Available online at www.sciencedirect.com

SciVerse ScienceDirect

Journal of Nutritional Biochemistry 23 (2012) 679–684

Journal of Nutritional Biochemistry

Effects of soy protein on alcoholic liver disease in rats undergoing ethanol withdrawal

Hsin-Yi Yang[a,b], Hsiao-Shan Lin[b], Jane C.J. Chao[b], Yi-Wen Chien[b], Hsiang-Chi Peng[b], Jiun-Rong Chen[b,c,*]

[a]Department of Medical Nutrition, I-Shou University, Kaohsiung 824, Taiwan
[b]School of Nutrition and Health Sciences, Taipei Medical University, Taipei 110, Taiwan
[c]Nutrition Research Center, Taipei Medical University Hospital, Taipei 110, Taiwan

Received 15 October 2010; received in revised form 15 March 2011; accepted 17 March 2011

Abstract

Objective: This investigation attempted to clarify the effects of soy protein on alcoholic liver disease (ALD) in rats undergoing ethanol withdrawal.
Methods: Alcoholic liver disease was induced in rats by administration of a low-carbohydrate ethanol liquid diet for 12 weeks, after which the ethanol was withdrawn and the rats were divided into two experimental groups: a control group (EC group) and a soy protein group (EP group) for 4 weeks.
Results: After the 12-week ALD-inducing period, the ethanol group had significantly higher hepatic lipid accumulation, oxidative stress and inflammation. We found that the EP group had significantly lower hepatic lipids, malondialdehyde, tumor necrosis factor-α, interleukin (IL)-1β, IL-6, hydroxyproline levels and myeloperoxidase activity compared to the EC group. Moreover, the fecal total cholesterol and total lipids were higher in the EP group. Expression of the hepatic cytochrome P450 2E1 (CYP2E1) protein in the EP group was significantly lower than that in the EC group, and the hepatic peroxisome proliferator-activated receptor (PPAR) α and cytochrome P450 4A (CYP4A) protein expressions in the EP group were significantly higher than those in the EC group. In the histopathological analysis, we also found that soy protein ameliorated fat accumulation in the liver.
Conclusion: These results suggest that soy protein may improve alcohol-induced lipid accumulation, oxidative stress and inflammation by decreasing proinflammatory cytokines and CYP2E1 protein expression and by increasing PPARα and CYP4A protein expressions and fecal lipid excretion, thereby producing beneficial effects on ALD during ethanol withdrawal.

Keywords: Alcoholic liver disease; Soy protein; Alcohol; Cytokines

1. Introduction

Alcoholic liver disease (ALD) is a result of long-term alcohol consumption [1] and involves a fatty liver, steatosis, fibrosis and cirrhosis [2]. Ethanol induces hepatic fat accumulation, tissue damage and liver dysfunction [3]. Recent studies indicated that ethanol may suppress peroxisome proliferator-activated receptor (PPAR) α expression, reduce the β-oxidation of fatty acids and lead to an accumulation of triglycerides in the liver [4]. In PPARα-knockout mice, ethanol treatment caused liver hypertrophy, impairment of hepatocytes, reduction of antioxidative enzyme activities and elevations of malondialdehyde (MDA) and steatosis [5]; PPARα agonists were able to ameliorate ethanol-induced liver damage and steatosis [6]. In addition, inhibition of PPARα expression by ethanol may also repress hepatic cytochrome P450 4A (CYP4A) expression and inhibit fatty acid oxidation of peroxisomes, which leads to lipotoxicity [7].

Ethanol-induced lipid peroxidation and liver pathological changes are highly related to hepatic cytochrome P450 2E1 (CYP2E1) expression. Ethanol consumption activates the microsomal ethanol-oxidizing system (MEOS) and induces the expression of CYP2E1 to produce free radicals that impair liver function [2]. In addition, ethanol impairs the intestinal barrier and increases circulatory endotoxin level that activates Kupffer cells to produce cytokines that promote inflammatory reactions [8]. Ethanol also increases myeloperoxidase (MPO) activity and the hydroxyproline concentration, a marker of liver fibrosis at an early stage, in rats [9].

Soybean and soy-related products are important sources of plant protein in Asia [10]. Recent studies indicated that soybean contains many bioactive components such as soy protein and soy isoflavones that possess biophysiological effects. Soy protein was demonstrated to improve lipid profiles, modulate inflammatory reactions, reduce lipid peroxidation, ameliorate steatosis and so on [11]. The objective of the present study was to investigate whether soy protein consumption has beneficial effects on recovery during alcohol withdrawal in rats administered a low-carbohydrate ethanol liquid diet to induced ALD.

2. Materials and methods

2.1. Animals, diets and experimental design

Forty-six male Wistar rats (6 weeks old) were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). Investigators at the Taipei Medical University Laboratory Animal Center followed the protocols described in the *Guide for the Care and Use of Laboratory Animals*. Animals were fed a standard rat chow diet (Rodent Laboratory Chow 5001, Purina Mills, St. Louis, MO, USA) for 1 week and then

* Corresponding author. Department of Nutrition and Health Sciences, Taipei Medical University, Taipei 110, Taiwan. Fax: +886 2 2737 3112.
E-mail address: syunei@tmu.edu.tw (J.-R. Chen).

