline without drug each time. The animals were sacrificed on day 29 (Fig. 1A). Each group consisted of five rats. Livers were excised, weighed, and underwent histopathological examination and determination of collagen content using the Sircol collagen assay kit (Biocolor, Belfast, Northern Ireland).

### 2.4. Serum biochemistry

To assess hepatotoxicity, we measured the serum activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using spectrophotometric diagnostic kits according to the manufacturer's recommendations (Sigma Chemical Co., St. Louis, MO, USA).

### 2.5. Determination of lipid peroxidation

Hepatic lipid peroxidation level was determined by measuring thiobarbituric acid reactive substances (TBARS) (Lila, 2004). Briefly, samples were mixed with TBA reagent consisting of 0.375% TBA and 15% trichloroacetic acid in 0.25 M HCl. The reaction mixture was boiled in a water bath for 30 min and centrifuged at 2,000 rpm for 10 min at 4 °C. Then, the TBARS concentration was determined based on the absorbance at 532 nm measured with a spectrophotometer (Varioskan, Thermo Electron Co., Finland). Control tests were performed to ensure that CK did not interfere with the lipid peroxidation assays. Protein concentrations were determined using the Bradford method with bovine serum albumin (BSA) as the standard (Bradford, 1976).

### 2.6. Histopathological examinations

The left lateral lobe of the liver was sliced, and tissue slices were fixed in 10% buffered-neutral formalin for 24 h. The fixed liver tissue slices were embedded in paraffin, sectioned, deparaffinized, and rehydrated using standard techniques. Sections 5 μm in thickness were subjected to hematoxylin and eosin (H&E) and Masson's trichrome staining prior to examination (Vyberg et al., 1987). An arbitrary scope was given to each microscopic field viewed at a magnification of 100×. A minimum of 10 fields were scored per liver slice. The extent of fibrosis was graded as 0, no increase; 1, slight increase; 2, moderate increase; 3, distinct increase; or 4, severe increase. The extents of periportal bridging, intralobular degeneration, portal inflammation, and fibrosis were also graded according to Knodell's scoring method (Moragas et al., 1998).

### 2.7. Immunohistochemical staining of α-SMA and collagen type I

The paraffin-fixed liver specimens were sliced into 5-μm-thick sections. Sections were deparaffinized, rehydrated, and dipped in 3% $H_2O_2$ for 30 min to quench endogenous peroxidase activity. Antigen retrieval was carried out in a citrate buffer (pH 6.0) at 95 °C for 60 min. BSA (5%) was used to block nonspecific staining. The histological sections were then incubated with an anti-α-SMA or collagen type I antibody at a dilution of 1:200 overnight at 4 °C. After washing with phosphate-buffered saline (PBS), sections were incubated with biotinylated secondary antibodies. The immunoreaction was then amplified with streptavidin–avidin-peroxidase, and the sections were visualized using 3,30-diaminobenzidine tetrahydrochloride (DAB) and lightly counter-stained with hematoxylin. An arbitrary scope was given to each microscopic field viewed at a magnification of 100×.

### 2.8. Hepatic collagen content

The right lobe of the liver (0.2 g) was homogenized in 0.5 M acetic acid containing 1 mg pepsin (at a concentration of 10 mg tissue/10 mL acetic acid solution). The resulting mixture was then incubated for 24 h at 4 °C with stirring. Liver collagen content was determined by assaying total soluble collagen using the Sircol collagen assay kit (Biocolor, Belfast, Northern Ireland), according to the manufacturer's instructions. Acid-soluble type I collagen supplied with the kit was used to generate a standard curve.

### 2.9. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from liver samples with the RNAiso reagent (Takara, Kyoto, Japan), according to the manufacturer's protocol, and stored at −80 °C until use. Then, 0.5 μg RNA was used for reverse transcription and amplified by PCR using the access RT-PCR system Takara thermal cycler (Takara, Seoul, Korea). The PCR amplification protocol was: 30 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 45 s. The termination cycle included a prolonged extension at 72 °C for 7 min. Amplified products were resolved by 2% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light. The coding sequences of the genes are presented in Table 1. The intensity of RT-PCR bands was measured using the NIH Image J program.

