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EXTRACT OF TRADITIONAL PICKING MELON PREVENTS ETHANOL-INDUCED LIVER INJURY IN RATS

Akiko Kojima-Yuasa[1]*, Eri Hirauchi[1], Takuya Sawada[1], Ayano Kasahara[1], Eri Yoshikawa[1], Akiko Tamura[1] and Isao Matsui-Yuasa[1]

*Corresponding Author: Akiko Kojima-Yuasa, ✉ kojima@life.osaka-cu.ac.jp

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Traditional vegetables have rarely been subjected to plant breeding for the selection of new trials and thus have retained their original components. Therefore, it is interesting to examine the effect of extracts of traditional vegetables on protection against disease. Here we examined the effect of an extract of *tamatsukurikuromon-sirouri* (ETS), a traditional vegetable in Japan, on ethanol-induced liver injury. In animal experiments, the plasma aminotransferases (AST and ALT) induced with ethanol plus CCl_4 were significantly decreased by treatment with a 0.3% ETS diet. Furthermore, histological analysis showed that hepatic necrosis and collagen accumulation in the liver were observed in the ethanol-plus-CCl_4-treated rats but ETS supplementation fully protected them. In cultured hepatocytes, treatment with ETS suppressed ethanol-induced increases in cell death via inhibiting CYP2E1 and reactive oxygen species and enhancing the levels of phosphorylated AMPK. These results suggest that ETS, an extract of traditional pickling melon, protects against liver injury.

Keywords: Traditional vegetable, Ethanol-induced liver injury, CYP2E1, AMPK, Hepatocytes, Amino transferase

INTRODUCTION

Chronic alcohol abuse is a significant cause of cirrhosis and liver failure in adults worldwide. Alcoholic liver disease is a pathological process characterized by progressive liver damage that leads to steatosis, steatohepatitis, fibrosis, and finally cirrhosis. Eventually, cirrhosis may progress to hepatic decompensation and hepatocellular cancer (Lieber, 1997; Lucey *et al.*, 2009; and Tsukamoto and Lu, 2011). Oxidative stress plays an important role in this process (Zima *et al.*, 2001; and Albano, 2006). Alcohol-induced oxidative stress is associated with the metabolism of ethanol. Ethanol metabolism occurs primarily in the liver, which sustains the greatest amount of organ damage from excessive drinking (Lieber, 1994; and Lieber, 2004). Alcohol dehydrogenase is the major enzyme responsible for oxidizing ethanol to aldehyde. Heavy ingestion of ethanol induces cytochrome p450 2E1 (CYP2E1) in hepatocytes. This enzyme complements the activity of constitutively expressed alcohol dehydrogenase in oxidizing ethanol to acetaldehyde (Lieber and De Carli, 1968). However, CYP2E1 is also an effective generator of Reactive Oxygen Species (ROS) (Guengerich, 1987; and Porter and Coon, 1991). Ethanol-induced oxidative stress appears to play a major role in ethanol-induced liver injury (Lieber and De Carli, 1968; and Guengerich, 1987). Therefore, possible strategies for preventing this stress may be effective in attempts to minimize the hepatotoxicity of ethanol in humans.

Animal models of liver fibrosis are important for research into the underlying mechanisms of the treatments employed for this disease. Currently, two models for the administration

[1] Department of Food and Human Health Sciences, Graduate School of Human Life Science, Osaka City University, Osaka, 558-8585, Japan.

of alcohol to animals have been developed: theLieber-De Carli liquid diet (Lieber *et al.*, 1989) and the Tsukamoto-French gastric model (Tukamoto *et al.*, 1984). In the Lieber-De Carli liquid diet, ethanol replaces the carbohydrates of a normal diet. Tsukamoto and French developed an in vivo animal model in which enteral ethanol is continuously administered via intragastric infusion. However, neither the Lieber-De Caarli nor the Tsukamoto-French feeding protocols result in cirrhosis in rats. Furthermore, Tipoeet al. combined these two diets and showed that an increase in profibrogenic mediators did not equate with the presence of fibrosis in histological evidence. On the other hand, experimental fibrosis was induced in rats within 4 weeks by the administration of low-dose carbon tetrachloride (CCl_4) and a 5% ethanol solution. The hepatic histological changes in the ethanol-CCl_4-induced fibrosis model were similar to those found in human alcoholic cirrhosis (Siegers *et al.*, 1986; Takahashi *et al.*, 2012; and Tamura *et al.*, 2013).

Melon (*Cucumismelo* L.) is one of the most important vegetables cultivated in tropical and temperate regions, and it has great variation in morphological and physiological characteristics. On the other hand, most vegetable crops have undergone genetic adaptation due to selective breeding for higher yield, fewer days to harvest, and disease resistance (Argyris *et al.*, 2015). Consequently, valuable components and tastes have been lost compared with the original vegetables (Kagawa, 1996). In contrast traditional vegetables have rarely been subjected to plant breeding for the selection of new traits and thus have retained their original components. Tamatsukurikuromonn-sirouri (TS), a kind of Japanese pickling melon (*Cucumismelo*var.*conomon; tamatsukurikoromon*), is a traditional vegetable in Osaka, Japan. It is also reported that *katsura-uri*, a kind of Japanese pickling melon and a traditional vegetable in Kyoto, Japan, has antimutagenic, differentiation-inducing, and antioxidative effects (Nakamura *et al.*, 2008). Therefore, it is interesting to examine the effect of extracts of traditional vegetables on protection against disease.

In this study, we examined the effect of extract of tamatsukurikuromon-sirouri (ETS) on the development of liver fibrosis in an in vitro alcohol-injury model in hepatocytes and an in vivo ethanol-CCl_4–induced cirrhosis model.