0955-2863/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2011.03.016

fed a control liquid diet for 1 week to acclimate them to a liquid diet. Alcoholic liver disease was induced in rats for 12 weeks, and then they were fed a diet containing soy protein for 4 weeks. In the ALD-inducing period of the study, rats were divided into two groups and pair-fed on an isoenergetic basis for 12 weeks with low-carbohydrate liquid diets that contained 40% carbohydrates (control liquid diet group, C group, $n=18$) or 5.5% carbohydrate plus 34.5% ethanol (ethanol liquid diet group, E group, $n=28$), as previously described [12] (Table 1). After the ALD-inducing period, eight rats of each group were sacrificed to confirm the animal model of ALD. In the withdrawal period, ethanol was withdrawn from rats of the E group, which was divided into two groups: rats fed the control liquid diet (EC group, $n=10$) and rats fed a control liquid diet containing soy protein isolate (containing 3.91 mg/g isoflavones; Fujioil Co., Osaka, Japan) as a substitute for casein (EP group, $n=10$) for 4 weeks (Table 1); the C group continued to be fed the control liquid diet. During this period, food was provided *ad libitum*. The body weight and food intake were recorded daily. At the end of the soy protein experimental period, all rats were sacrificed, and blood and liver samples were collected for analysis.

2.2. Blood collection and analysis

After 12 and 16 weeks, rats were killed after 12-h fasting by withdrawing blood from the interior vena cava into tubes containing an anticoagulant. Blood samples were immediately centrifuged, and the plasma was stored at −80°C until being analyzed. Plasma total cholesterol, triglycerides, free fatty acid, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using a Hitachi 7170 autoanalyzer (Tokyo, Japan).

2.3. Liver analysis

Liver lipids were extracted by chloroform and methanol. Total cholesterol, triglyceride and free fatty acid concentrations in the liver were determined using diagnostic kits (Randox Laboratories, Antrim, UK).

To determine MDA levels, the liver was homogenized in the buffer containing 0.25 M sucrose, 1 mM EDTA and 10 mM Tris-HCl. After centrifugation, the supernatants were stored at −80°C. The MDA in the liver was measured by the thiobarbituric acid reactive substance method [13].

To determine cytokine concentrations in the liver, samples were homogenized in buffer containing 50 mM Tris-base, 150 mM NaCl and 1% Triton-X 100. After centrifugation, the supernatants were stored at −80°C. The liver level of tumor necrosis factor (TNF)-α was measured with an enzyme-linked immunosorbent assay kit (ELISA; rat TNF-α/TNFSF1A, R&D Systems, USA). Liver interleukin (IL)-1β and IL-6 levels were measured using an ELISA kit (Rat IL-1β and Rat IL-6, DuoSet ELISA Development System; R&D systems).

To measure MPO activity, the liver was homogenized in buffer containing 0.5% hexadecyltrimethyl ammonium bromide. The homogenate was analyzed according to a previously described method [14]. After centrifugation, the supernatants were stored at −80°C. Hydroxyproline, an indicator of fibrosis, was also measured as previously described [15].

Table 1
Diet composition of different experimental diets

g/L	C	E	EC	EP
Casein	41.4	41.4	41.4	-
L-Cystine	0.5	0.5	0.5	0.5
DL-Methionine	0.3	0.3	0.3	0.3
Corn oil	17.3	17.3	17.3	17.3
Olive oil	28.4	28.4	28.4	28.4
Safflower oil	2.7	2.7	2.7	2.7
Dextrin–maltose	98	13.5	98	98
Choline bitartrate	0.53	0.53	0.53	0.53
Cellulose	10	10	10	10
Xanthan gum	3.0	3.0	3.0	3.0
Vitamin mixture	2.2	2.2	2.2	2.2
Mineral mixture	7.7	7.7	7.7	7.7
Ethanol	-	48.3	-	-
SPI	-	-	-	41.4

SPI, Soy protein isolate. C group: control liquid diet, E group: low-carbohydrate ethanol liquid diet, EC group: ethanol withdrawal control liquid diet, EP group: ethanol withdrawal soy protein isolate liquid diet. Casein (high-N), cellulose (nonnutritive bulk), mineral mixture (AIN-93M), vitamin mixture (AIN-93M), cystine, methionine and choline bitartrate were from the ICN Biochemicals (Aurora, OH, USA). Dextrin was from the Corn Products International, Inc. (Westchester, IL, USA). Corn oil and olive oil were from the God-Bene Co., Ltd. (Yunlin, Taiwan). Safflower oil was from Taiwan Sugar Co. (Taipei, Taiwan). Ethanol was from J.T. Backer. Xanthan gum was from Sigma. Soy protein isolate was from Fuji oil Co., Ltd. (Fujipro, Osaka, Japan).

2.4. Western blotting

2.4.1. CYP2E1 and CYP4A

The liver was homogenized in buffer containing 0.25 M sucrose, 10 mM Tri-HCl and 0.25 mM phenylmethylsulfonyl fluoride (pH 7.4). The homogenate was centrifuged at 10,000*g* for 20 min at 4°C. The supernatant was transferred to a new tube and centrifuged at 105,000*g* for 60 min at 4°C to separate the microsomes. The microsomal pellet was dissolved in 50 mM potassium phosphate buffer containing 1 mM EDTA and 1 mM DTT (pH 7.4).

2.4.2. PPARα

The liver was homogenized in RIPA buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.1% sodium dodecylsulfate (SDS) and 1% NP-40 (pH 7.5) and then placed in an ice bath for 30 min. After centrifugation, the supernatant was stored at −80°C.

Western blotting was performed to evaluate levels of CYP2E1, CYP4A and PPARα using appropriate antibodies, followed by a horseradish peroxidase (HRP)-conjugated secondary antibody.

Samples containing 50 μg protein were separated on a 12.5% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Amersham Biosciences, Little Chalfont, Bucks, UK). Nonspecific binding sites were blocked by overnight incubation of the membranes at 4°C in a 5% nonfat milk solution.

