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Protective Effect of Fucoidan against Acetaminophen-Induced Liver Injury

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Fucoidan, a sulfated polysaccharide extracted from various brown seaweeds, possesses a wide range of pharmacological properties. In this study, we investigated the protective effect of fucoidan on acetaminophen-induced acute liver injury in rats. Liver injury was induced by administration of acetaminophen (800 mg/kg, i.p.) and fucoidan was administered (100 mg kg, p.o.) 2 h before and after acetaminophen administration. After 24 h, co-treatment of fucoidan ameliorated liver damage and cell death induced by acetaminophen. Acetaminophen induced the overexpression of CYP2E1, one of the metabolizing enzymes of acetaminophen, but co-treatment with fucoidan suppressed its increased expression of CYP2E1. Fucoidan also reduced the hepatic apoptosis induced by acetaminophen exposure as shown in the protein expression of Bax, Bcl-2, and cleaved caspase-3. The anti-oxidative effect of fucoidan was observed from the increase of the production and expression of glutathione, superoxide dismutase, and glutathione peroxidase, both of which were decreased by acetaminophen. Also, fucoidan decreased the expression of inflammatory mediators, including tumor necrosis factor-alpha, interleukin 1 beta, and inducible nitric oxide synthase. Taken together, the data demonstrate the hepato-protective effects of fucoidan against acetaminophen-induced liver injury via anti-oxidant, anti-inflammatory, and anti-apoptotic mechanisms.

Key words: Fucoidan, Acetaminophen, Liver injury, Anti-oxidant

Selected by Editors

INTRODUCTION

Acetaminophen is one of the most widely used analgesic and antipyretic drugs in the USA. Acetaminophen is metabolized by cytochrome P450 (CYP) to form highly reactive species, such as *N*-acetyl-*p*-benzoquinone imine (NAPQI). At normal dose, NAPQI is readily detoxified by conjugation with glutathione (GSH) (Mitchell et al., 1973; Prescott, 1980; Ferret et al., 2001). Nevertheless, overdoses of acetaminophen leads to the depletion of GSH and excessive NAPQI causes oxidative stress and binds covalently to liver proteins (Jollow et al., 1974; Dahlin et al., 1984). It finally leads to mitochondrial dysfunction and hepatic cell death (Boyer and Rouff, 1971; Nelson, 1995).

Fucoidan is a sulfated polysaccharide from the cell wall of brown seaweed containing a substantial percentage of L-fucose and sulfate ester groups (Li et al., 2008). Fucoidan from *Fucus vesiculosus* is composed of variable amounts of fucose, uronic acids, galactose, xylose, and sulfate (Nishino et al., 1994). Fucoidan has various pharmacological properties, such as anti-coagulant, anti-tumor, anti-inflammatory and anti-oxidative activities, and promotes immune function (Angstwurm et al., 1995; Maruyama et al., 2003; Yang et al., 2006; Hu et al., 2010).

Fucoidan has also been reported to protect tissues from various types of injury by increasing the content of hepatic growth factor (HGF) (Bilan et al., 2006). It has recently been reported to reduce liver fibrogenesis by protecting from hepatic cell death and inducing apoptosis of hepatic stellate cell, as well as showing anti-oxidative properties against carbon tetrachloride-

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induced acute liver injury and dimethylnitrosamine-induced chronic liver fibrosis (Hayashi et al., 2008; Kang et al., 2008; Hong et al., 2011). Fucoidan is absorbed in the intestines and is distributed in the liver (Tokita et al., 2010). The degradation of fucoidan also occurs in the kidneys (Nakazato et al., 2010; Tokita et al., 2010). In our previous study, there were no effects of fucoidan on inflammation, oxidative stress and cell toxicity (Hong et al., 2011).

In this study, we investigated the hepato-protective effects of fucoidan in parallel with its anti-inflammatory and anti-oxidative properties against acetaminophen-induced liver damage in a rat model.