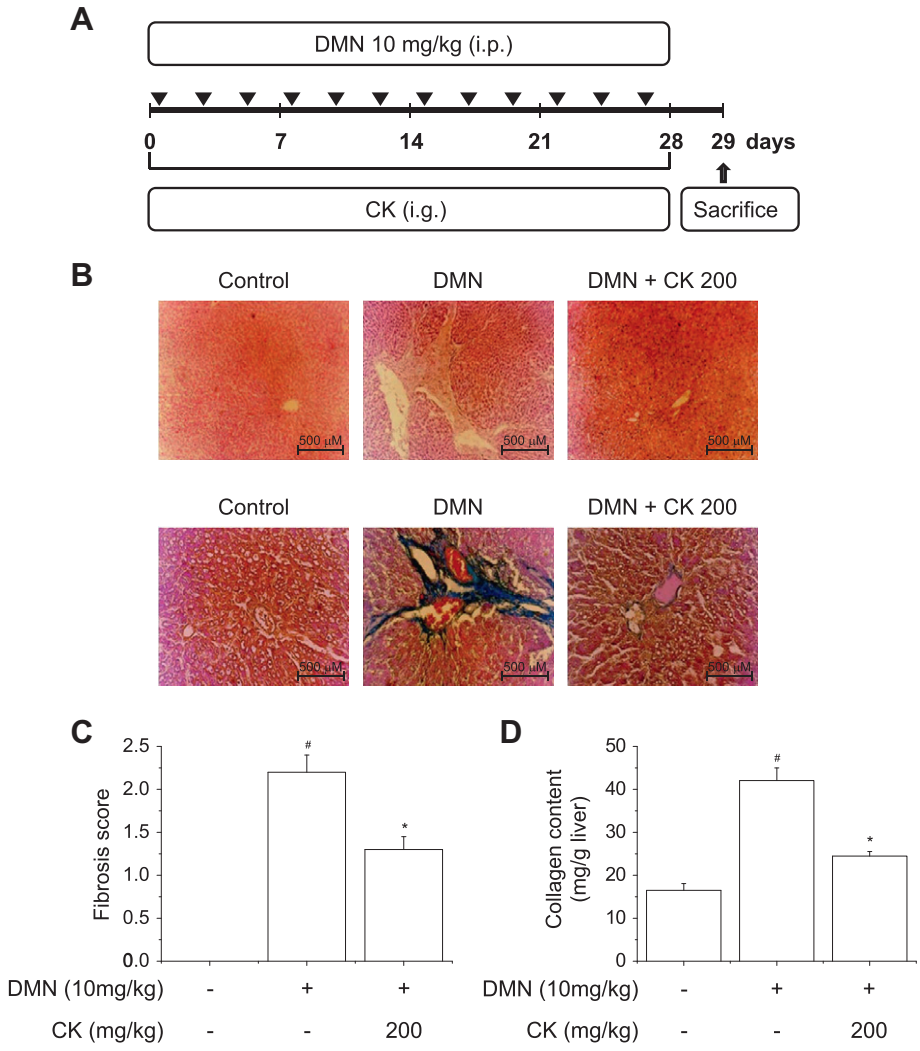


**Fig. 1.** Effects of CK on DMN-induced histopathological changes and hepatic collagen content. (A) Schematic diagram of experimental procedures. Rats were divided into three groups. To induce hepatic fibrosis, DMN was dissolved in sterile saline (10 mg/kg body weight) and administered by intraperitoneal (i.p.) injection three times per week for 4 weeks. CK was dissolved in saline. Rats were intragastrically (i.g.) administered 200 mg/kg/day of CK six times per week for 4 weeks. The control and DMN-treated groups were administered saline alone (i.g.) without CK. The animals were sacrificed on day 29. Each group consisted of five rats. (B) Liver tissue was collected and fixed in a 10% formaldehyde solution. Thin sections (5 μm) were cut and stained with hematoxylin and eosin (H&E) or Masson's trichrome. Liver tissue was obtained from rats administered saline (Control), DMN-treated rats administered saline (DMN), and DMN-treated rats administered 200 mg/kg CK (DMN + CK 200). (C) Fibrosis scores were evaluated in liver sections stained with Masson's trichrome in a blinded fashion. (D) Liver collagen content was determined by assaying total soluble collagen using the Sircol collagen assay kit, according to the manufacturer's directions. Results were obtained from three independent experiments. $^{\#}P < 0.05$, significantly different from the control group. $^{*}P < 0.05$, significantly different from the DMN-treated group.

**Table 1**
Primer sequences and the reaction conditions for semi-quantitative RT-PCR.

| Gene | Primer sequences (5′ → 3′) | Annealing temperature (°C) | Gene bank no. |
|---|---|---|---|
| MMP-13 | (F) TGA CTA TGC GTG GCT GGA A<br>(R) AAG CTG AAA TCT TGC CTT GGA | 57 | NM_133530.1 |
| TIMP-1 | (F) CAT GGA GAG CCT CTG TGG AT<br>(R) GTT CAG GCT TCA GCT TTT GC | 62 | NM_053819.1 |
| TNF-α | (F) GCC AAT GGC ATG GAT CTC AAA G<br>(R) CAG AGC AAT GAC TCC AAA GT | 53 | NM_012675.3 |
| β-actin | (F) GCC ATG TAC GTA GCC ATC CA<br>(R) GAA CCG CTC ATT GCC GAT AG | 55 | NM_031144.2 |

*2.10. Western blotting*

To analyze protein expression, liver homogenates or cellular proteins (50 μg) were normalized by the Bradford method (Bradford, 1976), resolved on 12% polyacrylamide gels, transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and probed with the appropriate primary and secondary antibodies. Nrf2, IκB-α, NF-κB p65, γ-GCSc, lamin B1, and β-actin (C4) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Collagen type I and HO-1 antibodies were obtained from Calbiochem (Merck KGaA, Darmstadt, Germany). NQO1 and GST antibodies were obtained from Abcam (Cambridge, MA, USA). The anti-α-SMA antibody was purchased from Dako (Glostrup, Denmark). The secondary antibody was a horseradish peroxidase-

coupled anti-rabbit or anti-mouse IgG (Beverly, MA, USA). Membranes were visualized using an enhanced chemiluminescence western blotting detection kit (iNtRON Biotechnology Co., Ltd., Korea). The intensity of western blot bands was measured using the NIH Image J program.

