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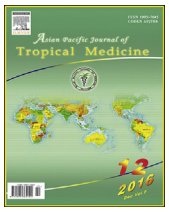

Original Research http://dx.doi.org/10.1016/j.apjtm.2016.10.009

Protective effects of a polymethoxy flavonoids-rich *Citrus aurantium* peel extract on liver fibrosis induced by bile duct ligation in mice

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ARTICLE INFO

Article history:
Received 26 Jul 2016
Accepted 9 Oct 2016
Available online 9 Nov 2016

Keywords:
Liver injury
Bile duct ligation
Cholestasis
Citrus aurantium peel extract
Hepatic fibrosis

ABSTRACT

Objective: To evaluate the possible protective effect of *Citrus aurantium* peel extract (CAE) against apoptosis in cholestatic liver fibrosis induced by bile duct ligation in mice.
Methods: Male ICR mice were divided to 5 groups: 1) Control group (Sham-operated mice), 2) Cholestatic liver injury group induced by bile duct ligation (BDL), 3) BDL mice treated with silymarin (200 mg/kg) for 4 weeks, 4) BDL mice treated with 50 mg/kg CAE for 4 weeks, 5) BDL mice treated with 200 mg/kg CAE for 4 weeks. Mice were sacrificed and liver fibrosis was evaluated by serum and hepatic tissue biochemistry tests and liver histopathological examination. Effects of CAE on inflammation and apoptosis gene regulation were investigated through real-time PCR. CAE effect on lipid metabolism related signaling was determined by western blot analysis.
Results: In BDL mice, administration of CAE for 4 weeks markedly attenuated liver fibrosis based on histopathological alteration. Serum and hepatic tissue biochemistry results revealed that CAE (50 and 200 mg/kg) decreased the levels of alanine transaminase, aspartate transaminase, gamma-glutamyl transferase, total bilirubin, nitric oxide, and thiobarbituric acid reactive substances. Real-time PCR and western blot analysis showed that CAE regulated inflammation, apoptosis, and lipid metabolism factors increased by BDL. Interleukin family, tumor necrosis factor α, and related apoptosis factors mRNA levels were increased by BDL treatment. However, these increases were suppressed by CAE administration. In addition, CAE effectively increased phosphorylation of AMP-activated protein kinase, nuclear factor E2-related factor 2, and related cytoprotective proteins.
Conclusions: CAE can efficiently regulate BDL-induced liver injury with antioxidant, anti-inflammatory, and anti-apoptotic activities.

1. Introduction

The pathogenesis of cholestatic liver injury caused by bile duct obstruction or functional defect in bile formation is thought to have a critical role in extra-hepatic damage [1]. Stagnant bile acid causes hepatotoxicity, disrupts hepatocyte cell membranes, and releases reactive oxygen species from peritoneal neutrophil in cholestatic liver diseases with lipid peroxidation [2,3]. Intrahepatic accumulation of bile acid and toxic materials causes pro-inflammation and oxidative stress, leading to fibrosis and cirrhosis [4].

Surgical bile duct ligation (BDL) experimental model has been well characterized to confirm obstructive cholestatic liver

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Peer review under responsibility of Hainan Medical University.
Foundation project: This work was supported by 'Cooperative Research Program for Agriculture Science & Technology Development' (No. PJ01132001) funded by Rural Development Administration, Republic of Korea.

injury. BDL stages (from acute to chronic liver damage) are represented by inflammation, fibrosis, cirrhosis, and eventually hepatocellular apoptosis progresses insidiously, resulting in histopathological symptom as in human cholestasis [5,6].

Citrus has abundant polymethoxy flavones. Nobiletin is a relatively common polymethoxy flavonoid (PMF) compound present in the peel of citrus [7]. Six methoxyl groups on the base structure of flavone of nobiletin have been intensively studied due to their biological properties [8]. It has been reported that nobiletin is metabolized and bio-transformed to metabolites with different activities, such as regulating adipogenesis, inflammation, and carcinogenesis [9,10].

Our previous studies have found that a polymethoxy flavonoids-rich *Citrus aurantium* peel extract (CAE) containing approximately 27% of nobiletin has hepatoprotective effects on binge drinking and carbon tetrachloride intoxication in mouse models [11,12]. However, the effect of CAE as an anti-cholestasis material remains unclear. Therefore, we used CAE, the same material used in previous *in vivo* studies, to investigate its potential effects on liver injury. Specifically, the objective of this study was to determine the possible protective effect of CAE against cholestatic liver fibrosis induced by BDL in mouse model.