MATERIALS AND METHODS

Animals

Animal care and all experimental procedures were performed in accordance with the Guide for Animal Experiments edited by the Korea Academy of Medical Science. The animals were housed in an air-conditioned room at 25°C with a 12-h dark/light cycle, and were fed with standard rodent chow and tap water ad libitum. Male Sprague-Dawley rats were obtained from the Orient-Bio Laboratory Animal Research Center (Gyeonggi-do). Fucoidan and acetaminophen were purchased from Sigma-Aldrich and dissolved in saline. Eighteen male 6-week-old rats were assigned to three groups of six each (Con, APAP, and APAP+FUCO groups). Acetaminophen (800 mg/kg) was intraperitoneally injected into the rats. The APAP+FUCO group was treated with fucoidan at a dose of 100 mg/kg by oral administration 2 h before and after acetaminophen administration without fasting. The APAP group was treated only with acetaminophen and the control group was treated only with saline equivalent to the treatment given to the other groups. The next day after acetaminophen treatment, all rats were sacrificed under ketamine anesthesia.

Biochemical parameters

Serum aspartate transaminase (AST), alanine transaminase (ALT), total bilirubin (T-Bilirubin), direct bilirubin (D-Bilirubin), blood urea nitrogen (BUN), and creatinine (CRE) levels were measured at Inha University Hospital (Incheon).

Liver histopathology

For histopathological analysis, liver slices were cut in equal parts, fixed in 10% formaldehyde, and embedded in paraffin. Five micrometer-thick sections were stained with hematoxylin and eosin (H&E) before observation under a light microscope.

Determination of glutathione (GSH), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GPx)

The liver tissues were homogenized in the same volume of a 50 mM Tris buffer (pH 7.4) for 2 min. MDA assay was performed according to the thiobarbituric acid method (Niehaus and Samuelsson, 1968) and the GSH, SOD, and GPx levels were measured using a commercial assay kit (Cayman).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the liver sample with the Trizol reagent (Invitrogen) according to the manufacturer's protocol. An aliquot of total RNA was reverse transcribed with MMuLV and amplified with Taq DNA polymerase (Promega). The expression level of each transcript was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA in the same tissue. The PCR product was electrophoresed on a 1.5% agarose gel and the results were recorded by an imaging system (Kodak Molecular Imaging Systems), and the bands were quantified by densitometry. Details of the primers are shown in Table I.

Western blotting

The liver samples were selected randomly from each group and homogenized in RIPA buffer. 50 μg of each liver protein were separated in 12% polyacrylamide gel, and the resolved proteins were transferred to a

Table I. Sequences of the PCR primers

Gene	Primer sequence (5'-3')	Product length (bp)
TNF-α	Sense: ACAAGGAGGAGAAGTTCCCAAAT Anti-sense: GACTTTCTCCTGGTATGAAATGG	332
IL-1β	Sense: AAATAGCAGCTTTCGACAGTGAG Anti-sense: GATTTTGTCGTTGCTTGTCTCTC	488
iNOS	Sense: CAGAGCCTCTAGACCTCAACAAA Anti-sense: GCTGAACTTCCAATCGTTGTACT	479
GAPDH	Sense: CCACTGGCGTCTTCACCAC Anti-sense: CCTGCTTCACCACCTTCTTG	501

nitrocellulose membrane (Amersham). The membrane was blocked overnight with 3% bovine serum albumin in 10 mM Tris-HCl containing 150 mM NaCl and 0.5% Tween 20 [Tris-buffered saline (TBS)-T]. After washing with TBS-T, the membrane was then incubated with 1:1000 dilutions of the following primary antibodies: inducible nitric oxide synthase (iNOS), Bax, Bcl-2 (Santa Cruz Biotechnology), interleukin-1 beta (IL-1β; Abcam), tumor necrosis factor-alpha (TNF-α), α-tubulin (Ab Frontier), and cleaved caspase-3 (Cell Signaling Technologies). After washing with TBS-T, 1:10000 dilutions of horseradish peroxidase (HRP)-conjugated secondary antibodies (New England Biolabs) in TBS-T were applied to the membrane, and each blot was developed using an enhanced chemiluminescence detection kit (Amersham) and exposure of the blots to X-ray film (Kodak).