### 2.11. Transient transfection and luciferase assay

To determine promoter activity, we used a dual-luciferase reporter assay system (Promega, WI, USA). The cells were seeded in 48-well plates and incubated at 37 °C. At 70–80% confluence, the cells were incubated with DMEM without serum or antibiotics for 6 h. The cells were transiently co-transfected with an HO-1-ARE-promoter luciferase construct and pRL-SV plasmid (Renilla luciferase expression for normalization) (Promega, WI, USA) using LipofectAMINE™ 2000 reagent (Invitrogen, CA, USA). The cells were then exposed to CK for 24 h, and relative luciferase activities were calculated by normalizing HO-1-ARE-promoter-driven firefly luciferase activity to Renilla luciferase activity (Luminoskan Ascent, Thermo Electron Co., Finland).

### 2.12. Statistical analyses

All experiments were repeated at least three times. Results are reported as means ± standard errors of the mean (SEM). Statistical significance was determined by a one-way analysis of variance (ANOVA), followed by the Tukey–Kramer multiple comparisons test. A significant value was defined as $P < 0.05$.

## 3. Results

### 3.1. Inhibitory effects of CK on DMN-induced hepatotoxicity

Long-term DMN treatment can induce hepatic fibrogenesis not only in humans, but also in rats. When the liver becomes damaged, the concentration of transaminases in the serum increases due to increased hepatocyte cell membrane permeability (Luo et al., 1998). In this study, we evaluated DMN-induced serum transaminase activity and hepatic lipid peroxidation. As shown in Table 2, repeated DMN application increased serum ALT and AST activities and hepatic lipid peroxidation. CK significantly inhibited the DMN-induced serum ALT and AST activities and hepatic lipid peroxidation. We also assessed changes in liver and body weight following DMN treatment. As shown in Table 3, the body and liver weights of the DMN-treated group were lower than those of the control group. The mean body and liver weights of rats in the DMN-treated group were approximately 57% and 53% that of the control group, respectively. However, CK prevented the decrease in body and liver weights of those rats treated with DMN.

### 3.2. Inhibitory effects of CK on DMN-induced histopathological changes and expression of α-SMA and collagen type I

DMN-induced liver injury results in activation of quiescent hepatic stellate cells into proliferating myofibroblast-like cells that cause liver fibrosis (Hsu et al., 2004). We determined the protective effect of CK on DMN-induced histopathological changes in liver tissue. In our histopathological analysis using hematoxylin and eosin and Masson's trichrome staining, the control group had intact lobular architecture with central veins and radiating hepatic cords. The DMN-treated group showed widespread destruction of liver architecture, which was characterized by massive and severe hepatic damage, collagen deposition, and sinusoidal congestion. However, CK treatment inhibited these DMN-induced pathological changes and deposition of collagen fibers (Fig. 1B). Moreover, the DMN-induced fibrosis score was decreased by CK treatment (Fig. 1C). Immunohistochemical staining revealed that DMN-treated liver exhibited increased expression of α-SMA and collagen type I. The CK-treated group showed weak α-SMA and collagen type I signal, suggesting that CK treatment effectively eliminated the activated HSCs induced by DMN (Fig. 2A and B). Similarly, chronic DMN treatment increased expression of α-SMA and collagen type I in the liver (Fig. 2C). Moreover, DMN induced severe liver fibrosis where a large amount of total collagen accumulated (Fig. 1D). CK treatment inhibited the DMN-induced expression of α-SMA and collagen type I and accumulation of total collagen in the liver.

**Table 3**
Effects of CK on DMN-induced body and liver weights in rats.

| | Body weight (g) | Liver weight (g) |
|---|---|---|
| Control | 330 ± 32.6 | 12.0 ± 1.5 |
| DMN 10 mg/kg | 188 ± 20.5[#] | 6.3 ± 0.8[#] |
| DMN + CK 200 mg/kg | 285 ± 26.5[*] | 9.1 ± 0.9[*] |

Hepatotoxicity was determined by examining body and liver weights. Results are the means ± standard errors of the mean (SEM) of five rats in each group. Results were obtained from three independent experiments.
[#] $P < 0.05$, significantly different from the control group.
[*] $P < 0.05$, significantly different from the DMN-treated group.

### 3.3. Inhibitory effects of CK on DMN-induced expression of MMP-13, TIMP-1, and TNF-α

The extent of liver fibrosis depends on the rates of hepatic extracellular matrix deposition, collagen synthesis and degradation (Mormone et al., 2011). Also, oxidative stress is attributable to an increase in free radical formation and activated inflammatory cells from the breakdown of DMN in the liver (Jung et al., 2009). To elucidate the possible molecular pathways by which CK suppressed hepatic fibrosis, we examined mRNA expression of several fibrogenesis-related genes. As shown in Fig. 3A, repeated DMN administration increased hepatic mRNA levels of several factors involved in fibrillar extracellular matrix overproduction, such as tissue inhibitor of metalloproteinase-1 (TIMP-1) and matrix metalloproteinase-13 (MMP-13). Also, the fibrotic-related cytokine, tumor necrosis factor-alpha (TNF-α), was increased. However, CK treatment inhibited the DMN-induced fibrogenesis-related gene expression in the liver.