2. Materials and methods

2.1. Chemicals and reagents

Nobiletin standard compound was purchased from Sigma–Aldrich (MO, USA). Assay kits for superoxide dismutase (SOD), thiobarbituric acid reactive substances (TBARS), and glutathione (GSH) were obtained from Cayman Chemical Company (MI, USA). Assay kit for nitric oxide (NO) was purchased from BioAssay Systems (CA, USA). Protein assay reagent and polyvinylidene difluoride membranes were purchased from Bio-Rad Laboratories (CA, USA). The following antibodies were purchased from Santa Cruz Biotechnology (CA, USA): anti-nuclear factor erythroid 2-related factor 2 (Nrf-2, sc-722), NAD(P)H: Quinone oxidoreductase-1 (NQO-1, sc-376023), Uridine 5′-diphospho-glucuronosyltransferase (UGT, sc-25847), γ-glutamylcysteine synthetase (γ-GCS, sc-28965), β-actin (sc-3700), AMP-activated protein kinase α (AMPKα, sc-25792), and phospho-AMPK (p-AMPKα, sc-33524). All other reagents were purchased from Sigma Chemicals (MO, USA).

2.2. CAE preparation

Ethanol extract of *Citrus aurantium* peel was supplied by KPLC group (Paris, France) and prepared as described previously [11,12]. The main flavonoid constituent in CAE was analyzed by high performance liquid chromatography. Chromatographic analysis was performed according to the method of Choi *et al.* (2015).

2.3. Animal treatment

Male ICR mice (5–6) weeks old were obtained from Orient Bio (Sungnam, Korea) and housed in animal facility of Chungnam National University. Mice were ad libitum fed with lab chow and water under stable condition of (22 ± 2) °C, 55% ± 5% humidity, and 12 h light/dark cycle. After acclimation, mice were randomly divided into 5 groups: 1) control group, 2) BDL alone group, 3) BDL + silymarin (200 mg/kg) group, 4) BDL + CAE (50 mg/kg) group, and 5) BDL + CAE (200 mg/kg) group. In all BDL groups, the common bile duct was identified by laparotomy, doubly ligated using 6–0 nylon sutures, and transected. After 24 h, all mice were given respective drugs for 4 weeks. Mice in the control group and BDL alone group were treated with distilled water. At the end of the 4-week treatment, mice were killed by cardiac puncture under carbon dioxide anesthesia. Serum and liver samples were collected and stored for further analyses. Tissues for histopathological study were fixed in neutral buffered formalin. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Institute of Chungnam National University (Daejeon, Korea, CNU-00374).

2.4. Serum biochemical examination

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), total bilirubin (TBIL), and total cholesterol (TCHO) were analyzed using a dry chemistry system (FUJI DRI-CHEM 4000i, Fuji Medical System, Tokyo, Japan).

2.5. Liver histopathology

Paraffin embedded liver tissues were sectioned at thickness of 5 μm and stained with hematoxylin and eosin (H&E), Masson's trichrome (MT), and Sirius red. Liver injury was scored in H&E stained tissue using a scale of 0–5 (0, none; 1, <10%; 2, 10%–25%; 3, 25%–50%; 4, 50%–70%; and 5, >75%) depending on the percentage of damaged area. Fibrosis score was determined for Masson's trichrome stained sections by using modified Ishak system as follows: 0, no fibrosis; 1, fibrous expansion of portal tracts, without short fibrous septa; 2, fibrous expansion with short fibrous septa; 3, portal to portal fibrous bridging; 4, portal to portal and portal to central fibrous bridging; 5, cirrhosis [13].

Collagen deposition in Sirius red-positive stained area was quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

2.6. Liver tissue biochemistry

The contents of glutathione (GSH), superoxide dismutase (SOD), nitric oxide (NO), and thiobarbituric acid reactive substances (TBARS) in liver tissues were determined using commercial kits from BioAssay Systems (CA, USA) according to the manufacturer's instructions.

2.7. Western blot analysis

Liver tissue was homogenized with radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor cocktail and centrifuged at 15000 ×*g* for 15 min at 4 °C. Supernatants were collected and subjected to protein assay. Adequate proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes followed by blocking with 5% of nonfat dried skim milk. After blocking, membranes were incubated with primary antibodies. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized with Chemiluminometer (CLINX Science Instruments, Shanghai, China).