After washing with PBS/Tween-20, membranes were incubated with a mouse CYP2E1 monoclonal antibody (mAb; 1:1000 dilution, Oxford PM32), a rabbit CYP4A polyclonal antibody (pAb; 1:1000 dilution, ABR PA3-033) or a mouse PPARα pAb (1:500 dilution, Santa Cruz, CA, USA) at room temperature for 2, 1 and 2 h, followed by incubation with an HRP-conjugated anti-mouse antibody (1:5000 dilution, Chemicon AP124P) for CYP2E1and PPARα and an anti-rabbit antibody (1:5000 dilution, Amersham) for CYP4A. Membranes were then washed, and the immune complex was developed using a chemiluminescence detection system (ECL, Western lighting, PerkinElmer). Equal loading of the total protein was verified using a commercially available mAb against GAPDH, and the results are expressed as the ratio of protein to GAPDH.

2.5. Fecal cholesterol, bile acid and total lipid levels

Fecal samples were collected for 1 day at 12 and 16 weeks, dried for 48 h and stored at −80°C until being analyzed. Fecal lipids were extracted by chloroform and methanol. Fecal bile acid was extracted by methanol. Fecal neutral steroids and bile acids concentrations were analyzed with commercial kits (Randox Laboratories, Antrim, UK). Total lipids were determined using commercial kits (Fortress BXC0263, UK).

2.6. Histological analysis

The dissected livers of the rats were fixed in formaldehyde. Samples were stained with hematoxylin and eosin. Biopsies were examined by a pathologist on a blinded basis. To evaluate the fatty change, the liver was observed at ×200 magnification with a scale. Degrees of fatty liver were assessed by a pathologist.

2.7. Statistical analysis

Data were analyzed by two-way analysis of variance and Tukey method using the Statistical Analysis System (SAS Institute, Cary, NC, USA). Results are expressed as the mean and S.E.M. A P value of $<.05$ was taken as the level of statistical significance.

3. Results

3.1. Ethanol intake, food intake and body weight

In the ALD-inducing period, the average energy intake of the C group was 81.9±0.4 kcal/day, and that of the E group was 82.8±0.8 kcal/day. There was no difference in the energy intake between these two groups. In the withdrawal period, the average energy of the three groups was as follow: C group (76.0±1.8 kcal/day), EC group (70.8±2.3 kcal/day) and EP group (73.8±1.8 kcal/day). There were no differences in the energy intake among the three groups, and this indicated that soy protein in the diet did not affect the food intake by the rats.

At the end of the ALD-inducing period, the average body weight of the C group was 581±23 g, and that of the E groups was 485±13 g. The body weight in the E groups was significantly lower than that of the C group. However, the average body weights of animals in the C, EC and EP groups did not significantly differ at the end of the withdrawal period.

3.2. Plasma AST, ALT and lipid profiles

The liver function of the rats was determined by the AST and ALT activities. At the end of the ALD-inducing period, the AST and ALT

Table 2
Plasma biochemical analysis of rats at the end of ALD-inducing period and ethanol withdrawal period for 4 weeks

	C	E	
		EC	EP
AST (IU/L)			
0	88.1±2.4[b]	225.9±40.5[a]	
4	121.0±5.7	115.1±4.2 *	128.1±7.3 *
ALT (IU/L)			
0	27.6±1.4[b]	130.0±35.1[a]	
4	33.5±1.8	29.9±1.7 *	31.3±1.2 *
Total cholesterol (mg/dl)			
0	48.0±2.3	55.9±7.0	
4	61.5±3.4[a]	53.9±2.8[ab]	51.8±2.3[b]
Triglycerides (mg/dl)			
0	59.9±5.8[a]	27.3±2.6[b]	
4	64.7±7.1	53.2±6.1 *	56.7±5.7 *
Free fatty acid (mmol/L)			
0	0.74±0.05[a]	0.42±0.03[b]	
4	0.76±0.06	0.71±0.08 *	0.82±0.04 *

Values are presented as the mean±S.E.M. C group: control liquid diet (n=8), E group: low-carbohydrate ethanol liquid diet (n=8), EC: ethanol withdrawal control liquid diet (n=10), EP: ethanol withdrawal soy protein isolate liquid diet (n=10). Values in a row with different letters in superscript ([ab]) mean significant difference at the same time (P<.05).
* Significantly different compared to the E group (P<.05).

activities in the E group were significantly higher than those in the C group. The AST and ALT activities in the EC and EP groups were significantly lower after ethanol withdrawal, and no difference was found among the C, EC and EP groups (Table 2). Plasma total cholesterol concentrations of the C and E groups did not differ at the end of the ALD-inducing period. Plasma triglyceride and free fatty acid concentrations of the E group were significantly lower than those of the C group at the end of the ALD-inducing period, and those of the EC and EP groups were significantly higher than that of the E group at the end of the withdrawal period. No difference was found among the C, EC and EP groups.

Table 3
Liver and feces lipid profiles of at the end of ALD-inducing period and ethanol withdrawal period for 4 weeks

	C	E	
		EC	EP
Liver			
Triglycerides (μmol/liver)			
0	148.7±15.3[b]	407.7±55.5[a]	
4	147.8±8.3[ab]	155.2±17.3[a,*]	120.8±5.3[b,*]
Total cholesterol (μmol/liver)			
0	58.8±6.7[b]	90.7±4.0[a]	
4	75.2±4.4	69.5±3.3 *	66.9±2.2 *
Free fatty acid (mmol/g liver)			
0	8.1±0.2[a]	6.7±0.2[b]	
4	7.6±0.1[b]	8.3±0.1[a,*]	7.4±0.1[b,*]
Feces			
Cholesterol (mg/day)			
0	7.6±0.8	7.2±0.6	
4	14.2±1.8	10.7±2.0	15.8±4.3 *
Bile acid (μmol/day)			
0	2.1±0.4	1.5±0.1	
4	2.9±0.3	2.6±0.7	2.2±0.5
Total lipids (mg/day)			
0	245.8±16.2[a]	155.8±11.4[b]	
4	340.0±34.9[a]	251.4±56.1[b,*]	416.5±45.9[a,*]

Values are presented as the mean±S.E.M. C group: control liquid diet (n=8), E group: low-carbohydrate ethanol liquid diet (n=8), EC: ethanol withdrawal control liquid diet (n=10), EP: ethanol withdrawal soy protein isolate liquid diet (n=10). Values in a row with different letters in superscript ([ab]) mean significant difference at the same time (P<.05).
* Significantly different compared to the E group (P<.05).