Cytotoxic effects in normal liver cells

HL-7702, human normal liver cell line was treated with various concentrations of acetaminophen and fucoidan, and cell viability was measured 24 h later using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based cell viability assay. Cells were seeded at a density of 3,000 cells/well in 96-well plates. Absorbance was read at 570 nm.

Statistical analysis

Data were expressed as mean ± S.D. and statistical analysis was performed using significant differences between the groups using Student's *t*-test. The differences between multiple groups were evaluated by a one-way analysis of variances (ANOVA), followed by a nonparametric post hoc test (LSD). A p value of 0.05 or less. Statistical calculations were performed using SPSS software for Windows operating system, Version 10.0 (SPSS).

Table II. Effects of fucoidan on serum parameters

	CON	APAP	APAP+FUCO
AST	189.57 ± 15.22	425.23 ± 36.52$^{\#\#}$	313 ± 31.11*
ALT	98.13 ± 9.89	132 ± 11.11$^{\#}$	98.14 ± 7.46*
T-Bil	0.013 ± 0.008	0.055 ± 0.006$^{\#\#}$	0.032 ± 0.005*
D-Bil	0.008 ± 0.007	0.038 ± 0.004$^{\#}$	0.026 ± 0.004*
BUN	14 ± 0.57	21.77 ± 2.68$^{\#}$	17.17 ± 0.55**
CRE	0.445 ± 0.021	0.563 ± 0.129$^{\#}$	0.453 ± 0.026*

Values are expressed as the mean ± S.D. (n = 6). $^{\#}p < 0.05$, $^{\#\#}p < 0.001$ *vs* control and $^{*}p < 0.05$, $^{**}p < 0.001$ *vs* APAP group, respectively. CON, control; APAP, acetaminophen-treated group; APAP+FUCO, acetaminophen plus fucoidan-treated group; AST, aspartate aminotransferase; ALT, alanine aminotransferase; T-Bil, total bilirubin; D-Bil, direct bilirubin; BUN, blood urea nitrogen.

RESULTS

Effects on serological parameters

A significant effect of acetaminophen on the serum parameters and interaction between acetaminophen and fucoidan were apparent (Table II). Acetaminophen overdose increased the levels of AST, ALT, T-Bilirubin, and D-Bilirubin, which are indicative of liver damage. However, the administration of fucoidan significantly lowered the effects of AST, ALT, T-Bilirubin, and D-Bilirubin ($p < 0.05$). Furthermore, co-administration of fucoidan decreased BUN and creatinine levels, suggesting that it also protected from acetaminophen-induced kidney injury ($p < 0.05$). H&E staining revealed normal liver architecture in control rats, whereas the APAP group exhibited massive and severe hepatic necrosis, as well as infiltration of inflammatory cells. In the APAP+FUCO group, hepatocytes were relatively well-preserved, compared with the APAP group (Fig. 1).