2.8. Quantitative real-time RT-PCR

Liver total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). For cDNA synthesis, oligo dT primers (1 μL, 0.5 pmole; Promega, WI, USA) were added to the reaction in a total volume of 9 μL. After denaturing at 70 °C for 5 min, the mixture was immediately cooled down at 4 °C for 5 min. The following components were then added to the mixture: $MgCl_2$ (4 μL of 25 mM), 5× reverse transcriptase enzyme buffer (4 μL), RNase inhibitor (1 μL), reverse transcriptase (1 μL), and dNTPs (1 μL, 10 mM). After gentle vortexing, the mixture was then incubated at 25 °C for 5 min, 42 °C for 60 min, and 70 °C for 15 min. SYBR Green-based real-time PCR was performed using SensiMix Plus SYBR (Quantace, London, UK) based on the manufacturer's protocol. PCR was performed using primers specific for Interleukin (IL) family genes, tumor necrosis factor α (TNF α), and apoptosis related genes as follows: IL-2, CCC ACT TCA AGC TCC ACT TC and ATC CTG GGG AGT TTC AGG TT; IL-4, TCA ACC CCC AGC TAG TTG TC and TGT TCT TCG CTG TGA GG; IL-6, AGC CCC CAG TCT GTA TCC TT and TCC ACG ATT TCC CAG AGA AC; TNF α, AGC CCC CAG TCT GTA TCC TT and CTC CCT TTG CAG AAC TCA GG; FAS, CCC TTG ATG AAG AGG GAT CA and ACT CCA CAG GTG GGA ACA AG; Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), GAA GAC CTC AGA AAG TGG C and GAC CAG CTC TCC ATT CCT A; Caspase-8, CCA GGA AAA GAT TTG TGT CTA GC and GGC CTT CCT GAG TAC TGT CAC CTG T. Real-time PCR was carried out on Roto-Gene 6000 (Corbett, Mortlake, AUS) with gradient schedules as follows: 10 min at 95 °C and 40 cycles of 95 °C (5 s), 60 °C (15 s), and 72 °C (25 s). The expression levels of mRNAs were normalized against the level of glyceraldehyde-3-phosphatedehydrogenase (GAPDH). Results were analyzed using the comparative cycle threshold method [14].

2.9. Statistical analysis

All data were expressed as mean ± SEM. Significance differences between experimental groups were determined using analysis of variance (ANOVA) with Tukey's test or Mann–Whitney U-test using the software, Origin 7 (Microcal Software, USA). Statistical significance was considered when P value was less than 0.05.

3. Results

3.1. Effect of CAE on serum profiles of BDL mice

Bile duct ligation caused severe hepatocellular injury. To assess the effect of CAE on liver physiology, serum levels of ALT and AST were measured. As expected, ALT and AST levels were significantly ($P < 0.05$) increased (22-fold and 7-fold, respectively) in BDL group compared to those of control group without BDL (Table 1). The administration of CAE (50 and 200 mg/kg) to BDL mice for 4 weeks reduced the increases of serum ALT and AST levels caused by BDL. Serum levels of ALT and AST of the treatment group with high dose CAE (200 mg/kg) were significantly ($P < 0.05$) decreased (reduced 38.9% and 35.2%, respectively) compared with BDL alone. They were decreased even more compared to the levels in the group treated with silymarin. Serum concentrations of GGT, TCHO, and TBIL indicating biliary cell damage and cholestasis caused by BDL are shown in Table 1. Serum levels of GGT, TCHO, and TBIL in BDL alone group were significantly ($P < 0.05$) higher compared to those in the control group. In BDL mice treated with CAE (200 mg/kg), serum levels of GGT, TCHO, and TBIL were significantly ($P < 0.05$) reduced (by 38.4%, 27.0%, and 18.2%, respectively) compared to those of the control group.