3.3. Liver lipid profiles

The liver total cholesterol and triglyceride in the E group had significantly increased compared to the C group by the end of ALD-inducing period. Liver total cholesterol and triglycerides of the EC and EP groups were both lower than those in the E group after ethanol withdrawal. Moreover, liver triglycerides of the EP group were significantly lower than those of the EC group. The liver free fatty acid concentration of the E group was significantly lower than that of the C group at the end of the ALD-inducing period (P<.05). After the withdrawal period, the liver free fatty acids in the EC and EP groups were significantly higher than those in the E group (Table 3).

3.4. Liver MDA levels

The E group had significantly higher MDA levels in the liver compared to the C group at the end of the ALD-inducing period, and only the EP group had significantly lower MDA levels in the liver compared to the EC groups (Fig. 1A).

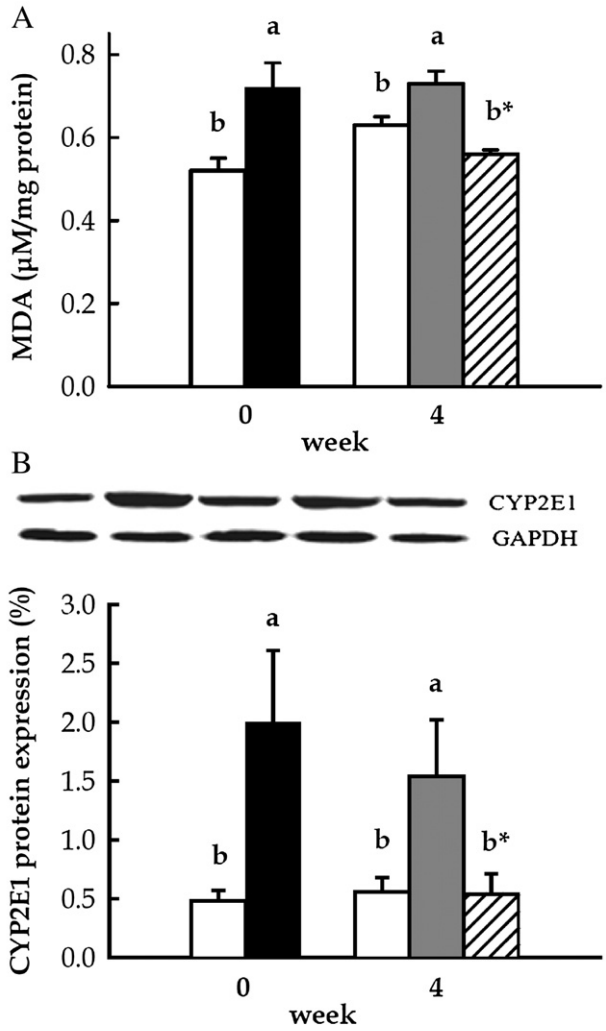

Fig. 1. Liver MDA (A) level and CYP2E1 protein expression (B) of rats at the end of ALD-inducing period and ethanol withdrawal period for 4 weeks. C group: control liquid diet (□, n=8), E group: low-carbohydrate ethanol liquid diet (■, n=8), EC group: ethanol withdrawal control liquid diet (▬, n=10), EP group: ethanol withdrawal soy protein isolate liquid diet (▨, n=10). Values with different letters ([ab]) mean significant difference at the same time (P<.05). *Significantly different compared to the E group (P<.05).

3.5. Liver TNF-α, IL-1β and IL-6 levels

Liver TNF-α, IL-1β and IL-6 levels of the E group were significantly higher than those of the C group at the end of the ALD-inducing period, and those of the EC and EP groups were significantly lower than those of the E group after the ethanol withdrawal period. Furthermore, the liver IL-6 level in the EP group was significantly lower than that in the EC group (Table 4).

3.6. Liver MPO activity and hydroxyproline levels

Liver MPO activity and the hydroxyproline level significantly increased in the E group after the ALD-inducing period ($P<.05$). After the ethanol withdrawal period, MPO activity and the hydroxyproline level in the EP group were significantly lower than those in the E and EC groups, and did not differ from those of the C group (Table 4).

3.7. CYP2E1, CYP4A and PPARα protein expression

The E group had significantly higher CYP2E1 protein expression in the liver compared to the C group at the end of the ALD-inducing period. The EP group had lower CYP2E1 protein expression in the liver compared to the E and EC groups, and there was no difference compared to the C group at the end of the withdrawal period (Fig. 1B).

The PPARα and CYP4A protein expressions in the E group were also lower than those in the C group at the end of the ALD-inducing period. After ethanol withdrawal for 4 weeks, the EC and EP groups had significantly higher PPARα and CYP4A protein expressions in the liver compared to those of the E group, and those protein expressions in the EP group were higher than those in the EC group and did not differ from those in the C group (Fig. 2).

3.8. Fecal cholesterol, bile acid and total lipids levels

At the end of the ALD-inducing period, fecal cholesterol excretion of the E group did not differ from that of the C group, but only the EP diet significantly increased fecal cholesterol excretion compared to the E group after ethanol withdrawal. However, fecal bile acid excretion showed no significant difference among all groups. The fecal total lipid concentration of the E group was significantly lower than that of the C group at the end of the ALD-inducing period, and they were higher in the EC and EP groups compared to the E group after the withdrawal period. Moreover, the EP group had significantly higher fecal total lipids excretion than the EC group.