MATERIALS AND METHODS

Fetal bovine serum was purchased from Nichirei Biosciences, Inc. (Tokyo, Japan). Biotinylated goat anti-mouse immunoglobulin, biotinylated goat anti-rabbit immunoglobulin, biotinylated goat anti-goat immunoglobulin, and peroxidase-conjugated streptavidin antibodies were obtained from DAKO A/S (Glostrup, Denmark). Rabbit anti-cytochrome P450 2E1 polyclonal antibodies were obtained from Enzo Life Science, Inc. (Farmingdale, NY, USA). Anti-phospho-AMPK (pAMPK) and anti-AMPK were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). Rabbit anti-human â-tubulin antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The other chemicals used in this study were special-grade commercial products purchased from WAKO Pure Chemical Co., LTD. (Osaka, Japan).

Preparation of ETS or Extract of Sirouri (ESU)

Tamatsukurikuromon-sirouri (TS) (*Cucumismelo*L. var.*conomon;tamatsukuriKuromon*) was supplied directly from farmers at Kanan-cho, Osaka. Sirouri (SU), *Cucumismelo*L. var. *conomon*) is a popular pickled cucumber and was obtained commercially at the market. TS or SU were separated into edible portions, and the peel and seeds of each portion were frozen and dried. The powder of the portions was extracted in 20 vol of 50% ethanol for 2 h, and the extracts were frozen and dried.

Animals

Male Wistar rats were purchased from Japan SLC Inc. (Shizuoka, Japan). The rats were housed at a constant temperature and were allowed free access to water and standard rat chow (Labo MR stock, Japan SLC, Inc.). Animal experiments followed our institution's criteria for the care and use of laboratory animals in research, which are in accordance with the guidelines for animal experimentation at Osaka City University.

Animal Experiments

Male Wistar rats weighing 180-210 g were fed a standard laboratory diet and water ad libitum until 3 days prior to the experiment. The rats were fed a control diet for 3 days and were divided into 5 groups. Group 1 was the control, Group 2 was treated with ethanol plus CCl_4, Group 3 was treated with CCl_4 alone, Group 4 was treated with ethanol, CCl_4, and 0.3% ESU, and Group 5 was treated with ethanol, CCl_4, and 0.3% ETS. The composition of the diets is presented in Table 1. CCl_4 (0.1 ml/kg of body weight diluted with olive oil to 25%) was administered by intraperitoneal injection twice

Table 1: Composition of Diets

Components (g)	Control	0.3% ESU	0.3% ETS
Casein	200	200	200
L-Cystine	3	3	3
Cornstarch	397.486	394.486	394.486
α-Cornstarch	132	132	132
Sucrose	100	100	100
Soybean oil	70	70	70
Cellulose powder	50	50	50
Mineral mix (AIN-93G-MX)[1]	35	35	35
Vitamin mix (AIN-93VX)[2]	10	10	10
Choline hydrogen tartrate	2.5	2.5	2.5
t-Butylhydroquinone	0.014	0.014	0.014
ESU	0	3	0
ETS	0	0	3
Total	1000	1000	1000

Note: [1] Composition in g/kg diet: calcium carbonate, 357; potassium phosphate, monobasic, 196; potassium citrate?H2O, 70.78; sodium chloride, 74; potassium sulfate, 46.6; magnesium oxide, 24; ferric citrate, 6.06; zinc carbonate, 1.65; manganese carbonate, 0.63; cupric carbonate, 0.324; potassium iodate, 0.01; sodium selenate, 0.01025; chromium K sulfate o $12H_2O$, 0.275; ammonium molybdate o $4H_2O$, 0.00795; sodium silicate o $9H_2O$, 1.45; lithium chloride, 0.0174; boric acid, 0.0815; sodium fluoride, 0.0635; nickel carbonate o $4H_2O$, 0.0306; ammonium vanadate, 0.0066; sucrose, 221.0032. [2] Composition in g/kg diet:vitamin A acetate (500,000 IU/g), 0.8; vitamin D3 (400,000 IU/g), 0.25; vitamin E acetate (500 IU/g), 15; phylloquinone, 0.075; biotin, 2; cyanocobalamin, 2.5; folic acid, 0.2; nicotinic acid, 3; calcium pantothenate, 1.6; pyridoxine-HCl, 0.7; riboflavin, 0.6; thiamin HCl, 0.6; sucrose, 974.655.

a week (on Monday and Thursday), and 5% ethanol in the drinking water was administered ad libitum. The rats were euthanized at 3 weeks.

Histological analysis

Liver samples were collected from each rat, fixed in 10% buffered formalin fixative and then dehydrated in a graded alcohol series. Following xylene treatment, the specimens were embedded in paraffin blocks and cut into 5 μm sections. Consecutive sections were stained with hematoxylin and eosin or Weigerts elastic van Gieson (EVG) stain. The pathologist was blinded to the rats group designations.

Liver Damage Biomarkers

The activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in plasma were estimated using a transaminase CII-test kit (Wako, Japan).

Hepatocyte Preparation and Culture

Hepatocytes were isolated by collagenase perfusion following their removal from 10-week-old male Wistar rats anesthetized with sodium pentobarbital (Moldeus *et al.*, 1978). The viability of the isolated hepatocytes was greater than 90%, as determined by 0.2% trypan blue exclusion. The cells were plated on 35 mm plastic dishes at a density of 2.5×10^5 cells/ml in 2 ml of Williams' Medium E supplemented with 10% Fetal Bovine Serum (FBS). The cells were cultured in a humidified atmosphere (5% CO_2/95% air) at 37 °C overnight. After preincubation, the cells were cultured in 10% FBS containing fresh Williams' Medium E with 100 mM of ethanol with or without samples in DMSO for 0-24 h.