3.2. Effect of CAE on levels of antioxidants in BDL mice

Enhanced oxidative status is associated with liver fibrosis. The oxidative status of the liver in BDL-induced mouse model was evaluated by measuring the levels of GSH, SOD, TBARS, and NO in the liver (Figure 1). The levels of GSH and SOD showed marked ($P < 0.05$) reduction in the BDL group compare to those of the control group. However, administration of CAE (50 and 200 mg/kg) recovered GSH and SOD levels. Treatment with CAE 200 mg/kg significantly ($P < 0.05$) increased their levels compared to BDL group alone. Levels of TBARS were monitored to determine lipid peroxidation levels. TBARS levels in BDL group were significantly ($P < 0.05$) higher than that of the normal control group. Administration with CAE administration to BDL treated mice decreased the increase of hepatic TBARS concentration in a dose-dependent manner. The levels of NO were also significantly ($P < 0.05$) reduced in the group treated with 200 mg/kg of CAE compared to that of the BDL group without CAE treatment.

3.3. Effect of CAE on mRNA levels in BDL mouse model

To investigate whether CAE has anti-inflammatory or anti-apoptosis effects, the mRNA expression levels of pro-inflammatory cytokines and tumor necrosis factor-related apoptosis genes in liver tissues were determined by quantitative real-time PCR. As shown in Figure 2A, mRNA expression levels of interleukins and TNF α in BDL mice treated with CAE (50 and 200 mg/kg) were significantly ($P < 0.05$) decreased in a dose-dependent manner compared to those in BDL alone group.

Table 1
Effect of *Citrus aurantium* extract on serum profiles in bile duct-ligated mouse model.

Group	ALT (U/L)	AST (U/L)	GGT (U/L)	TCHO (mg/dL)	TBIL (mg/dL)
Control	23.0 ± 1.4	84.8 ± 4.8	11.1 ± 3.8	139.0 ± 8.3	1.1 ± 0.2
BDL alone	507.7 ± 57.5#	604.0 ± 65.5#	31.5 ± 3.5#	239.7 ± 25.3#	17.9 ± 1.8#
BDL + silymarin (200 mg/kg)	375.2 ± 52.6*	452.2 ± 116.2*	22.5 ± 2.3*	134.1 ± 6.9*	15.5 ± 1.1#
BDL + CAE (50 mg/kg)	320.2 ± 33.1	418.6 ± 16.2	32.6 ± 3.4	296.0 ± 20.7	18.1 ± 2.2#
BDL + CAE (200 mg/kg)	310.4 ± 48.8*	391.6 ± 52.3*	19.4 ± 2.3*	174.8 ± 13.5*	14.6 ± 1.6#

#$P < 0.05$ compared with control group.
*$P < 0.05$ compared with BDL alone group.