Effects on hepatic CYP2E1 activity and oxidative stress

To investigate the protective effect of fucoidan against

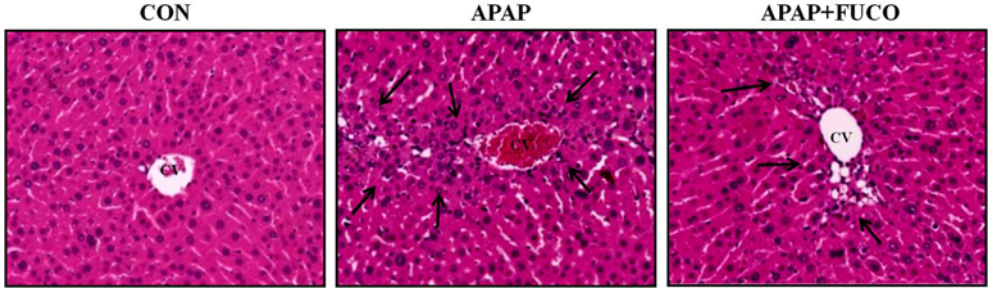

Fig. 1. Histological analysis of the liver sections. The liver sections were stained with H&E. Original magnification ×200. CON, control; APAP, acetaminophen-treated group; APAP+FUCO, acetaminophen plus fucoidan-treated group; CV, central vein; Arrows indicate infiltrated inflammatory cells.

acetaminophen-induced hepato-toxicity, its metabolism and anti-oxidant capacity were measured. Bioactivation of acetaminophen by CYP2E1 results in the formation of the reactive intermediate, NAPQI, which form covalent protein adducts with GSH (Potter et al., 1973). Immunoblot analysis was performed to examine the effect of fucoidan on the protein expression of CYP2E1. CYP2E1 protein levels were suppressed by fucoidan treatment (Fig. 2A). Acetaminophen administration significantly depleted the GSH levels, whereas co-treatment with fucoidan significantly improved the level (Fig. 2B). The liver SOD level of the APAP group became significantly lower than that of the control, whereas the SOD level of the APAP+FUCO group was improved more than that of APAP group ($p < 0.05$). Furthermore, the liver GPx level in the APAP+FUCO showed a 2.5-fold increase compared to that of the APAP group ($p < 0.05$). The level of MDA, a product of lipid peroxidation, was increased in the liver of the APAP group compared with the control group, but it was suppressed in the APAP+FUCO group ($p < 0.05$). These results show that fucoidan suppressed the oxidative stress induced by acetaminophen.

Expression of inflammatory mediators

To identify whether fucoidan could suppress progression of the inflammation, we investigated the expression of TNF-α, IL-1β, and iNOS in rats with acetaminophen-induced acute liver injury. As shown in Fig. 3A, the mRNA expression of TNF-α, IL-1β, and iNOS were significantly decreased by fucoidan treatment ($p < 0.05$). Also, their protein expression were similar to the results of mRNA expression (Fig. 3B). These results indicate that fucoidan protected against the inflammation accompanied with acetaminophen-induced liver injury.

Effects on apoptosis-related proteins

To focus on the hepato-cellular mechanism altered by acetaminophen treatment, the involvement of pro-apoptotic and anti-apoptotic proteins during acetaminophen-induced cell death was studied. Acetaminophen treatment decreased the level of Bcl-2, an anti-apoptotic protein, as well as increasing the expression of Bax, a pro-apoptotic protein, compared with untreated rats (Fig. 4). Consequently, activation of caspase-3 was enhanced by acetaminophen treatment. In comparison, fucoidan showed efficient protection against acetamino-

