Published in final edited form as:

Alcohol Clin Exp Res. 2016 October ; 40(10): 2076–2084. doi:10.1111/acer.13172.

Dietary fisetin supplementation protects against alcohol-induced liver injury in mice

Qian Sun^{1,2}, Wenliang Zhang², Wei Zhong², Xinguo Sun², and Zhanxiang Zhou^{1,2}

¹Department of Nutrition, University of North Carolina at Greensboro, North Carolina Research Campus, Kannapolis, NC, USA 28081

²Center for Translational Biomedical Research, University of North Carolina at Greensboro, North Carolina Research Campus, Kannapolis, NC, USA 28081

Abstract

Background—Overproduction of reactive oxygen species (ROS) is associated with the development of alcoholic liver disease (ALD). Plant polyphenols have been used as dietary interventions for multiple diseases including ALD. The objective of the present study was to determine whether dietary supplementation with fisetin, a novel flavonoid, exerts beneficial effect on alcohol-induced liver injury.

Methods—C57BL/6J mice were pair-fed with the Lieber-DeCarli control or ethanol diet for four weeks with or without fisetin supplementation at 10 mg/kg/d.

Results—Alcohol feeding induced lipid accumulation in the liver and increased plasma ALT and AST activities, which were attenuated by fisetin supplementation. The ethanol concentrations in the plasma and liver were significantly elevated by alcohol exposure but were reduced by fisetin supplementation. Although fisetin did not affect the protein expression of alcohol metabolism enzymes, the aldehyde dehydrogenase activities were significantly increased by fisetin compared to the alcohol alone group. In addition, fisetin supplementation remarkably reduced hepatic NADPH oxidase 4 (NOX4) levels along with decreased plasma hydrogen peroxide and hepatic superoxide and 4-hydroxynonenal (4HNE) levels after alcohol exposure. Alcohol-induced apoptosis and upregulation of Fas and cleaved caspase-3 in the liver were prevented by fisetin. Moreover, fisetin supplementation attenuated alcohol-induced hepatic steatosis through increasing plasma adiponectin levels and hepatic protein levels of p-AMPK, ACOX1, CYP4A, and MTTP.

Conclusion—The present study demonstrated that the protective effect of fisetin on ALD is achieved by accelerating ethanol clearance and inhibition of oxidative stress. The data suggest that fisetin has a therapeutic potential for treating ALD.

Keywords

Alcoholic liver disease; Fisetin; Oxidative stress; Apoptosis; Fatty liver

*Corresponding author: Zhanxiang Zhou, Center for Translational Biomedical Research and Department of Nutrition, University of North Carolina at Greensboro, North Carolina Research Campus, 500 Laureate Way Suite 4226, Kannapolis, NC 28081. Phone: 704-250-5800. Fax: 704-250-5809. z_zhou@uncg.edu.

1. Introduction

Prolonged alcohol consumption leads to alcoholic liver disease (ALD) and exerts severe outcomes. It has been shown that ALD progress from hepatic steatosis to hepatitis, cirrhosis, and even carcinoma (O'Shea et al., 2010). The adverse effects of alcohol consumption on the liver result from the formation of toxic metabolites and byproducts after alcohol oxidation, for instance acetaldehyde and lipid aldehyde (Cederbaum, 2012, Zhu and Li, 2012). Previous study demonstrated that aldehyde dehydrogenase 2 (ALDH2) knockout mice had higher levels of acetaldehyde in the liver after alcohol exposure and were prone to alcohol-induced inflammation and fibrosis (Kwon et al., 2014). In addition, our laboratory demonstrated that pharmacological activation of ALDH2 by Alda-1 accelerates acetaldehyde clearance in plasma and liver, which resulted in amelioration of alcohol-induced liver injury and oxidative stress (Zhong et al., 2015b).

Excessive formation of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and lipid peroxides is a key component in the development of ALD (Sun et al., 2015). Overproduction of reactive oxygen species (ROS) and inactivation of antioxidants lead to damage of cellular organelles and proteins, which can promote hepatic lipid accumulation and apoptosis (Sun et al., 2015, Sun et al., 2016). Previous studies have shown that increased expression of hepatic cytochrome P450 2E1 (CYP2E1) and NADPH oxidase, as well as dysfunctional mitochondrial are the main factors that contribute to alcohol-induced oxidative stress (Dey and Cederbaum, 2006, Lu and Cederbaum, 2008). Knockout of CYP2E1 (Wang et al., 2013) or NADPH oxidase (Kono et al., 2000) protected against alcohol-induced hepatic lipid accumulation, inflammation, and apoptosis in the liver of mice. In contrast, Cu,Zn-superoxide dismutase (SOD1) knockout mice had severely increased oxidative stress and liver injury compared to SOD1^{+/+} mice after alcohol exposure (Kessova et al., 2003).

Fisetin is a bioactive flavonoid molecule found in vegetable and fruits, such as strawberry, apple, persimmon, lotus root, and onion (Khan et al., 2013). The biological activities of fisetin include antioxidant and anti-inflammatory effects (Khan et al., 2013). The powerful antioxidant property of fisetin is due to the presence of phenolic hydroxyl group in the flavonoid structure (Prasath and Subramanian, 2013). Fisetin has shown promising treatment effects in cancer, inflammatory, neural, and metabolic diseases (Jeon et al., 2013, Syed et al., 2013). Previous study has demonstrated that polyphenol, for instance resveratrol, protects against alcohol-induced liver injury (Ajmo et al., 2008). However, whether fisetin, as a novel antioxidant and health food supplement, also has beneficial effects on ALD is not clear. Therefore, the current study was undertaken to investigate the effect of fisetin supplementation on alcohol-induced fatty liver in mice and explore the potential molecular mechanisms through which the protective effect of fisetin may work.