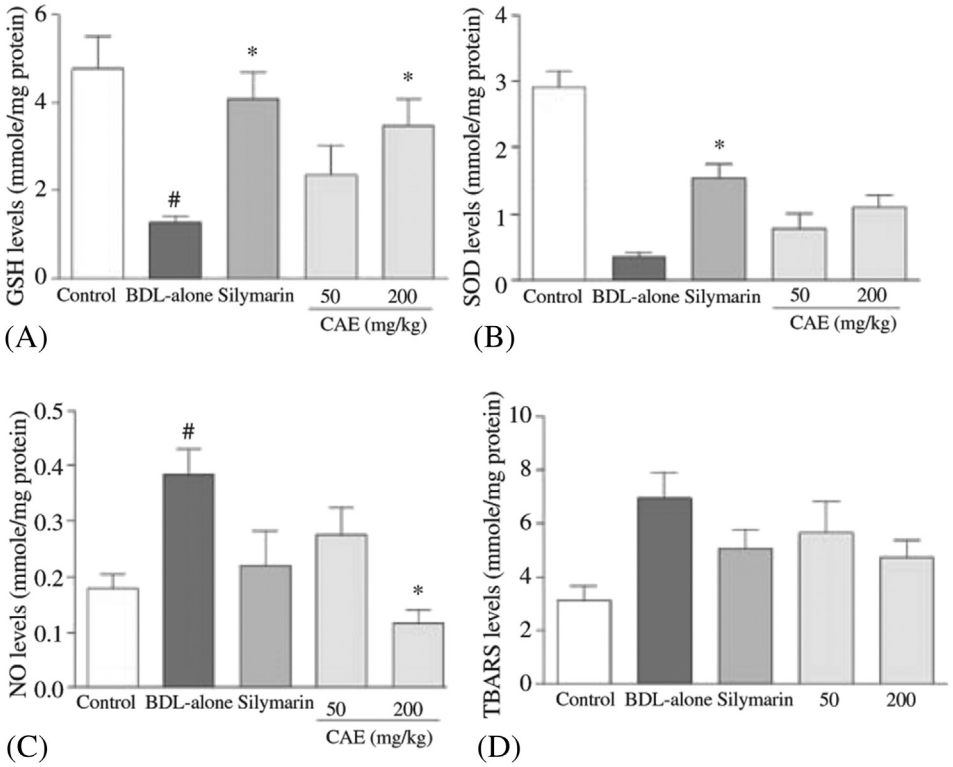

Figure 1. Effect of *Citrus aurantium* extract on liver oxidative states of BDL mice. At 24 h after BDL operation, mice were orally administered with distilled water, silymarin (200 mg/kg), or CAE (50 or 200 mg/kg) daily for 4 weeks. Levels of glutathione (GSH) (A), superoxide dismutase (SOD) (B), nitric oxide (NO) (C), and thiobarbituric acid reactive substances (TBARS) (D) in the liver tissue were determined. #$P < 0.05$ compared with control group; *$P < 0.05$ compared with BDL alone group.

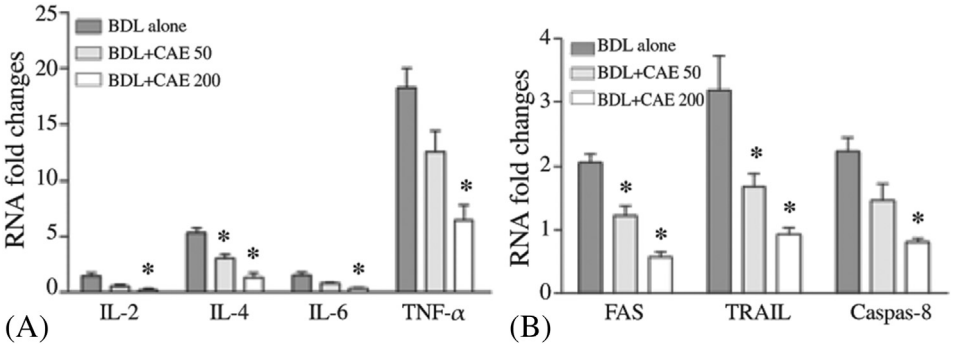

Figure 2. Effect of CAE on inflammation (A) and apoptosis (B) related signals in the livers of BDL mice. At 24 h after BDL operation, mice were orally administered with distilled water, silymarin (200 mg/kg), or CAE (50 or 200 mg/kg) daily for 4 weeks. Total RNA was extracted from liver tissues and cytokines expression levels were quantified by SYBR Green-based real-time PCR using mouse-specific cytokine primers. *$P < 0.05$ compared with BDL alone group.

The expression levels of apoptosis related genes after treatment with CAE are shown in Figure 2B. The mRNA levels of apoptosis related genes such as FAS, TRAIL, and Caspase-8 were significantly ($P < 0.05$) decreased in BDL mice treated with CAE (50 and 200 mg/kg) compared to those in BDL alone group.

3.4. Effect of CAE on histopathological alteration in BDL mice

Four weeks after CAE administration to BDL mice, liver histological changes were observed. Results are shown in Figure 3. After liver tissues were stained with hematoxylin and eosin and compared to those of the control group, BDL caused dissociation and lytic necrosis of hepatocytes. The necrosis and neutrophilic infiltration observed in the BDL alone group were reduced in BDL mice treated with CAE. Based on Ishak's score of statistical analysis, the pathologic alteration score of the BDL alone group was significantly ($P < 0.05$) increased compared to that of the normal control group. However, treatment with CAE significantly ($P < 0.05$) decreased the injury score compared to BDL alone.