Cell Viability

The cell viability of the hepatocytes was measured by a neutral red assay as described previously (Zhang *et al.*, 1990). Neutral red stock solution (0.4% neutral red in water) was diluted 1:80 in Phosphate-Buffered Saline (PBS). Hepatocytes were incubated with the neutral red solution for 2 h at 37 °C to allow the uptake of the lysosomal dye into viable cells. The neutral red solution was then removed, and the cultures were washed rapidly (for less than 2.5 min) with a mixture of 1% formaldehyde and 1% calcium chloride. A mixture of 1% acetic acid and 50% ethanol was added to the cells at room temperature for 30 min to extract the neutral red from the hepatocytes. The optical density of each sample was then measured at 540 nm with a spectrophotometer. Cell viability was estimated as a percentage of the value obtained for untreated controls.

Intracellular ROS Formation

A relatively specific probe for hydrogen peroxide, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), was used to analyze the formation of intracellular ROS (Royall and Ischiropoulos, 1990; and Thannickal and Fanburg, 2000). Cells were incubated with 2.4 mM of DCFH-DA (5 μl) for the final 30 min of the treatment. After the cells were washed twice with PBS, the dish was covered with a coverslip. Images were obtained using FSX 100 (Olympus, Tokyo, Japan).

Western Blot Analysis

CYP2E1 expression was assessed by Western blotting in cell lysates or homogenate of liver samples. Livers were

homogenized with 9 volumes of 50 mM of Tris-HCl (containing 0.1 mM of EDTA·2Na and 0.2% SDS, pH 7.4). Homogenates were centrifuged at 2000 × *g* for 10 min at 4 °C, and the supernatant was collected. The supernatant was mixed with lysis buffer Y (150 mM of NaCl, 50 mM of Tris (pH 7.2), 1 mM of EDTA, 1% Nonidet P40, 10 µg/ml of leupeptin, 10 µg/ml of pepstatin A, and 100 µg/ml of phenylmethylsulfonyl fluoride). Equal amounts of protein were loaded into each lane of a 10% SDS-PAGE gel. The separated proteins were blotted onto 0.45 µm of polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Inc., Uppsala, Sweden). After blocking overnight with 0.1% Tween-20 and 5% nonfat dry milk in Tris Buffered Saline (TBS), the membrane was incubated with an antibody for 1 h at room temperature. The membrane was stained with anti-AMPK and anti-phosphorylated AMPK. After washing, the membrane was incubated with biotinylated immunoglobulin antibody (diluted 1:1000) for 1 h at room temperature. The membrane was washed several times and incubated with horseradish peroxidase–coupled streptavidin (1:1000) for 1 h at room temperature. After several washing steps, the color reaction was developed with 3,3'-diaminobenzidine tetrahydrochloride. A densitometric analysis of the protein bands was performed using the software Scion Image (Scion Corporation, Frederick, MD, USA).

Statistical Analysis

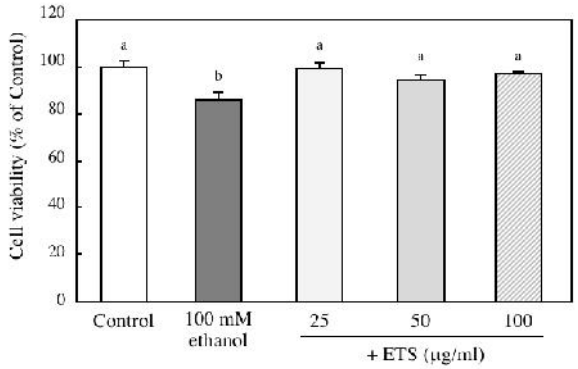
Statistical comparisons were performed between groups by one-way analysis of variance and post hoc multiple comparisons using Tukey's test. A *p*-value of less than 0.05 was considered significant.

RESULTS

The Effect of ETS on Cell Viability of Ethanol-Treated Hepatocytes

We demonstrated previously that the cell viability of ethanol-treated hepatocytes decreased significantly at concentrations greater than 100 mM of ethanol, which is a serum ethanol concentration in heavy drinkers (Takahashi *et al.*, 2012). Therefore, we measured the cell viability of hepatocytes treated with 100 mM of ethanol with or without various concentrations of extracts of edible portion in ETS. As shown in Figure 1, doses of 25 µg/ml of extract of edible portions in ETS significantly prevented cell death in hepatocytes treated with ethanol.

Figure 1: The Effect of ETS on Cell Viability of Ethanol-Treated Hepatocytes

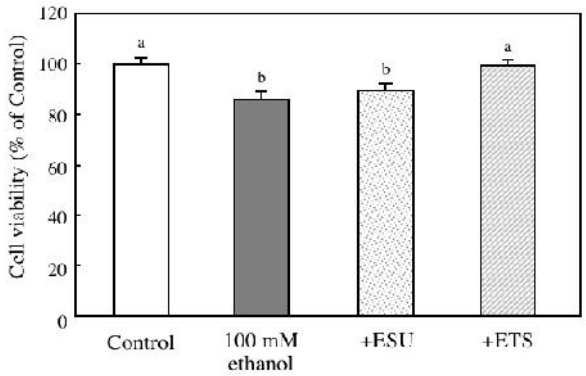


Note: Hepatocytes were incubated with 100 mM of ethanol with or without various ETS concentrations. Cell viability was measured by the neutral red assay, as described in the Materials and Methods section. Data are presented as the mean ± SD of 5 experiments. Values without a common letter are significantly different ($p < 0.01$).