### 3.4. Inhibitory effects of CK on DMN-induced activation of NF-κB-regulated COX-2 expression

Cyclooxygenase-2 (COX-2) is associated with inflammatory response, tissue remodeling, and carcinogenesis (Zhu et al., 2011; Tanigawa et al., 2011). Nuclear factor-kappa B (NF-κB) is involved

**Table 2**
Effects of CK on DMN-induced hepatotoxicity in rats.

| | ALT (U/L) | AST (U/L) | MDA (nmole/g liver) |
|---|---|---|---|
| Control | 35 ± 4.3 | 26 ± 3.1 | 3.1 ± 0.4 |
| DMN 10 mg/kg | 2175 ± 221.4[#] | 1472 ± 151.5[#] | 8.6 ± 0.9[#] |
| DMN + CK 200 mg/kg | 661 ± 26.4[*] | 241 ± 15.6[*] | 3.6 ± 0.4[*] |

Hepatotoxicity was determined by quantifying serum ALT and AST activities and by determining hepatic lipid peroxidation. Results are the means ± standard errors of the mean (SEM) of five rats in each group. Results were obtained from three independent experiments.
[#] $P < 0.05$, significantly different from the control group.
[*] $P < 0.05$, significantly different from the DMN-treated group.

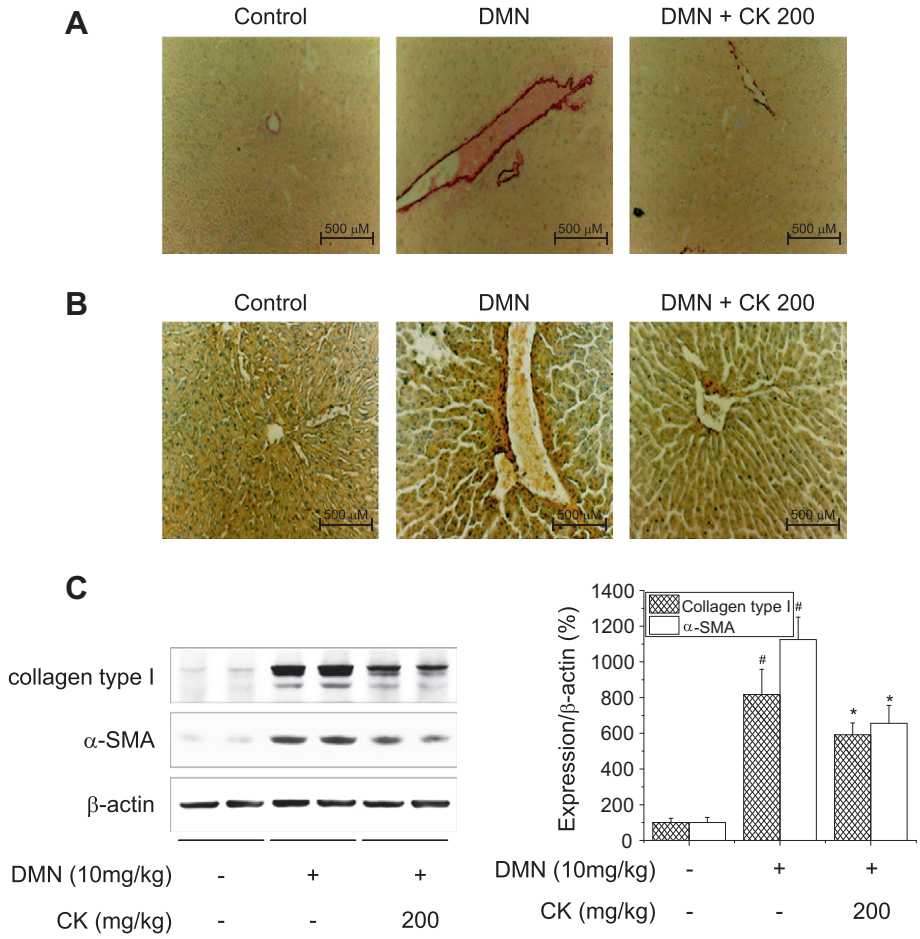


**Fig. 2.** Effects of CK on DMN-induced α-SMA and collagen type I immunohistochemical detection and expression. (A and B) Liver tissue was collected and fixed in a 10% formaldehyde solution. Thin sections (5 μm) were cut and incubated with antibodies against α-SMA or collagen type I. Liver tissue was obtained from rats administered saline (Control), DMN-treated rats administered saline (DMN), and DMN-treated rats administered 200 mg/kg CK (DMN + CK 200). (C) Total protein was extracted from liver tissue, and α-SMA, collagen type I, and β-actin protein levels were determined by western blotting. Results shown are representative of five observations. The intensity of western blot bands was measured using the NIH Image J program. Results were obtained from three independent experiments. $^{\#}P < 0.05$, significantly different from the control group. $^{*}P < 0.05$, significantly different from the DMN-treated group.