Table 4
Liver TNF-α, IL-1β, IL-6, MPO and hydroxyproline levels of rats at the end of ALD-inducing period and ethanol withdrawal period for 4 weeks

	C	E	
		EC	EP
TNF-α (pg/mg protein)			
0	25.4 ± 1.4^{b}	34.3 ± 3.4^{a}	
4	25.0 ± 2.1^{a}	$24.1\pm1.2^{ab,*}$	$19.7\pm1.6^{b,*}$
IL-1β (pg/mg protein)			
0	78.7 ± 3.7^{b}	136.6 ± 13.2^{a}	
4	75.3 ± 3.3	$80.4\pm4.3^{*}$	$72.0\pm3.5^{*}$
IL-6 (pg/mg protein)			
0	12.4 ± 1.2^{b}	18.9 ± 1.9^{a}	
4	13.3 ± 1.5^{a}	$13.0\pm0.6^{a,*}$	$8.8\pm0.6^{b,*}$
MPO (U/mg protein)			
0	0.44 ± 0.05^{b}	2.14 ± 0.70^{a}	
4	0.57 ± 0.18^{b}	1.85 ± 0.22^{a}	$0.54\pm0.14^{b,*}$
Hydroxyproline (μg/ mg protein)			
0	3.7 ± 0.2^{b}	5.0 ± 0.3^{a}	
4	2.6 ± 0.2^{b}	$3.5\pm0.2^{a,*}$	$2.4\pm0.1^{b,*}$

Values are presented as the mean±S.E.M. C group: control liquid diet ($n=8$), E group: low-carbohydrate ethanol liquid diet ($n=8$), EC: ethanol withdrawal control liquid diet ($n=10$), EP: ethanol withdrawal soy protein isolate liquid diet ($n=10$). Values in a row with different letters in superscript (^{ab}) mean significant difference at the same time ($P<.05$).

* Significantly different compared to the E group ($P<.05$).

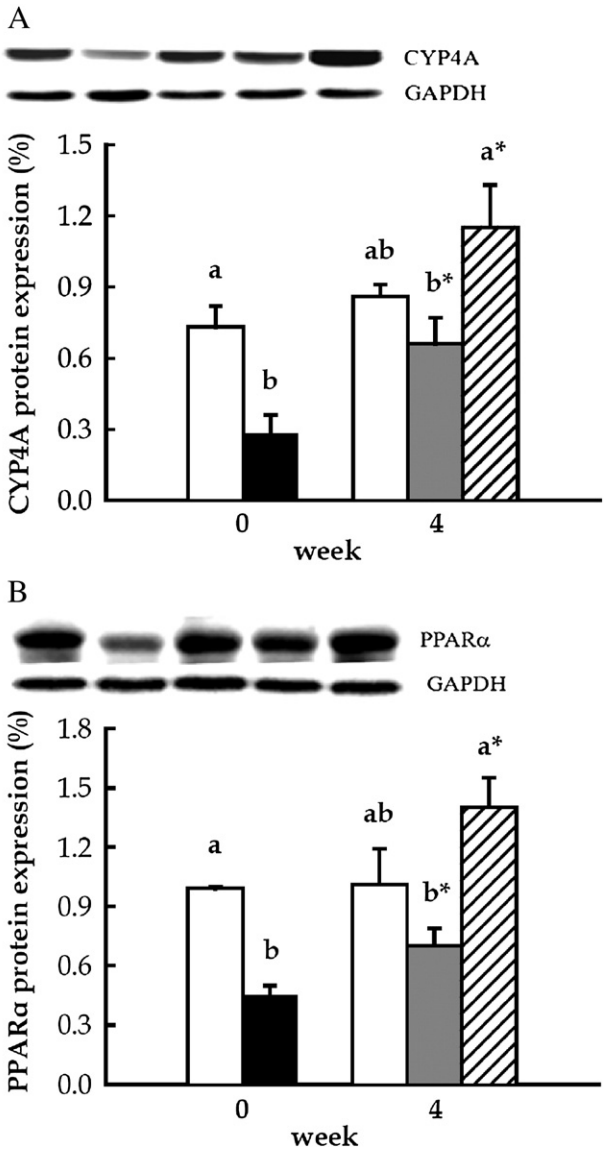

Fig. 2. Hepatic CYP4A (A) and PPARα protein expression (B) of rats at the end of ALD-inducing period and ethanol withdrawal period for 4 weeks. C group: control liquid diet (□, $n=8$), E group: low-carbohydrate ethanol liquid diet (■, $n=8$), EC group: ethanol withdrawal control liquid diet (■, $n=10$), EP group: ethanol withdrawal soy protein isolate liquid diet (▨, $n=10$). Values with different letters (^{ab}) mean significant difference at the same time ($P<.05$). * Significantly different compared to the E group ($P<.05$).

3.9. Histological analysis of the liver

Rats treated with ethanol were found to have fat accumulation in the liver (Fig. 3A, B). We also found that soy protein ameliorated ethanol-induced steatosis (Fig. 3 C, D) and that the EP group showed better effects than did the EC group.

4. Discussion

We induced ALD in rats with a low-carbohydrate ethanol liquid diet to investigate the effects of dietary soy protein during the withdrawal period. After we fed rats the liquid diet for 12 weeks, we found