The Specificity of ETS in the Protection on the Cell Viability of Ethanol-Treated Hepatocytes

To know whether the effect of ETS on ethanol-treated hepatocytes is specific, we compared the effect of ETS with that of ESU, an oriental pickling melon (*Cucumismelo.* var. *conomon, utissimus*). As shown in Figure 2, the protective effects were not observed in extracts of edible portions in ESU.

Figure 2: Specificity of ETS in the Protection on Cell Viability of Ethanol-Treated Hepatocytes

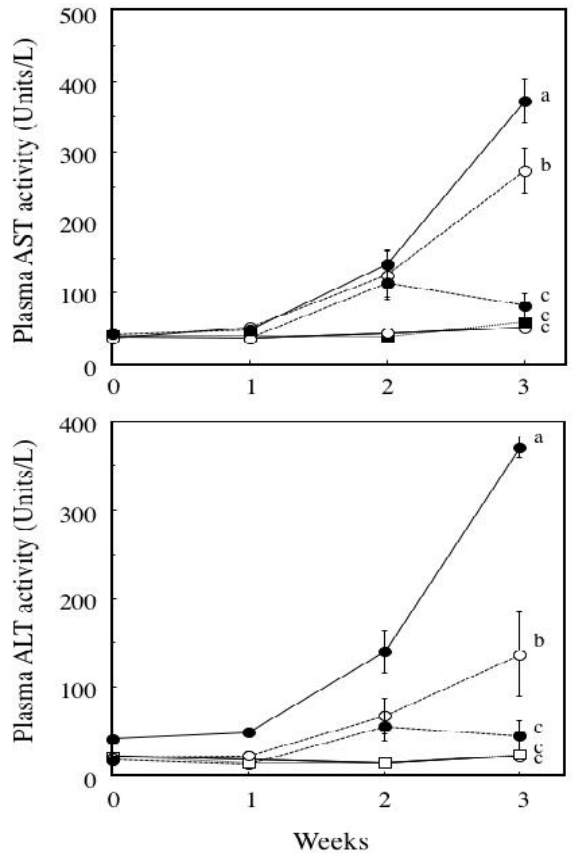


Note: Hepatocytes were treated with 100 mM of ethanol with or without ETS or ESU (25 µg/ml). Cell viability was measured by the neutral red assay, as described in the Materials and Methods section. Data are presented as the mean ± SD of 5 experiments. Values without a common letter are significantly different ($p < 0.01$).

The Effect of ETS and ESU Treatments on Plasma AST and ALT Activities in Ethanol-Plus-CCl_4–Treated Rats

The hepatic histological changes in an ethanol-CCl_4–induced fibrosis model are similar to those found in human alcoholic cirrhosis (Siegers *et al.*, 1986). We examined the effect of ESU and ETS treatment on plasma AST and ALT activities and liver fibrosis in rats treated with ethanol plus CCl_4 (0.1 ml/kg of body weight). As shown in Figure 3, the plasma ALT and AST activities in these rats significantly increased, by 4.7- and 9.7-fold, respectively, compared to those of the control group. In contrast, treatment with CCl_4 or ethanol alone maintained the levels at those of the control rats. On the other hand, the increased activities with the treatments of ethanol plus CCl_4 were significantly decreased in the ETS- and ethanol-plus-CCl_4–treated rats. However, the activities in ESU- and ethanol-plus-CCl_4–treated rats were not decreased significantly.

Figure 3: The Effect of ETS and ESU Treatments on Plasma AST and ALT Activities in Ethanol-Plus-CCl_4-Treated Rats



Note: Effect of ETS and ESU on (A) plasma AST activity and (B) plasma ALT activity. Data are presented as the mean ± SEM of the activity of 5 rats. Values without a common letter are significantly different ($p < 0.01$). --○--: Control, --■--: control diet with CCl_4, -●-: control diet with 5% ethanol plus CCl_4, --○--: 0.3% ESU diet with 5% ethanol plus CCl_4, -●-: 0.3% ETS diet with 5% ethanol plus CCl_4.

The Effect of ETS and ESU Treatments on Liver Fibrosis in Ethanol-Plus-CCl_4–Treated Rats

Histological analysis was performed by EVG staining to assess liver damage (Figure 4a). No histological abnormalities were observed in the control rats, the CCl_4-treated rats, or the ethanol-treated rats. However, hepatic necrosis and collagen accumulation in the liver were observed in the ethanol-plus-CCl_4–treated rats. ETS treatment fully protected the rats against ethanol-plus-CCl_4–induced liver fibrosis and necrosis. Moreover, ETS treatment also significantly suppressed the expression of a-SMA, a marker of liver fibrosis, in the ethanol-plus-CCl_4–treated rats (Figure 4b).

The Effect of ETS on ROS and MDA Levels of Ethanol-Treated Hepatocytes

Ethanol-induced liver injury is characterized by increased ROS formation (Zima *et al.*, 2001; and Albano, 2006). Therefore, we measured intracellular ROS levels in the media of ethanol-treated hepatocytes using DCHF-DA, which is converted to highly fluorescent 2',7'-dichlorodihydrofluorescin in the presence of intracellular ROS. Hepatocytes were incubated for 9 h with 100 mM of ethanol, with or without ETS (Figure 5). Exposure to 100 mM of ethanol caused an increase in ROS. However, intracellular ROS levels of ETS- and ethanol-treated cells were maintained at the levels of the control, ethanol-untreated cells.

We also examined the effects of ETS on ethanol-induced increased in lipid peroxidation using the TBARS assay. Ethanol significantly increased MDA levels after a 24-h incubation. Treatment of ETS maintained the MDA levels below the levels of ethanol-treated cells (Figure 6).