in regulating many aspects of cellular activity: stress, injury and especially in pathways of the immune and inflammatory response. NF-κB is composed of two subunits, p65 and p50, and is normally sequestered in the cytosol by an inhibitory protein, IκBα. Exposure of cells to a variety of extracellular stimuli leads to the rapid phosphorylation, ubiquitination, and ultimately proteolytic degradation of IκBα, resulting in the release of NF-κB from its inhibitory protein to translocate to the nucleus where it regulates transcription of various genes (Aggarwal and Shishodia, 2004). The effect of CK on DMN-induced COX-2 expression, NF-κB p65 nuclear translocation, and IκBα degradation in fibrotic liver tissues was investigated by western blotting. As shown in Fig. 3B and C, repeated DMN administration increased hepatic COX-2 expression and NF-κB p65 nuclear translocation. However, CK treatment inhibited DMN-induced COX-2 expression and NF-κB p65 nuclear translocation in rats. DMN reduced IκBα by degrading phosphorylated IκBα, while CK prevented IκBα degradation in fibrotic liver tissue (Fig. 3D). These results suggest that CK inhibited NF-κB p65 from entering the nucleus where NF-κB regulates genes, including pro-inflammatory cytokines.

### 3.5. The effects of CK on the levels of γ-GCSc, HO-1, NQO1, GST, and Nrf2 in rats with DMN-induced liver injury

Nuclear erythroid 2-related factor 2 (Nrf2) is a cellular sensor of oxidative stress. Nrf2 is sequestered in the cytosol by Kelch-like Ech-associated protein (Keap1). Nrf2 is crucial for antioxidant responsive element (ARE)-mediated induction of detoxifying enzymes, anti-oxidative stress genes, and other target genes involved in cellular protection (Asghar et al., 2007; Chan et al., 2001; Ishii et al., 2000). Activation of these gene targets serves to decrease cellular oxidative stress. To determine whether Nrf2-regulated activation was induced by CK in DMN-induced liver injury, we examined the γ-glutamylcysteine synthetase (γ-GCS), heme oxygenase-1 (HO-1), NA(D)PH quinine oxidoreductase 1 (NQO1), and glutathione-S-transferase (GST) expression and Nrf2 translocation by western blotting. As shown in Fig. 4A, DMN decreased phase II enzymes such as γ-GCSc, HO-1, NQO1, and GST in the injured liver. DMN treatment also decreased hepatic Nrf2 translocation (Fig. 4B). CK treatment markedly increased γ-GCSc, HO-1, NQO1, and GST expression and Nrf2 activation.

### 3.6. The effects of CK on the levels of γ-GCSc, HO-1, NQO1, GST, and Nrf2/ARE in HepG2 cells

To obtain a suitable concentration range to investigate the effects of CK on cell viability in HepG2 cells, we treated cells with CK concentrations ranging from 10 to 400 μg/mL for 24 h. We observed no significant alterations in cell viability following CK treatment at these concentrations (data not shown). We further examined the effect of CK on the expression of Nrf2/ARE-dependent phase II detoxifying and anti-oxidant enzymes in cells. CK