Liver fibrosis and collagen depositions in liver tissues induced by BDL were determined by MT staining and Sirius red staining, respectively (Figure 3B and C). The control group showed normal lobular architecture with central veins. However, the BDL group showed markedly ($P < 0.05$) widespread destruction of liver architecture with increased connective tissue, extensive collagen deposition, pseudolobular formation, and fibrosis. Treatment with CAE (50 and 200 mg/kg) significantly ($P < 0.05$)

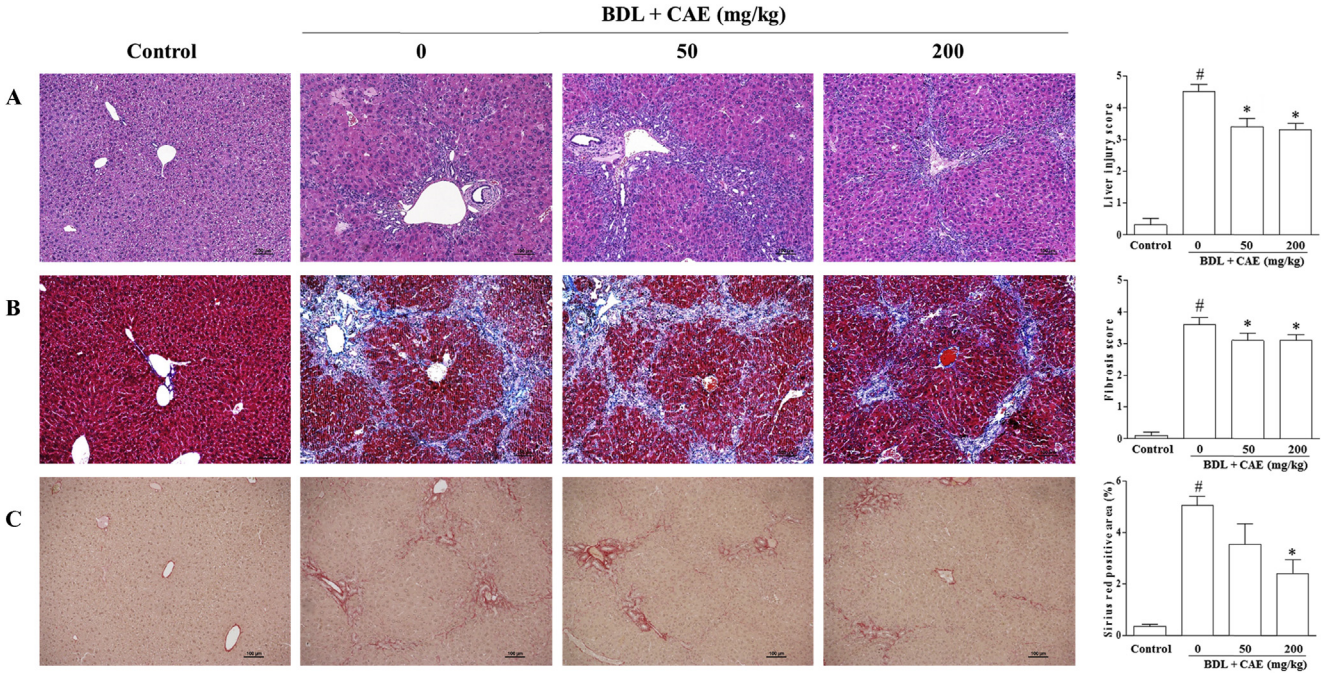

Figure 3. Effect of CAE on pathological changes in the livers of BDL mice. At 24 h after BDL operation, mice were orally administered with distilled water, silymarin (200 mg/kg), or CAE (50 or 200 mg/kg) daily for 4 weeks. Representative photomicrographs of liver sections were processed for hematoxylin & eosin (H&E) staining (A), Masson's trichrome staining (B), and Sirius red staining (C). Bar: 100 μm. $^{\#}P < 0.05$ compared with the control group; $^{*}P < 0.05$ compared with BDL alone group.