The Effect of ETS on CYP2E1 Expression of Ethanol-Treated Hepatocytes

We examined the effect of ETS on ethanol-induced increases in CYP2E1 expression using Western blotting. Hepatocytes were incubated for various time periods with 100 mM of ethanol. The ethanol-treated hepatocytes significantly

Figure 4: The Effect of ETS and ESU Treatments on Liver Fibrosis in Ethanol-Plus-CCl_4-Treated Rats

(A)

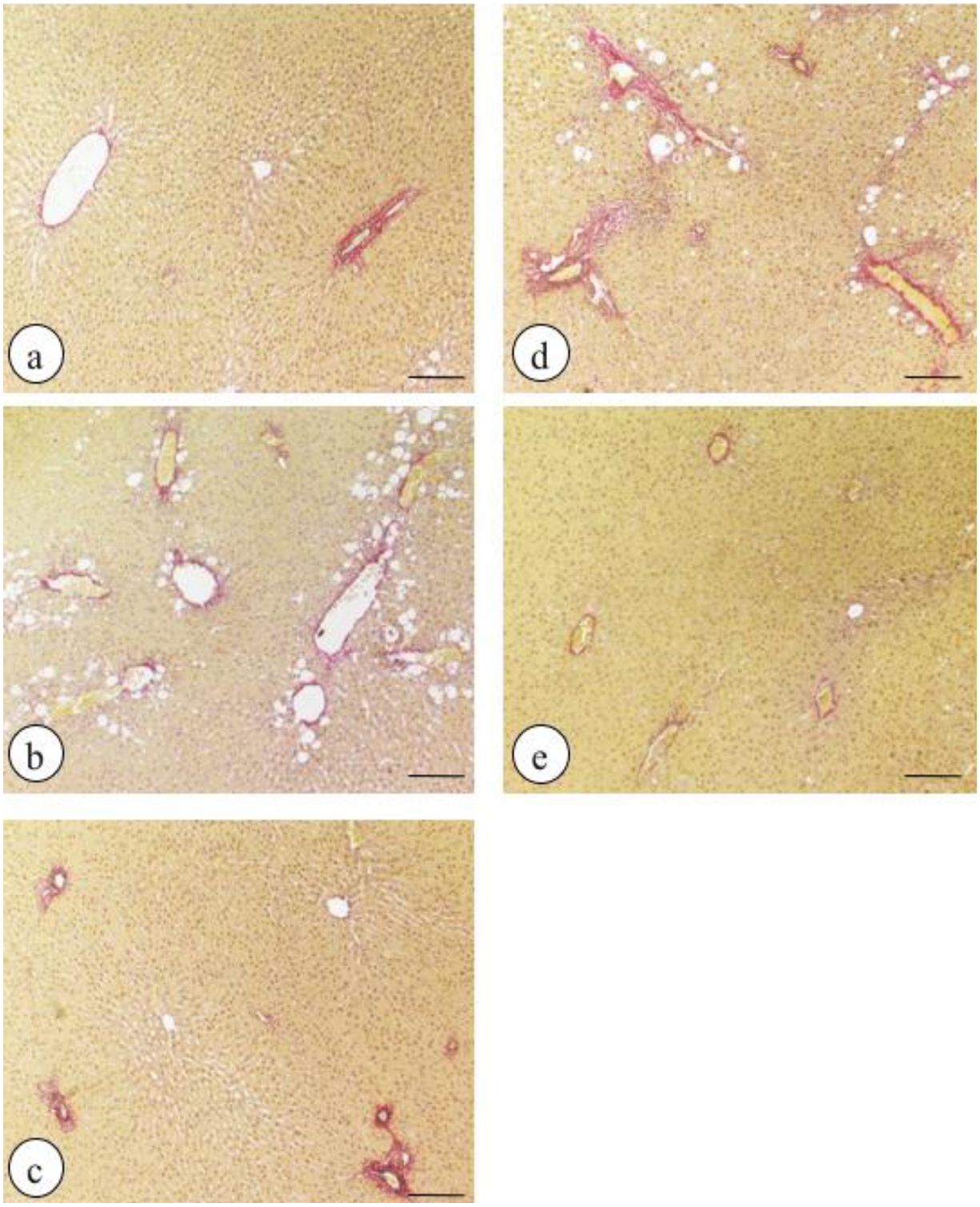
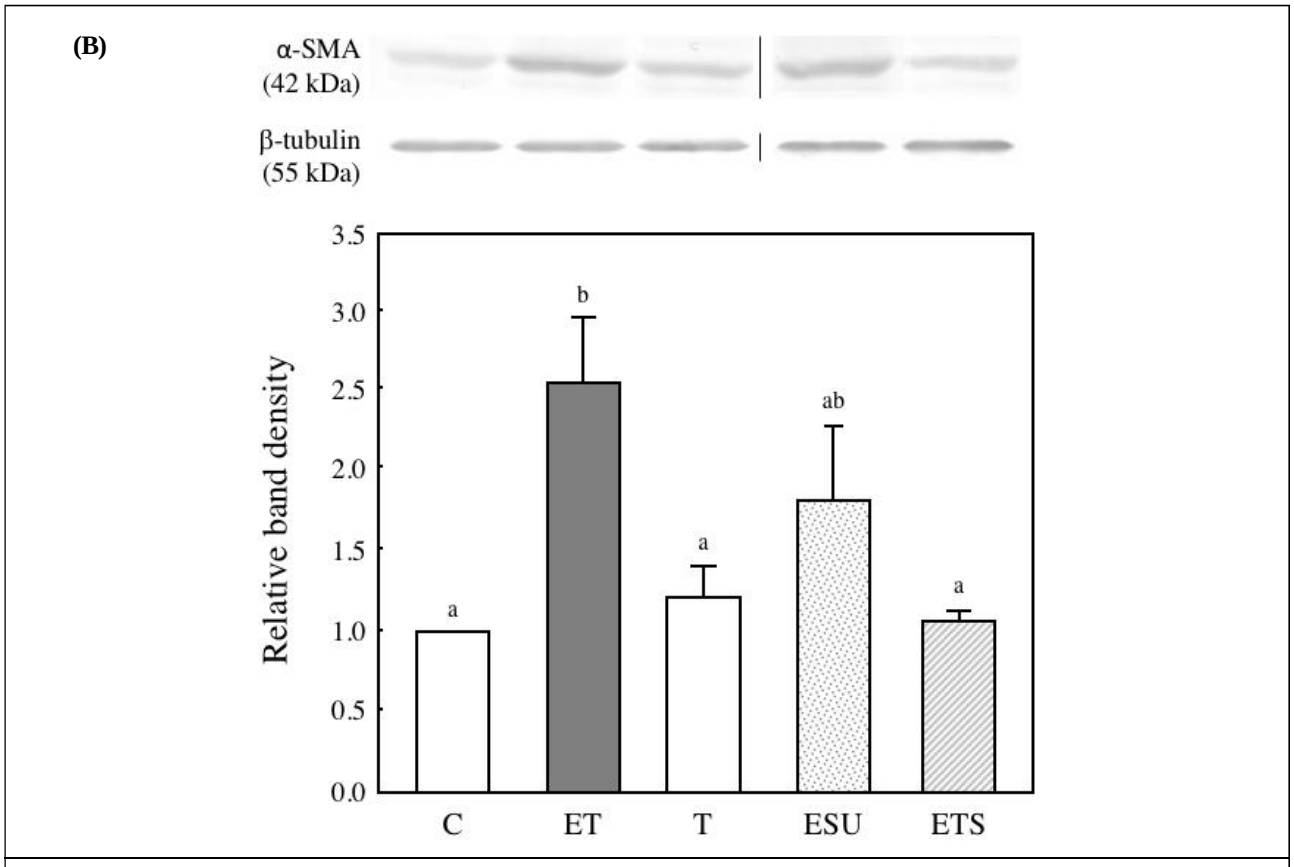