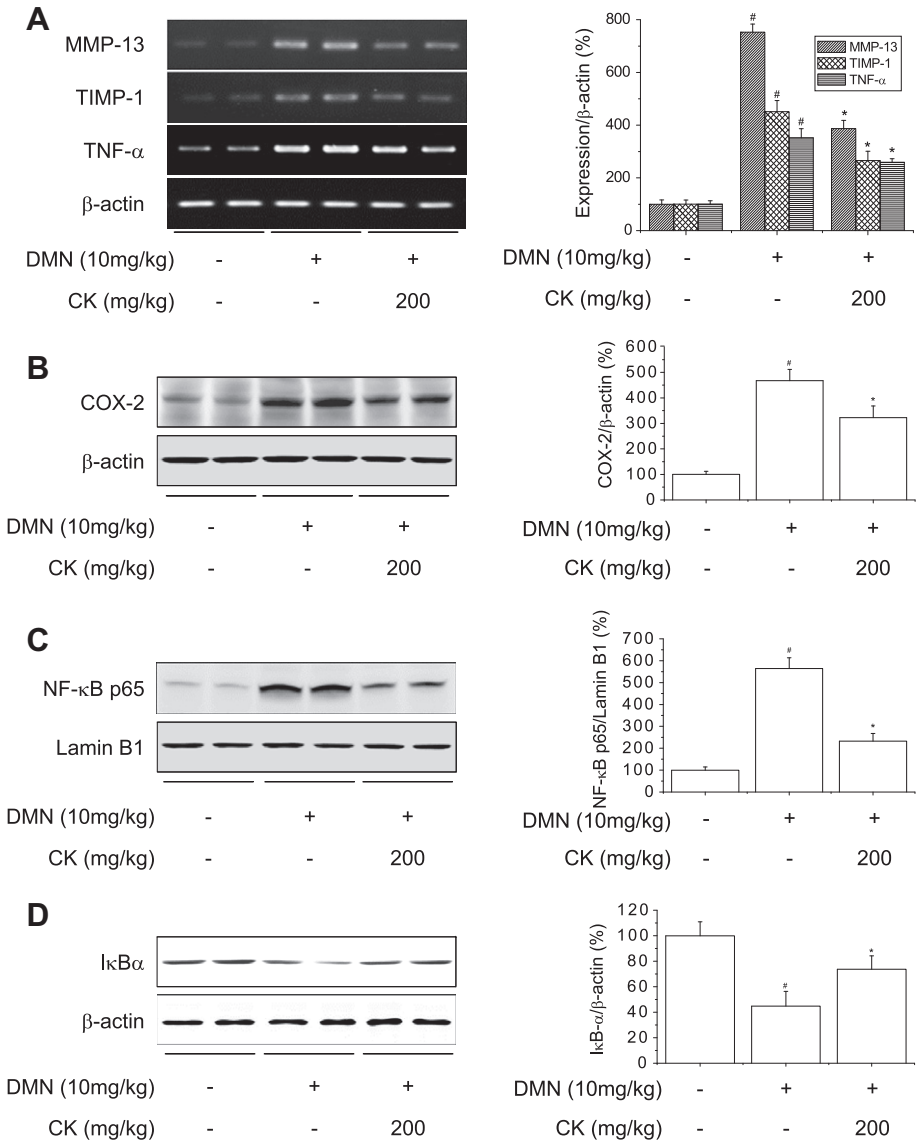


**Fig. 3.** Effects of CK on DMN-induced liver fibrosis-related gene and protein expression. (A) Total RNA was extracted from liver tissue, and mRNA expression of MMP-13, TIMP-1, TNF-α, and β-actin was determined by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). (B) Total protein was extracted from liver tissue and COX-2 and β-actin protein levels were determined by western blotting. (C) Nuclear NF-κB protein was extracted from liver tissue and NF-κB nuclear translocation was determined by western blotting. (D) Total protein was extracted from liver tissue and IκBα and β-actin protein levels were determined by western blotting. Results shown are representative of five observations. The intensity of RT-PCR and western blot bands was measured using the NIH Image J program. Results were obtained from three independent experiments. $^{\#}P < 0.05$, significantly different from the control group. $^{*}P < 0.05$, significantly different from the DMN-treated group.

treatments significantly and concentration-dependently increased γ-GCSc, HO-1, NQO1, and GST expression, Nrf2 translocation, and ARE luciferase activity in cells (Fig. 5A–C). These results suggest that CK up-regulates detoxifying and anti-oxidant enzymes via Nrf2/ARE activation in hepatocytes, which may be associated with its anti-fibrotic effect.

## 4. Discussion

Liver fibrosis is the end result of chronic inflammatory reactions induced by a variety of stimuli, including persistent infections, autoimmune reactions, allergic responses, chemical insults, radiation, and tissue injury. Liver fibrosis is the overgrowth, hardening, and scarring of liver and is attributed to excess deposition of extracellular matrix components including collagen. Chronic liver disease can progress to cirrhosis of the liver and end-stage liver disease, which manifests as portal hypertension, synthetic dysfunction, hepatopulmonary syndrome, and encephalopathy and hepatocellular carcinoma. Cirrhosis of the liver causes suffering, hospital costs, and death. Better therapies for combating hepatic fibrosis and cirrhosis are thus needed. Inhibiting and preventing the development of liver fibrosis might be an effective strategy to improve the prognosis of patients with chronic liver injury.

Recently, natural agents have been used in an attempt to discover better therapies. Platycodi Radix possesses powerful anti-inflammatory, anti-allergic, and anti-obesity properties (Choi et al., 2009; Han et al., 2000, 2009; Lee et al., 2004a,b). Our previous studies reported that an aqueous extract of the Platycodi Radix root (Changkil: CK) possesses potent hepatoprotective effects in the carbon tetrachloride ($CC1_4$)-induced liver injury model. CK treatment blocked cytochrome P450 2E1-mediated $CC1_4$ bioactivation, hepatic glutathione depletion, and free radical scavenging (Lee and Jeong, 2002). However, the mechanisms underlying the