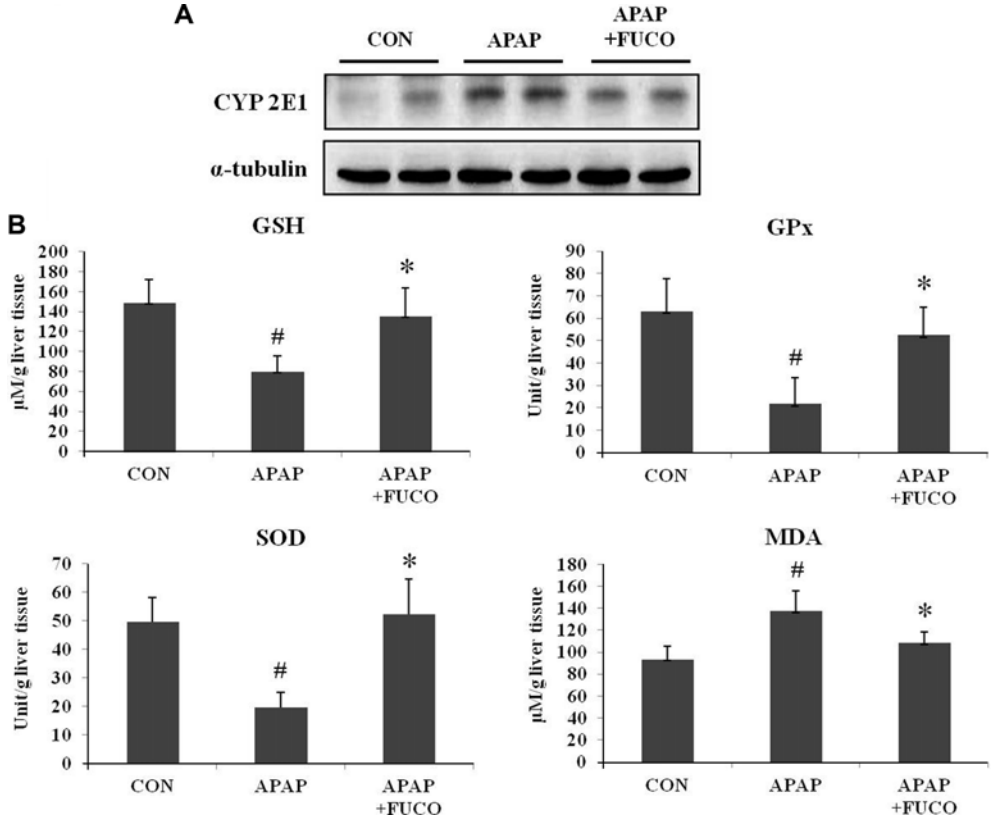

Fig. 2. Effect of fucoidan on the amount of CYP2E1 and glutathione. (**A**) The protein expression of CYP2E1 was analyzed by Western blotting. (**B**) Effect of fucoidan on hepatic GSH, MDA, SOD, and GPx levels. CON, control; APAP, acetaminophen-treated group; APAP+FUCO, acetaminophen plus fucoidan-treated group. Values are expressed as the mean ± S.D. (n = 6). $^{\#}p < 0.05$ *vs* control and $^{*}p < 0.05$ *vs* APAP group, respectively.