Figure 4 (Cont.)



Note: (A) The effect of ETS and ESU on the changes in liver morphology. Liver sections were processed for EVG staining. (a) control diet; (b) control diet with 5% ethanol plus CCl_4; (c) control diet with CCl_4; (d) 0.3% ESU diet with 5% ethanol plus CCl_4; and (e) 0.3% ETS diet with 5% ethanol plus CCl_4. Scales indicate100 μm. (B) The effect of ETS and ESU on a-SMA expression in the liver of ethanol-plus-CCl_4–treated rats. Data are presented as the mean ± SD of 5 experiments. Values without a common letter are significantly different ($p < 0.01$). Cell lysis and Western blot analysis were performed, as described in the Materials and Methods section. C: control diet; ET: control diet with 5% ethanol plus CCl_4; T: control diet with CCl_4; ESU: 0.3% ESU diet with 5% ethanol plus CCl_4, and ETS: 0.3% ETS diet with 5% ethanol plus CCl_4.

increased the levels of CYP2E1 over the 9-h incubation. In contrast, ETS treatment suppressed ethanol-induced increases in the levels of CYP2E1 to the levels of the control cells.

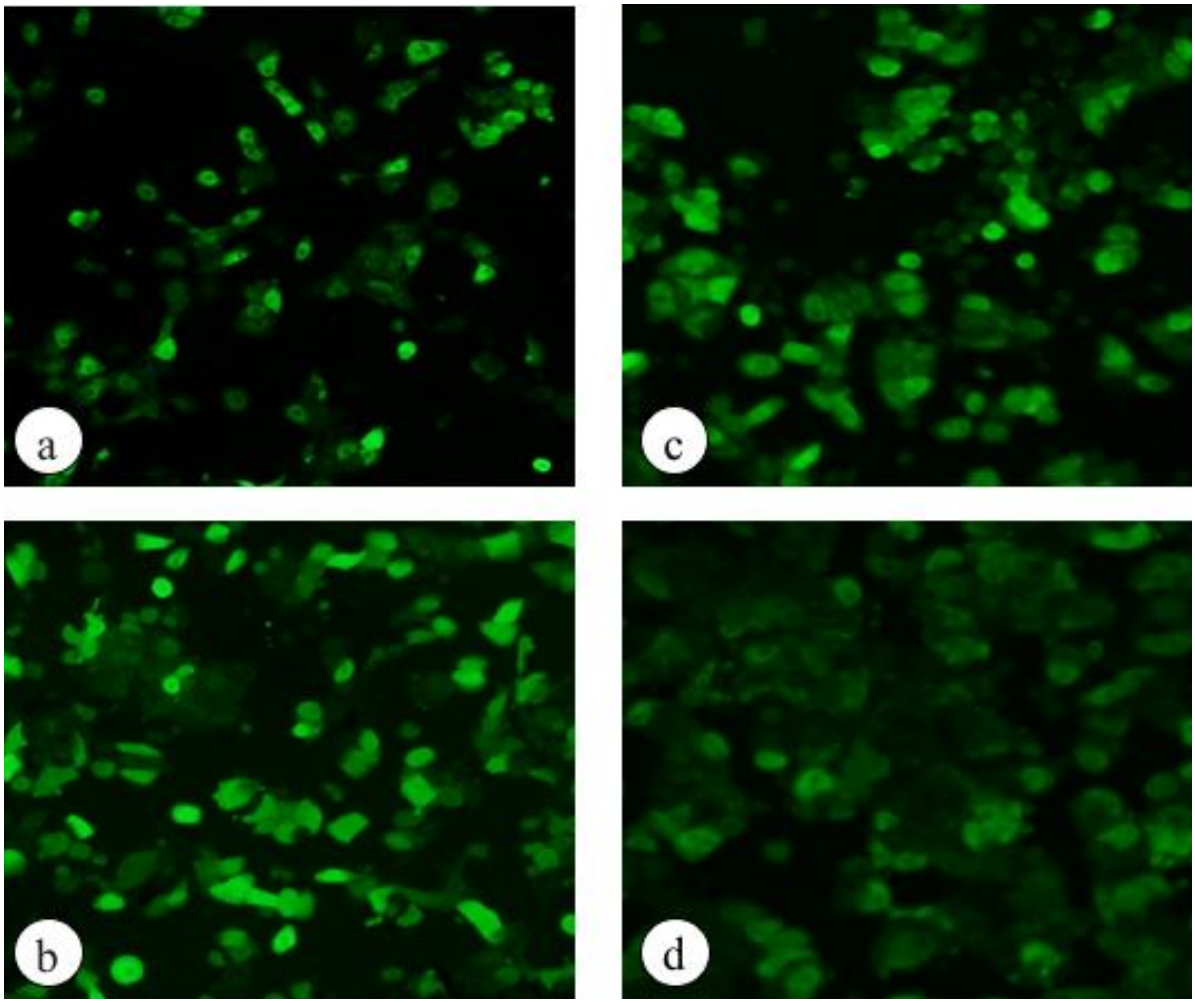
The Effect of ETS on AMPK Activation in Ethanol-Treated Hepatocytes