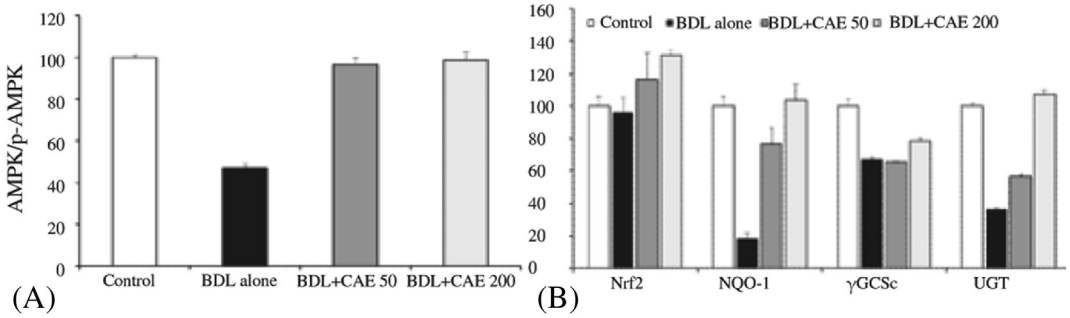

Figure 4. Effect of CAE on protein expression in the livers of BDL mice. At 24 h after BDL operation, mice were orally administered with distilled water, silymarin (200 mg/kg), or CAE (50 or 200 mg/kg) daily for 4 weeks. Levels of phospho-AMPKα (A) and Nrf2 related signals (B) were assessed by western blot analysis. Levels of AMP-activated protein kinase (AMPK), nuclear factor E2-related factor 2 (Nrf2), NAD (P) H: Quinone oxidoreductase-1 (NQO-1), γ-glutamylcysteine synthetase heavy subunit (γGCSc), and Uridine 5'-diphospho-glucuronosyltransferase (UGT) were measured. $^{*}P < 0.05$ compared with BDL alone group.

protected BDL liver and ameliorated fibrosis. The control group had a normal distribution of collagen. However, collagen deposition was increased in the BDL alone group. On the other hand, CAE treatment (200 mg/kg) significantly ($P < 0.05$) reduced collagen deposition compared to BDL alone group.

3.5. Effect of CAE on AMPK and Nrf-2 related protein expression in BDL mice

The effect of CAE on BDL-induced AMPK and Nrf-2 related protein levels in liver tissues were investigated by western blotting. As shown in Figure 4, administration with CAE (50 and 200 mg/kg) significantly ($P < 0.05$) enhanced p-AMPK expression levels compared to BDL alone group (Figure 4A). The expression levels of Nrf2, NQO1, γGCSc, and UGT in the BDL alone group were markedly ($P < 0.05$) decreased compared to those in the control group (Figure 4B). However, CAE treatment significantly ($P < 0.05$) increased the expression levels of hepatic Nrf2 related proteins, especially NQO1 and UGT whose expression levels were significantly ($P < 0.05$) increased by CAE treatment in a dose-dependent manner.

4. Discussion

Liver is self-regenerative organ with highly replicative hepatocytes [15]. Cholestatic liver injury is caused by disrupted bile secretion from liver to duodenum. Stagnant bile acid and bile salt could not convert to bile acid molecule or extract lipids from hepatocytes into bile fluid in the gut. The role of bile acid in the liver and intestine has been well established. Its main role is enterohepatic circulation in cholesterol metabolism, such as eliminating cholesterol through fecal route [16,17]. Therefore, abnormal concentrations of bile compounds are toxic to hepatic and biliary.

Cholestasis causes cellular damage, subsequently causing inflammation and fibrogenesis in the liver [6]. Cholestasis can be

classified as intra-hepatic and extra-hepatic. Chronic cholestatic liver injury is mostly intra-hepatic. It affects intra- and extra-hepatic bile ducts. Impairment of bile formation of bile flow can cause primary biliary cirrhosis, which is a chronic inflammatory intra-hepatic liver disorder. When these ducts are extended, intracellularly they cause hepatocyte cholestasis, extracellularly they cause bile acid malabsorption in the colon, thus disturbing bile acid metabolism [10,18].

Our previous studies have found that an ethanol extract of *Citrus aurantium* peel contains polymethoxy flavones with potent hepatoprotective effects against binge alcohol and carbon tetrachloride induced liver injury [11,12]. CAE treatment can regulate the oxidative status and lipid peroxidation with cytoprotective signals in pathologic hepatic injuries and intoxication. We investigated the potential protective effects of CAE (containing approximately 27% of nobiletin) against liver injury induced by obstructive cholestasis in BDL administrated mice. Bile duct ligation in rodents has been well established. BDL can induce high yields of stereotypical cirrhosis, fibrogenesis, and cholestasis with morphological and biochemical changes of liver and serum comparable to human hypercholesterolemia [5,19]. Our results demonstrated that the progression of liver fibrosis and cholestasis in mice underwent BDL for 4 weeks was attenuated by CAE.