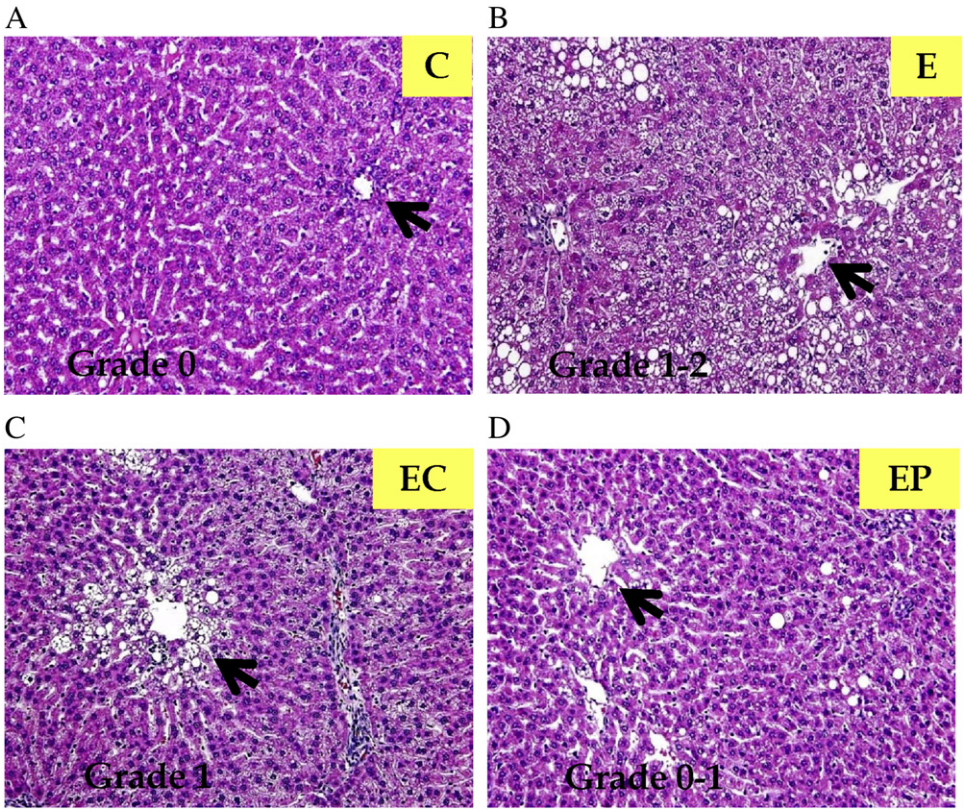

Fig. 3. Fatty change at the central vein zone in liver of rats at the end of ALD-inducing period and ethanol withdrawal period for 4 weeks. C: control liquid diet group (n=10), E: low carbohydrate ethanol liquid diet group (n=8), EC: ethanol withdrawal-control liquid diet group (n=10), EP: ethanol withdrawal-soy protein isolate liquid diet group (n=10), ⬆: Central vein (H&E stain ×200).

significant elevation of fat accumulation in the liver and reductions of plasma triglyceride and free fatty acid concentrations. Triglycerides can be carried from the liver to other peripheral tissues by very-low-density lipoprotein (VLDL). However, ethanol may inhibit the secretion of VLDL, thus suppressing the transportation of triglycerides and release of free fatty acids from the lipoprotein. Alcohol consumption also promotes liver uptake of circulatory lipids and hepatic triglyceride synthesis, and decreases fatty acid oxidation [16]. These may explain the changes in lipid profiles in the plasma and liver in our study.

Steatosis is a common pathological change in the early stage of ALD. In the present study, PPARα and CYP4A protein expressions were lower in the E group than the C group. Both acute and chronic ethanol feeding leads to impairment of β-oxidation by liver mitochondria and an increase in triglyceride synthesis; other pathways will be needed to metabolize the lipids [17]. PPARα is the main transcriptional factor for regulating hepatic lipid metabolism, such as fatty acid oxidation and transportation, and it promotes the activity of peroxisomes to increase fatty acid oxidation when fat overaccumulates in the liver [18]. Ethanol inhibits PPARα mRNA and protein expressions, and activation of PPARα improves ethanol-induced steatosis, inflammation and necrosis of the liver [19]. In PPARα-null mice, ethanol also induced steatosis and mitochondrial impairment [5]. Aldehyde produced from the metabolism of ethanol may also inhibit the expression of hepatic CYP4A [20], one of the target genes of PPARα, decrease fatty acid oxidation and lead to fatty liver [7]. In our study, soy protein consumption was more effective in normalizing PPARα and CYP4A expressions and the fatty liver than simply undergoing alcohol withdrawal. Soy protein was found to activate PPARα expression and ameliorate lipotoxicity in obese rats through the combined increases of PPARα and PPRE promoter response elements and CYP4A and acyl CoA oxidase expressions [21,22]. In addition, CYP2E1 can also affect PPARα. PPARα and its target gene expression increased, while CYP2E1 gene expression was suppressed [23]. These results suggested that soy protein's suppression of CYP2E1 expression may increase fatty acid oxidation and improve the lipid profile and steatosis via activation of PPARα and CYP4A.

Ethanol intake not only led to elevation of cholesterol and triglycerides levels in the liver but also resulted in a reduction in lipid excretion. Soy protein consumption during the withdrawal period helped to increase lipid excretion. Soy protein inhibits pancreatic lipase activity to reduce lipid absorption [24]. Recent studies indicated that soy protein acts similarly to dietary fiber to increase the excretion of fecal steroids and bile acids [25] and reduces fat accumulation in the liver [26]. Soy protein also activates cholesterol 7α-hydroxylase to promote cholesterol metabolism [27] and hepatic steroid synthesis [28]. We also found a decrease in the hepatic TNF-α concentration in rats that consumed the soy-protein-containing diet after the withdrawal period. TNF-α may be another factor in steatosis [29]. TNF-α induces the expression of the liver sterol-regulatory element-binding protein, which is involved in the synthesis of triglycerides and cholesterol [30]. In addition, TNF-α indirectly decreases PPARα expression through inhibiting AMP-activated protein kinase and results in a fatty liver [31]. These results suggest that soy protein may have beneficial effects on alcohol withdrawal by ameliorating liver injury via promoting lipid metabolism and excretion.