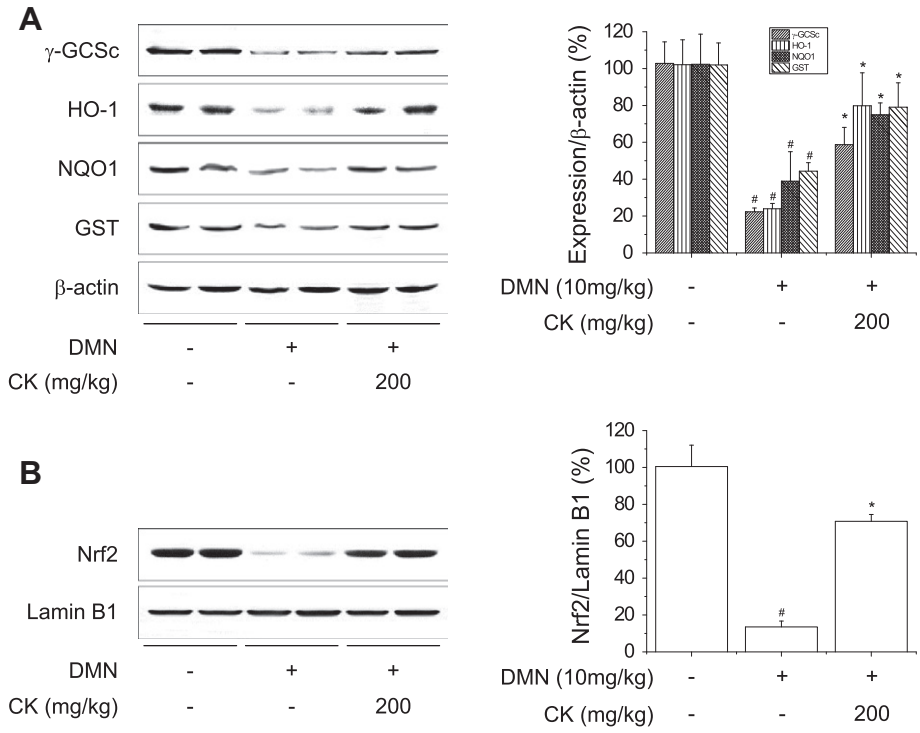


**Fig. 4.** Effects of CK on detoxifying enzyme expression and Nrf2 activation in rats with DMN-induced liver fibrosis. (A) Total protein was extracted from liver tissue and γ-GCSc, HO-1, NQO1, GST, and β-actin protein levels were determined by western blotting. (B) Nuclear Nrf2 protein was extracted from liver tissue and Nrf2 nuclear translocation was determined by western blotting. Results shown are representative of five observations. The intensity of western blot bands was measured using the NIH Image J program. Results were obtained from three independent experiments. $^{\#}P < 0.05$, significantly different from the control group. $^{*}P < 0.05$, significantly different from the DMN-treated group.

anti-fibrotic effect and antioxidant induction by CK remain unclear. Here, we investigated the anti-fibrotic effects of CK on DMN-induced liver fibrosis in rats.

DMN is a potent hepatotoxin, carcinogen, and mutagen that can cause fibrosis of the liver. A rat model of liver fibrosis induced by chronic, discontinuous DMN treatment reportedly reproduces a number of liver disease characteristics such as mortality, ascites, hepatic parenchymal cell destruction, formation of connective tissue, and nodular regeneration (George et al., 2001; Bataller and Brenner, 2005). This model provides a preclinical platform for evaluating the therapeutic efficacy of drugs and the underlying mechanisms of its mode of action (Kang et al., 2002). The present study examined the inhibitory effect of CK on the developing liver fibrosis model using clinical parameters. CK significantly inhibited DMN-induced plasma ALT and AST activity and hepatic lipid peroxidation and led to a recovery of reduced body and liver weight. Furthermore, liver fibrosis is a pathological process involving multiple cellular and molecular events that ultimately lead to deposition of excess matrix proteins in the extracellular membrane by liver damage (Bataller and Brenner, 2005). Histological examination of liver morphology with H&E and Masson's trichrome staining showed that CK inhibited extensive changes and collagen deposition. Immunohistochemical staining and western blotting showed that CK attenuated α-SMA and collagen type I expression. CK also inhibited DMN-induced MMP-13 and TIMP-1 expression. These results suggest that the improvement of DMN-induced hepatic fibrosis by CK may partially result from attenuation of hepatocyte injury.

Liver fibrosis is a chronic inflammatory response to liver injury. Oxidative stress, which favors mitochondrial permeability transition, promotes hepatocyte injury. Chronic DMN treatment induces hepatocyte damage through increased generation of oxidative stress (Lu et al., 2004; Shimizu et al., 1999). We previously reported that DMN increases COX-2 expression via transcription factor activation, including NF-κB (Hwang et al., 2011). CK has strong anti-inflammatory effects (Lee et al., 2004a,b; Choi et al., 2009). We thus examined the anti-inflammatory effect of CK on DMN-induced liver fibrosis in a rat model. CK treatment elicited anti-inflammatory effects by inhibiting NF-κB activation, IκB degradation, and COX-2 and TNF-α expression. These results suggest that CK may act as a therapeutic agent for inflammatory disease through regulating NF-κB activity and pro-inflammatory cytokine expression.

The induction of the chemopreventive and antioxidant enzyme system is an important event in the cellular stress response, during which a diverse array of electrophilic and oxidative toxicants can be eliminated or inactivated before they damage critical cellular macromolecules (Rushmore and Kong, 2002). Antioxidant agents can either scavenge ROS or stimulate detoxification mechanisms within cells, which results in ROS removal. Many natural and synthetic compounds can induce phase II detoxifying and anti-oxidant responsive genes. The induction of these genes is a highly effective strategy for protection against oxidative stress (Chen and Kong, 2005; Yu and Kensler, 2005). Recent reports suggested a key role of ARE in the regulation of phase II and antioxidant gene expression, such as HO-1, GST, and NQO1 (Kong et al., 2001; Chen et al., 2004; Itoh et al., 1997). Nrf2 is also an important regulator of cellular oxidative stress. Nrf2 normally binds to Keap1 molecules or is retained in the cytoplasm; however, Nrf2 splits from Keap1 under oxidative stress and other stimulating factors and then accumulates in the nucleus, subsequently binding to AREs to activate transcription of ARE-mediated genes, including HO-1, NQO1, GST, and GCL (Gu et al., 2011). We investigated the CK-induced expression of these antioxidant genes using western blotting analysis and luciferase activities. CK increased Nrf2 translocation-activated γ-GCSc, HO-1, NQO1, and GST expression, and ARE luciferase activity, suggesting that CK indeed promotes