Serum concentrations of ALT, AST, GGT, TCHO, and TBIL are known to increase during the development of cholestatic liver injury in mice with BDL. Oral administration of CAE markedly reduced the levels of these hepatic enzymes, the major biomarkers of hepatic injury. GGT is the highest in case of intra- or post-hepatic biliary obstruction in all forms of liver disease [20]. Elevated serum GGT can lead to increased free radical production and glutathione depletion, thus generating superoxide radicals with hepatic steatosis [21–23]. Our result demonstrated that serum markers of hepatic tissue injury were significantly attenuated by CAE (200 mg/kg) treatment in BDL-induced serum hepatic enzymes. The extent of attenuation by CAE was higher than that of silymarin (200 mg/kg).

Furthermore, it has been known that oxidative stress is one of the main contributors in cholestatic liver disease [24]. During liver cholestasis, oxidative stress occurs. In cholestasis induced liver injury, lipid peroxidation is increased. It has been reported that increased oxidative stress contributes to toxic bile acid stagnant, thus stimulating reactive oxygen species [25]. In the present study, BDL accelerated oxidative stress and lipid peroxidation as evidenced by increased NO and TBARS concentrations. However, oral administration with CAE protected BDL mice against liver injury with hepatic cholestasis.

GSH and SOD are important antioxidant and detoxification enzymes in intracellular protective mechanisms. These enzymes are rapidly cleared from circulation in serum. They act as scavengers of reactive oxygen species [26,27]. Our results also revealed the depletion of GSH and SOD levels in BDL-induced hepatic injury. Administration of CAE restored serum GSH and SOD levels, indicating that CAE could prevent the accumulation of free radicals in chronic liver injury induced by BDL.

Recently, it has been reported that cholestatic liver injury can result in inflammatory necrosis [28]. We also demonstrated that serum levels of pro-inflammatory cytokines and apoptosis factors were increased in BDL-induced chronic hepatic injury. Plasma biomarkers of pro-inflammatory cytokines such as IL-2, IL-4, IL-6, TNF-α, and AMPK were significantly reduced by CAE treatment, implying that CAE has anti-inflammatory activity in cholestasis. It has been reported that activation of phospho-AMPK can inhibit pro-inflammatory mediators against stress and collagen deposition in liver fibrosis [29]. These results are in agreement with previous reports that CAE treatment can protect against alcohol and CCl_4 intoxication induced hepatic injury in a mouse model [11,12].

This study revealed that CAE + BDL mice had significantly lower level of FAS and Caspase-8 compared to BDL alone mice. Hepatocytes apoptosis in obstructive cholestatic liver injury can be induced by toxic bile salts such as glycochenodeoxycholic acid that can induce apoptosis in mitochondria-controlled Caspase-8 pathway [1]. Caspase-8 is a protease abundant in the liver. It initiates apoptosis by FAS and tumor necrosis factor (TNF) [30,31]. It has been reported that toxic bile salts such as glycochenodeoxycholic acid can induce Caspase-8 activation through direct activation of FAS, which activates cell death in FAS receptor-dependent manner [32]. TRAIL is also a member of the TNF family. It induces apoptosis with tumor selectivity [33,34]. Seol *et al.* have reported that TRAIL-induced apoptosis requires Caspase-8 [35]. In this study, we also demonstrated that CAE could inhibit FAS, TNF, Caspase-8, and TRAIL. Therefore, CAE could inhibit toxic bile salts induced apoptosis in the liver.

To redox an oxygen radical-dependent liver pathogenesis, specific pathway can be targeted to prevent oxidative liver injury [36]. As previous described, toxic bile acid can lead to oxidative stress in obstructive cholestasis. To restore redox balance, specific pathways can be activated to protect liver from damage, including fibrosis and chronic liver injury [37,38]. In the liver, BDL activated oxidative injury that inhibiting regulator of cellular oxidative stress, Nrf-2, which binding to NQO1, γGCSc, and UGT with superoxide scavenging activity [39,40]. The highly inducible enzyme NQO1 and rate-limiting enzyme UGT can recover their cellular redox status in oxidative condition after liver damage. We found that CAE induced the expression of antioxidant protein, suggesting that CAE indeed can induce transcription factor Nrf2, thus triggering antioxidant proteins.

In conclusion, this study showed that nobiletin rich extract CAE could protect liver against inflammation, cholestasis, and apoptosis induced by BDL stimulating antioxidant and anti-apoptotic mechanisms in mice. Based on these findings, we suggest that CAE should be avoided as therapeutic application in clinical settings for cholestatic disorders caused by bile duct obstruction.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgement

This work was supported by ‘Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ01132001)’ funded by Rural Development Administration, Republic of Korea.

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