Several studies have reported that alcohol consumption exacerbated alcoholic liver injury by attenuating the activation of AMPK (Gao and Bataller, 2000; and O'Shea *et al.*, 2010). To determine whether ETS acts on the activation of AMPK, we measured the levels of phosphorylated AMPK. The Western blot analysis revealed that the ethanol exposure decreased the levels of phosphorylated AMPK in cultured hepatocytes. However, ETS suppressed the decrease in the levels of phosphorylated AMPK.

DISCUSSION

The present study has shown that ETS, an extract of a traditional pickling melon (*Cucumismelo*var. *conomon*, *tamatsukuri*) has protective effects against ethanol-induced injury using an in vitro ethanol-induced injury model in hepatocytes. We also demonstrated that the liver damage biomarkers, ALT and AST, were increased in an in vivo ethanol-plus-CCl_4–induced liver injury model in rats, but were reduced by ETS supplementation. However, ESU, an oriental pickling melon (*Cucumismelo*var. *conomon*, *utissimus*) does not have protective effects against ethanol-induced injury in in vitro and in vivo models. *Katsura-uri*, a kind of Japanese pickling melon and a traditional vegetable in Kyoto, Japan, also did not protect against ethanol-induced injury of hepatocytes inan in vitro model. Furthermore, the significant finding in this study is that

Figure 5: The Effect of ETS and ESU on Intracellular ROS Levels in Ethanol-Treated Hepatocytes

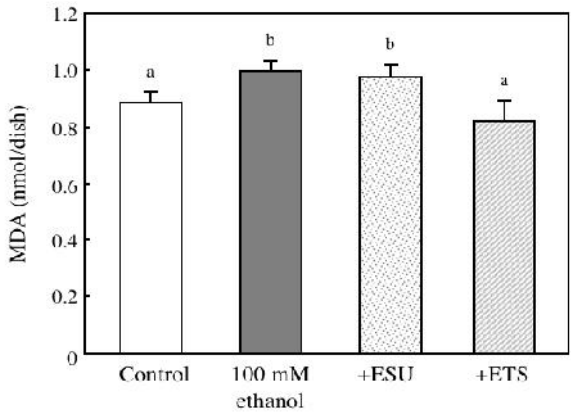


Note: Hepatocytes were treated with 100 mM of ethanol with or without ETS or ESU (25 μg/ml) for 9 h. During the final 30 min of the incubation, the cells were incubated with 2.4 mM of DCFH-DA. (a) control; (b) 100 mM of ethanol; (c) 100 mM of ethanol plus ESU and (d) 100 mM of ethanol plus ETS.

ETS prevents ethanol-induced liver injury by decreasing CYP2E1 levels and increasing AMPK activation.

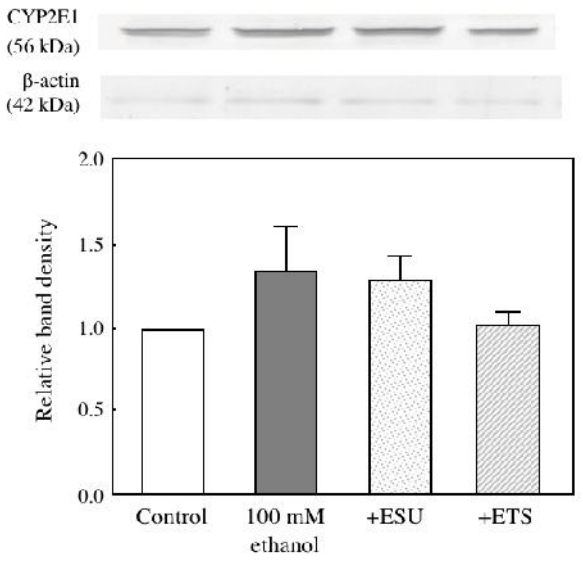
Alcohol-induced liver injury is induced by heavy drinking and is accompanied by the degeneration or necrosis of hepatocytes, which disrupt normal liver function by oxidative stress (Albano, 2006). CYP2E1 is one of the major sources of ethanol-induced ROS (Cederbaum *et al.*, 2001; and Caro and Cederbaum, 2004). Therefore, the decrease in CYP2E1 activity or the inhibition of CYP2E1 may be a feasible strategy for minimizing the hepatotoxicity of ethanol. In recent studies, we demonstrated that *Ecklonia cava* polyphenol treatment maintained CYP2E1 activity in ethanol-treated hepatocytes below that of control cells (Yamashita *et al.*, 2015). We also reported that treatment with the extract of yerba mate tea suppressed ethanol-induced increases in CYP2E1 activity to the levels of the control cells in an in vitro alcohol-induced model in hepatocytes and an in vivo ethanol-plus-CCl_4–induced liver injury model (Tamura *et al.*, 2013). In the present study, we have shown that ETS treatment suppressed ethanol-induced increases in CYP2E1 activity to the levels of the enzyme of control cells. In the present study, we also demonstrated that the increase in intracellular ROS with exposure to ethanol was inhibited in ETS-treated hepatocytes.