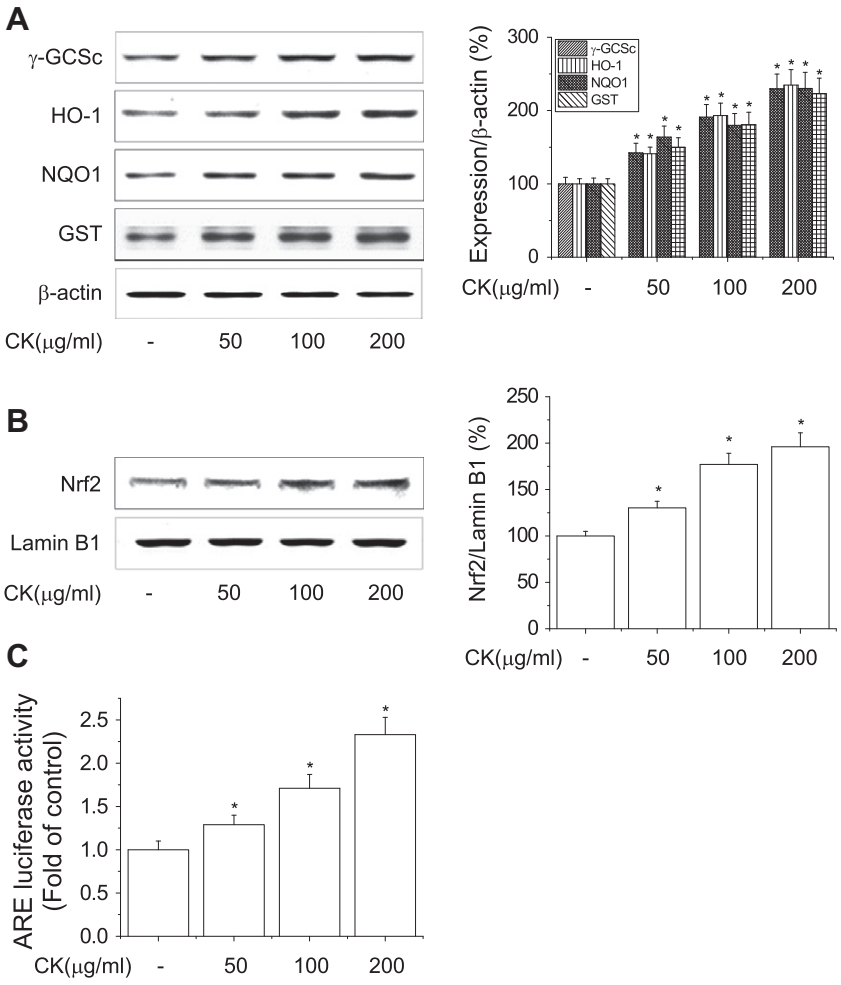


**Fig. 5.** Effect of CK on antioxidant enzyme expression and Nrf2 activation in cells. (A) Total protein was extracted from cells and γ-GCSc, HO-1, NQO1, GST, and β-actin protein levels were determined by western blotting. (B) Nuclear Nrf2 protein was extracted from cells and Nrf2 nuclear translocation was determined by western blotting. (C) Cells were transfected with the HO-1-ARE reporter plasmid and treated with CK. After 24 h of CK treatment, luciferase activity was determined. Results were obtained from three independent experiments. The intensity of western blot bands was measured using the NIH Image J program. $^{*}P < 0.05$, significantly different from the control group.

the transcription of key antioxidant genes by triggering the translocation of Nrf2 into the nucleus.

In conclusion, we demonstrated a novel mechanism of CK-mediated protection following DMN-induced liver damage. CK significantly inhibited both the increases in ALT and AST serum activity and hepatic lipid peroxidation induced by DMN. CK significantly suppressed the expression of α-SMA, collagen type I, inflammatory mediators, and cytokines. In addition, CK treatment induced the upregulation of antioxidant enzyme expression through Nrf2 activation. This, in turn, may inhibit DMN-induced inflammation in rats. These results imply that CK manifests effective hepatocellular protective action and ameliorative effects against chronic liver damage and developing liver fibrosis induced by DMN treatment. However, further studies are needed to elucidate the molecular mechanism underlying the preventive or therapeutic potential of CK in liver fibrosis.

## Conflict of Interest

The authors declare that there are no conflicts of interest.

## Acknowledgement

This work was supported by grant from the Priority Research Centers Program through the National Research Foundation of Korea funded by Ministry of Education, Science and Technology (2009-0093815), Republic of Korea.

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