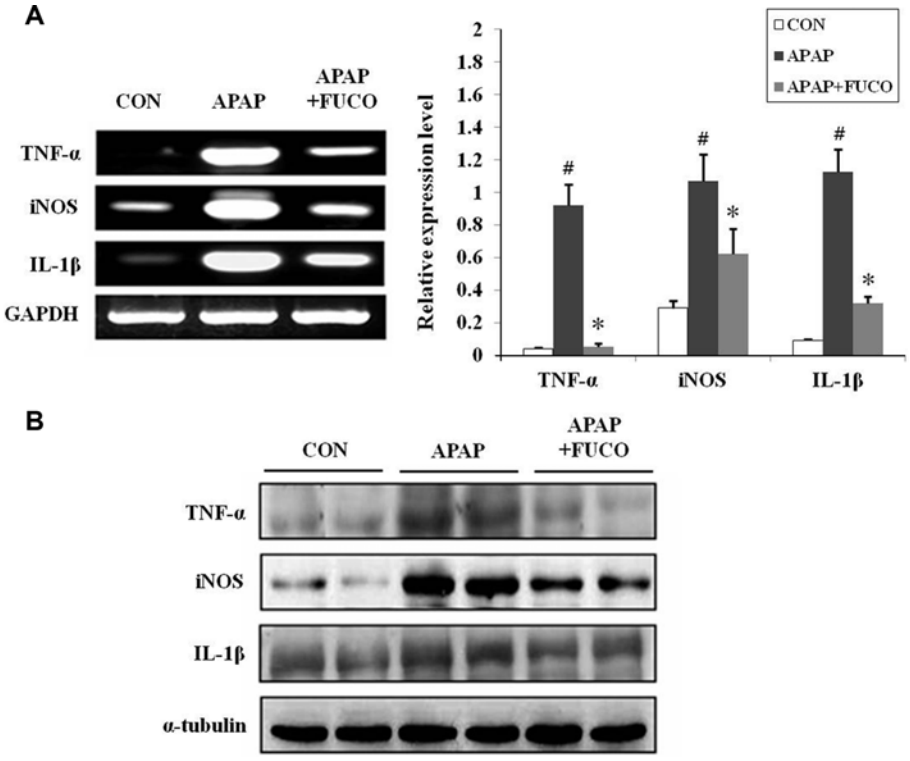

Fig. 3. Effect of fucoidan on the expression of inflammatory mediators. (**A**) The mRNA expression of TNF-α, IL-1β, and iNOS was measured by RT-PCR. (**B**) Protein expression of TNF-α, IL-1β, and iNOS was measured by Western blotting. CON, control; APAP, acetaminophen-treated group; APAP+FUCO, acetaminophen plus fucoidan-treated group. Values are expressed as the mean ± S.D. (n = 6). $^{\#}p < 0.05$ *vs* control and $^{*}p < 0.05$ *vs* APAP group, respectively.

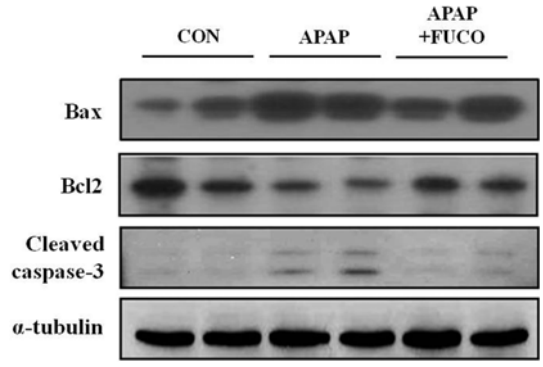

Fig. 4. Effect of fucoidan on the apoptosis induced by acetaminophen. The protein expressions of Bcl-2, Bax, and cleaved caspase 3 were measured by Western blotting. CON, control; APAP, acetaminophen-treated group; APAP+FUCO, acetaminophen plus fucoidan-treated group.

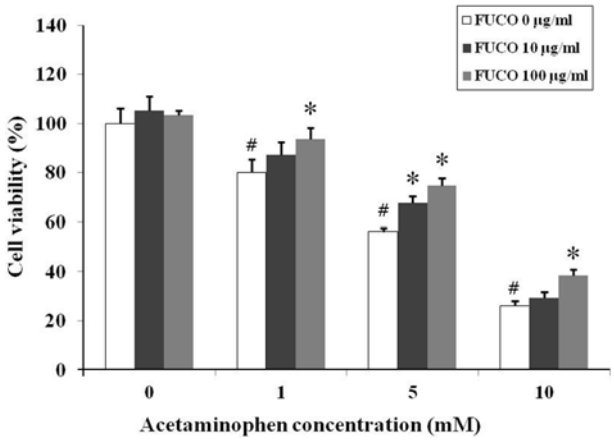

Fig. 5. Viability of human normal liver hepatocytes. Hepatocytes were treated with various concentrations of acetaminophen and fucoidan for 24 h prior to the MTT-based viability assay. Data values are expressed as the mean ± S.D. (n = 3). $^{\#}p < 0.05$ *vs* control and $^{*}p < 0.05$ *vs* APAP group, respectively.

phen-induced apoptotic events in that it decreased the enhanced expression of Bax and cleaved caspse-3, as well as increasing the expression of Bcl-2 that had been down-regulated by acetaminophen.

Hepatoprotective effect of fucoidan in normal liver cells

To investigate whether fucoidan can prevent acetaminophen-induced hepato-toxicity *in vitro*, we measured cell viability of human *HL-7702 hepatocytes* using the MTT assay in cells exposed to various concentration of acetaminophen with or without fucoidan. As shown in Fig. 5, treatment with acetaminophen resulted in significant cell death, whereas co-treatment with

fucoidan provided significant protection against acetaminophen-induced cytotoxicity.