Oxidative stress plays an important role in ALD, especially CYP2E1-induced oxidative stress [32]. We found increases in both CYP2E1 expression and the MDA concentration in the livers of the ethanol-consuming group after the induction period. Metabolism of ethanol by the MEOS can activate CYP2E1 with NADPH oxidase activity and produce free radicals such as H_2O_2, O_2^- and $OH^\cdot$, which lead to lipid peroxidation and further hepatic damage [2]. In our study, soy protein

consumption during the withdrawal period helped normalize CYP2E1 expression compared to the casein group. A previous animal study showed that rats fed a soy protein diet had a higher antioxidative capacity and lower lipid peroxidation metabolites [33]. Soy isoflavones also reduces the production of plasma and tissue lipid peroxidation [34], and H_2O_2-induced DNA damage *in vivo* [35]. Soy isoflavones are heterocyclic phenols that can bind to free radicals [36], and their estrogen-like structures may help regulate extracellular regulated kinase 1/2 and the nuclear factor (NF)-κB signal transduction pathway to increase antioxidative enzyme activities [37].

Ethanol-induced oxidative stress damages the intestinal barrier and causes bacterial translocation, thus stimulating the secretion of cytokines (TNF-α, IL-6 and IL-1β) and free radicals, which results in liver inflammation [38]. Then, neutrophils enter the liver and activate MPO, which produces more free radicals that injure hepatic tissues [39]. We found that soy protein consumption during alcohol withdrawal can reduce MPO activities, cytokines and hydroxyproline levels, and pathological improvement was seen. A PPARα deficiency may prolong the inflammatory response in mice [40]. PPARα activation may modulate the repression of NF-κB signaling and reduce inflammatory cytokine production [41]. Therefore, soy protein may also diminish liver inflammation by normalizing PPARα expression in ALD.

Reports about the effects of soy protein on ethanol-induced hepatic injuries are very few, and thus we designed the study to clarify whether soy protein can exhibit beneficial effects. In our study, we found that consuming soy protein during alcohol withdrawal reduced liver fat accumulation and cytokine and MDA concentrations and modulated CYP2E1, CYP4A and PPARα expressions. Therefore, we concluded that use of soy protein as a dietary protein source during alcohol withdrawal may be beneficial in reducing oxidative stress, inflammation and steatosis in ALD. Effects of different dosage of soy protein intervention and underlying mechanisms will need to be clarified in future studies.