Figure 6: The Effect of ETS and ESU on TBARS Levels in Ethanol-Treated Hepatocytes



Note: Hepatocytes were treated with 100 mM of ethanol with or without ETS or ESU (25 μg/ml) for 24 h. Intracellular TBARS levels were determined using the TBARS assay, as described in the Materials and Methods section. Data are presented as the mean ± SD of 5 experiments. Values without a common letter are significantly different ($p < 0.01$).

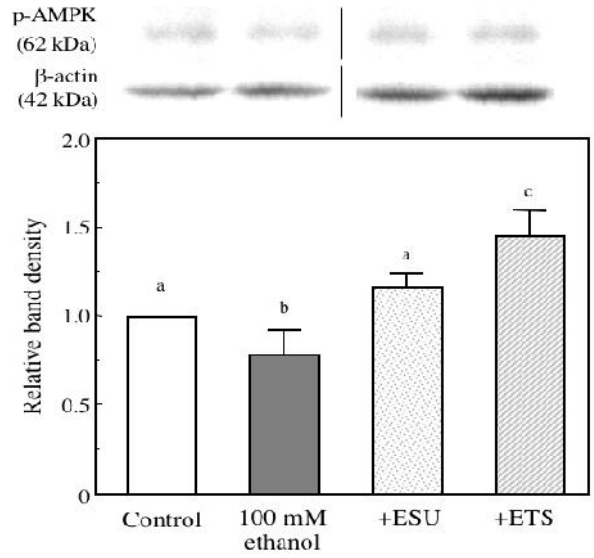
Figure 7: The Effect of ETS and ESU on CYP2E1 Expression in Ethanol-Treated Hepatocytes



Note: Hepatocytes were treated with 100 mM of ethanol with or without ETS or ESU (25 μg/ml) for 9 h. CYP2E1 expression was analyzed using Western blotting as described in Materials and Methods. Data are presented as the mean ± SD of 5 experiments.

Phosphorylation of AMPK is required for the activity of AMPK. The inhibition of phosphorylation of AMPK by

Figure 8: The Effect of ETS on p-AMPK Expression in Ethanol-Treated Hepatocytes



Note: Hepatocytes were treated with 100 mM of ethanol with or without ETS or ESU (25 μg/ml) for 4 h. p-AMPK expression was analyzed using Western blotting as described in Materials and Methods. Data are presented as the mean ± SD of 5 experiments. Values without a common letter are significantly different ($p < 0.01$).

ethanol plays a key role in the development of steatosis induced by chronic alcohol consumption (Sid *et al.*, 2013). The inhibition of AMPK leads to activation of acetyl-CoA carboxylase, which enhances the synthesis of malonyl CoA and thereby inhibits fatty acid uptake and oxidation in mitochondria (You *et al.*, 2004; and Garcia-Villfranca *et al.*, 2008). Many studies have shown a close relationship between oxidative stress and AMPK activation in various disorders such as liver diseases. Recently Choi et al. have demonstrated that pAMPK was markedly decreased in ethanol-treated mice but that treatment of *Citrus aurantium* extract recovered pAMPK expression (Choi *et al.*, 2015). On the other hand, ethanol metabolism produces ROS. ROS are critical controlling factors in AMPK activation. In the present study, decreased levels of phosphorylated AMPK and increased ROS levels in ethanol-treated hepatocytes were observed. Furthermore, treatment of ETS increased the level of phosphorylated AMPK and decreased ROS levels in ethanol-treated hepatocytes. Everittet al. also demonstrated that AMPK phosphorylation was inhibited in the livers of chronically ethanol-fed ob/ob mice by a ROS-independent pathway (Everitt *et al.*, 2013). Thus although many studies suggest that ethanol inhibits AMPK,

some reports do not show its inhibitory effects (Shen *et al.*, 2010; and Xu *et al.*, 2011). Therefore, it is necessary to do further research to resolve the exact relationship between oxidative stress and AMPK activation.

In a preliminary experiment, we measured the polyphenol contents and DPPH radical-scavenging activities in ETS and ESU. There was no difference in the polyphenol contents of ETS and ESU. However, the DPPH free radical activity of ETS was higher than that of ESU. To date it is not known which component(s) in ETS have a protective effect against ethanol-induced liver injury. Therefore, it is necessary to do further research to purify the active component(s) from ETS.

In the present study, we observed that a dose of 25 μg/ml of ETS prevented cell death in hepatocytes treated with 100 mM of ethanol. In animal experiments, the plasma AST and ALT activities induced with ethanol plus CCl_4 (0.1 ml/kg of body weight) were significantly decreased by treatment with a 0.3% ETS diet.

CONCLUSION

These results suggest treatment with ETS protects hepatocytes from ethanol-induced cell injury or ethanol-plus-CCl_4–induced liver injuries.

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