DISCUSSION

Our present studies indicate that fucoidan ameliorates liver toxicity induced by acetaminophen overdose in a rat model of liver injury and human hepatocytes. Fucoidan displayed a hepato-protective effect as demonstrated by a significant decrease in serological parameters such as ALT, AST, and bilirubin. H&E staining also showed that fucoidan treatment preserved liver tissue architecture and prevented severe hepatic necrosis.

Acetaminophen at high doses is metabolized to NAPQI predominantly by CYP and especially by CYP2E1 (Potter et al., 1973). GSH conjugates with NAPQI-reactive toxic metabolites of acetaminophen and depletion of GSH leads to severe hepato-toxicity (Dahlin et al., 1984). In this study, fucoidan treatment (100 mg/kg) decreased the expression of CYP2E1 and prevented depletion of GSH by reduction of CYP2E1. Furthermore, fucoidan had a hepato-protective effect as demonstrated by significant decreases in the serological parameter levels and by reduction of hepatic histopathological changes.

Accumulation of NAPQI causes mitochondrial dysfunction, uncoupling of the oxidative phosphorylation, and formation of pores in the mitochondrial inner membrane (Jaeschke and Lemasters, 2003; Masubuchi et al., 2005). Bax propagates apoptosis by its insertion into the outer mitochondrial membrane and formation of pores through oligomerization, which induces the release of pro-apoptotic factors. Inhibition of Bax can prevent acetaminophen-induced cell death *in vitro* and *in vivo* (El-Hassan et al., 2003; Bajt et al., 2008). In our study, enhanced Bax and decreased Bcl-2 levels were evident in rats with acetaminophen-induced liver injury, whereas fucoidan prevented acetaminophen-induced mitochondrial dysfunction. Consequentially, cleaved casapase-3 was decreased in fucoidan-treated rats.

A number of studies have implicated reactive oxygen species (ROS) in the oxidative stress produced by acetaminophen (Rajkapoor et al., 2008; Yan et al., 2009). Fucoidan showed anti-oxidative properties by increasing anti-oxidative enzymes such as SOD and GPx against carbon tetrachloride-induced acute liver injury and dimethylnitrosamine-induced chronic liver fibrosis, respectively (Kang et al., 2008; Hong et al., 2011). In agreement, fucoidan presently increased SOD and GPx levels with concomitant reduction of MDA production in a liver homogenate and in the plasma of rats with acetaminophen-induced liver injury.

ROS cause inflammation, which is a contributing factor in the pathogenesis of various acute and chronic liver diseases (Jaeschke, 2000; Novo and Parola, 2008). ROS induce inflammation by up-regulating the expression of inflammatory mediators. The inflammatory response is mediated by the action of pro-inflammatory cytokines such as TNF-α and IFN-γ. These cytokines contribute to the intra-hepatic recruitment and activation of granulocytes that are characteristically found in hepatic inflammation. iNOS also has an inflammatory action through its mediated production of a large amount of NO (Hierholzer et al., 1998; Vidali et al., 2008). We therefore investigated whether fucoidan would have an anti-inflammatory effect on acetaminophen-induced liver injury. As expected, fucoidan decreased the expression of the inflammation mediators TNF-α, IL-1β, and iNOS in rats with acetaminophen-induced liver injury.

In summary, we have shown that fucoidan exhibits hepato-protective activity against acetaminophen-induced liver injury in rats. This may be partially because fucoidan prevents GSH depletion by suppressing the expression of CYP2E1 and the subsequent formation of a toxic metabolite of acetaminophen. Fucoidan also possesses anti-oxidant and anti-inflammation activities, and prevents mitochondrial dysfunction. The results show the potential of fucoidan as a hepato-protective agent against acetaminophen-induced liver injury.

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