References

[1] Albano E. Oxidative mechanisms in the pathogenesis of alcoholic liver disease. Mol Aspects Med 2008;29:9–16.
[2] Nagata K, Suzuki H, Sakaguchi S. Common pathogenic mechanism in development progression of liver injury caused by non-alcoholic or alcoholic steatohepatitis. J Toxicol Sci 2007;32:453–68.
[3] Lieber CS. Ethanol metabolism, cirrhosis and alcoholism. Clin Chim Acta 1997;257: 59–84.
[4] Ringseis R, Muschick A, Eder K. Dietary oxidized fat prevents ethanol-induced triacylglycerol accumulation and increases expression of PPARalpha target genes in rat liver. J Nutr 2007;137:77–83.
[5] Nakajima T, Kamijo Y, Tanaka N, Sugiyama E, Tanaka E, Kiyosawa K, et al. Peroxisome proliferator-activated receptor alpha protects against alcohol-induced liver damage. Hepatology 2004;40:972–80.
[6] Fischer M, You M, Matsumoto M, Crabb DW. Peroxisome proliferator-activated receptor alpha (PPARalpha) agonist treatment reverses PPARalpha dysfunction and abnormalities in hepatic lipid metabolism in ethanol-fed mice. J Biol Chem 2003;278:27997–8004.
[7] Purohit V, Gao B, Song BJ. Molecular mechanisms of alcoholic fatty liver. Alcohol Clin Exp Res 2009;33:191–205.
[8] Wheeler MD. Endotoxin and Kupffer cell activation in alcoholic liver disease. Alcohol Res Health 2003;27:300–6.
[9] Ibanez P, Solis N, Pizarro M, Aguayo G, Duarte I, Miquel JF, et al. Effect of losartan on early liver fibrosis development in a rat model of nonalcoholic steatohepatitis. J Gastroenterol Hepatol 2007;22:846–51.
[10] Kurahashi N, Iwasaki M, Sasazuki S, Otani T, Inoue M, Tsugane S. Soy product and isoflavone consumption in relation to prostate cancer in Japanese men. Cancer Epidemiol Biomarkers Prev 2007;16:538–45.
[11] Anderson JW, Johnstone BM, Cook-Newell ME. Meta-analysis of the effects of soy protein intake on serum lipids. N Engl J Med 1995;333:276–82.
[12] Lindros KO, Jarvelainen HA. A new oral low-carbohydrate alcohol liquid diet producing liver lesions: a preliminary account. Alcohol Alcohol 1998;33:347–53.
[13] Griesmacher A, Kindhauser M, Andert SE, Schreiner W, Toma C, Knoebl P, et al. Enhanced serum levels of thiobarbituric-acid-reactive substances in diabetes mellitus. Am J Med 1995;98:469–75.
[14] Bradley PP, Priebat DA, Christensen RD, Rothstein G. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. J Invest Dermatol 1982;78:206–9.
[15] Reddy GK, Enwemeka CS. A simplified method for the analysis of hydroxyproline in biological tissues. Clin Biochem 1996;29:225–9.
[16] Lieber CS. Metabolism of alcohol. Clin Liver Dis 2005;9:1–35.
[17] Fromenty B, Pessayre D. Inhibition of mitochondrial beta-oxidation as a mechanism of hepatotoxicity. Pharmacol Ther 1995;67:101–54.
[18] Mello T, Polvani S, Galli A. Peroxisome proliferator-activated receptor and retinoic x receptor in alcoholic liver disease. PPAR Res 2009;2009:748174.
[19] Nanji AA, Dannenberg AJ, Jokelainen K, Bass NM. Alcoholic liver injury in the rat is associated with reduced expression of peroxisome proliferator-alpha (PPARalpha)-regulated genes and is ameliorated by PPARalpha activation. J Pharmacol Exp Ther 2004;310:417–24.
[20] Cowpland C, Su GM, Murray M, Puddey IB, Croft KD. Effect of alcohol on cytochrome p450 arachidonic acid metabolism and blood pressure in rats and its modulation by red wine polyphenolics. Clin Exp Pharmacol Physiol 2006;33:183–8.
[21] Ronis MJ, Chen Y, Badeaux J, Badger TM. Dietary soy protein isolate attenuates metabolic syndrome in rats via effects on PPAR, LXR, and SREBP signaling. J Nutr 2009;139:1431–8.
[22] Badger TM, Ronis MJ, Wolff G, Stanley S, Ferguson M, Shankar K, et al. Soy protein isolate reduces hepatosteatosis in yellow Avy/a mice without altering coat color phenotype. Exp Biol Med (Maywood) 2008;233:1242–54.
[23] Wan YY, Cai Y, Li J, Yuan Q, French B, Gonzalez FJ, et al. Regulation of peroxisome proliferator activated receptor alpha-mediated pathways in alcohol fed cytochrome P450 2E1 deficient mice. Hepatol Res 2001;19:117–30.
[24] Roy DM, Schneeman BO. Effect of soy protein, casein and trypsin inhibitor on cholesterol, bile acids and pancreatic enzymes in mice. J Nutr 1981;111:878–85.
[25] Chen JR, Chiou SF, Suetsuna K, Yang HY, Yang SC. Lipid metabolism in hypercholesterolemic rats affected by feeding cholesterol-free diets containing different amounts of non-dialyzed soybean protein fraction. Nutrition 2003;19: 676–80.
[26] Vijaimohan K, Jainu M, Sabitha KE, Subramaniyam S, Anandhan C, Shyamala Devi CS. Beneficial effects of alpha linolenic acid rich flaxseed oil on growth performance and hepatic cholesterol metabolism in high fat diet fed rats. Life Sci 2006;79:448–54.
[27] Lin Y, Meijer GW, Vermeer MA, Trautwein EA. Soy protein enhances the cholesterol-lowering effect of plant sterol esters in cholesterol-fed hamsters. J Nutr 2004;134:143–8.
[28] Tovar AR, Murguia F, Cruz C, Hernandez-Pando R, Aguilar-Salinas CA, Pedraza-Chaverri J, et al. A soy protein diet alters hepatic lipid metabolism gene expression and reduces serum lipids and renal fibrogenic cytokines in rats with chronic nephrotic syndrome. J Nutr 2002;132:2562–9.
[29] Bode C, Bode JC. Activation of the innate immune system and alcoholic liver disease: effects of ethanol per se or enhanced intestinal translocation of bacterial toxins induced by ethanol? Alcohol Clin Exp Res 2005;29:166S–71S.
[30] Endo M, Masaki T, Seike M, Yoshimatsu H. TNF-alpha induces hepatic steatosis in mice by enhancing gene expression of sterol regulatory element binding protein-1c (SREBP-1c). Exp Biol Med (Maywood) 2007;232:614–21.
[31] Tsukamoto H. Conceptual importance of identifying alcoholic liver disease as a lifestyle disease. J Gastroenterol 2007;42:603–9.
[32] Bradford BU, Kono H, Isayama F, Kosyk O, Wheeler MD, Akiyama TE, et al. Cytochrome P450 CYP2E1, but not nicotinamide adenine dinucleotide phosphate oxidase, is required for ethanol-induced oxidative DNA damage in rodent liver. Hepatology 2005;41:336–44.
[33] Takenaka A, Annaka H, Kimura Y, Aoki H, Igarashi K. Reduction of paraquat-induced oxidative stress in rats by dietary soy peptide. Biosci Biotechnol Biochem 2003;67:278–83.
[34] Yousef MI, Kamel KI, Esmail AM, Baghdadi HH. Antioxidant activities and lipid lowering effects of isoflavone in male rabbits. Food Chem Toxicol 2004;42:1497–503.
[35] Sierens J, Hartley JA, Campbell MJ, Leathem AJ, Woodside JV. Effect of phytoestrogen and antioxidant supplementation on oxidative DNA damage assessed using the comet assay. Mutat Res 2001;485:169–76.
[36] Tikkanen MJ, Wahala K, Ojala S, Vihma V, Adlercreutz H. Effect of soybean phytoestrogen intake on low density lipoprotein oxidation resistance. Proc Natl Acad Sci U S A 1998;95:3106–10.
[37] Borras C, Gambini J, Gomez-Cabrera MC, Sastre J, Pallardo FV, Mann GE, et al. Genistein, a soy isoflavone, up-regulates expression of antioxidant genes: involvement of estrogen receptors, ERK1/2, and NFkappaB. FASEB J 2006;20:2136–8.
[38] Keshavarzian A, Farhadi A, Forsyth CB, Rangan J, Jakate S, Shaikh M, et al. Evidence that chronic alcohol exposure promotes intestinal oxidative stress, intestinal hyperpermeability and endotoxemia prior to development of alcoholic steatohepatitis in rats. J Hepatol 2009;50:538–47.
[39] Omurtag GZ, Guranlioglu FD, Sehirli O, Arbak S, Uslu B, Gedik N, et al. Protective effect of aqueous garlic extract against naphthalene-induced oxidative stress in mice. J Pharm Pharmacol 2005;57:623–30.
[40] Devchand PR, Keller H, Peters JM, Vazquez M, Gonzalez FJ, Wahli W. The PPARalpha-leukotriene B4 pathway to inflammation control. Nature 1996;384:39–43.
[41] Poynter ME, Daynes RA. Peroxisome proliferator-activated receptor alpha activation modulates cellular redox status, represses nuclear factor-kappaB signaling, and reduces inflammatory cytokine production in aging. J Biol Chem 1998;273:32833–41.