2. Materials and Methods

2.1. Animal and alcohol feeding experiments

Male C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were treated according to the experimental procedures approved by Institutional Animal Care and Use Committee of North Carolina Research Campus (Protocol Number:

13016). The mice were pair-fed a stepwise and modified Lieber-DeCarli ethanol (EtOH group) or control (Ctrl group) liquid diet for four weeks (n=6) with or without 10 mg/kg/d Fisetin (Selleck Chemicals, Houston, TX) supplementation (E/Fisetin group). The ethanol content (% w/v) in the diet was gradually increased from 3.2 (22.4% of total calories) to 4.2 (29.4% of total calories). The EtOH and E/Fisetin groups were fed *ad libitum* because ethanol rather than fisetin affected food intake. The control mice were fed the control diet in the average amount of diet consumed by the EtOH and E/Fisetin groups in the previous day. Food intake all the 3 groups was measured daily. The mice were gavaged with ethanol (3g/kg) or isocaloric dextrin (5.2g/kg) once a week and the last gavage was given 4 hr before tissue collection. At the end of 4-week of feeding, mice were anesthetized with inhalational isoflurane and tissue were collected.

2.2. Liver histopathology

Liver tissues were fixed in 10% formalin, and processed for paraffin embedding. Paraffin sections were cut in 5 µm and processed with hematoxylin and eosin (H&E) staining.

2.3. Measurement of ethanol and acetaldehyde

Plasma and hepatic ethanol and acetaldehyde levels were determined by GC-MS (Agilent Technologies, Santa Clara, CA). One hundred milligram NaCl and 10 µl n-propanol (internal standard) were added to 100 µl plasma. One hundred milligram livers were homogenized with 300 µl water and 10 µl n-propanol using Next Advance Bullet Blender for two cycles and centrifuged at 1500g for 5min at 4°C. Liver homogenate were then centrifuged at low speed for 30s. Then the supernatant was quantitatively transferred into a new headspace vial. The vials were then capped with a crimper. The plasma and liver samples were then subject to GC-MS analysis.

2.4. Measurement of hepatic ALDH activity

Hepatic ALDH activity was measured with aldehyde dehydrogenase activity colorimetric assay kit (Bio Vision) following the manufacturer's instruction.

2.5. Immunohistochemical staining of hepatic 4-hydroxynonenal (4HNE)

Hepatic 4-HNE levels were detected by immunohistochemical staining. Briefly, liver tissue paraffin sections were incubated with 3% hydrogen peroxide for 10 minutes to inactivate endogenous peroxidases. The endogenous mouse IgG was blocked by incubation with a mouse-to-mouse blocking reagent (ScyTek Laboratories, Logan, UT). Tissue sections were then incubated with a monoclonal mouse anti-4-HNE antibody (Northwest Life Science Specialties, Vancouver, WA) at 4 °C overnight, followed by incubation with EnVision+ Labeled Polymer-HRP-conjugated anti-mouse IgG (DAKO, Carpinteria, CA) at room temperature for 30 minutes. Diaminobenzidine (DAB) was used as HRP substrate for visualization.

2.6. Dihydroethidium (DHE) staining

Superoxide levels in the liver of mice were detected with DHE fluorescent microscopy. DHE can be oxidized by superoxide anions to ethidium which intercalates into DNA and gives

nucleus a bright red fluorescent. Cryostat sections of liver were cut in 5µm and incubated with DHE at a concentration of 5 µM (Life technologies, Carlsbad, CA) for 30 min. The red fluorescence was detected with fluorescence microscope.

2.7. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST)

The ALT and AST activity in the serum were measured with Infinity kit (Thermo Scientific, Waltham, MA).

2.8. Immunoblot analysis

Tissue extraction reagent (Thermo scientific, Waltham, MA) containing protease inhibitors (Sigma-Aldrich, St. Louis, MO) were used for extraction of proteins from liver tissue. Aliquots containing 30µg liver protein were loaded onto a 10%–15% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). After electrophoresis, proteins were transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was probed with polyclonal antibodies against cytochrome P450 2E1 (CYP2E1), aldehyde dehydrogenase 2 (ALDH2), NADPH oxidase 4 (NOX4) (Abcam, Cambridge, MA), superoxide dismutase 1 (SOD1), microsomal triglyceride transfer protein (Mtp) (Santa Cruz Biotechnologies, Santa Cruz, CA), alcohol dehydrogenase (ADH), glutathione peroxidase 1(Gpx1), cytochrome P450 4A (CYP4A) (Novus biological, Littleton, CO), catalase, superoxide dismutase 2 (SOD2) (Millipore, Billerica, MA), Fas, Fas ligand (FasL), caspase-3, cleaved caspase-3, AMP-activated protein kinase (AMPK), p-AMPK (Cell Signaling Technology, Danvers, MA) Acyl-Coenzyme A oxidase 1 (Acox1) (Proteintech, Chicago, IL), and β-actin (Sigma-Aldrich), respectively. The membrane was then incubated with HRP-conjugated goat anti-rabbit IgG, goat anti-mouse IgG, or rabbit anti-goat IgG antibody. The protein bands were visualized by an Enhanced Chemiluminescence detection system (GE Healthcare, Piscataway, NJ) and quantified by densitometry analysis.

2.9. Quantitative assay of hepatic triglycerides and free fatty acid

The levels of triglycerides (TG) and free fatty acids (FFA) in liver tissues were measured with assay kits (BioVision, Milpitas, CA) following the manufacturer's instruction.

2.10. Hydrogen peroxide (H₂O₂)

Serum H₂O₂ levels were measured with an Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (Invitrogen, Waltham, MA). Amplex Red reagent reacts with H₂O₂ to produce the red oxidation product resorufin which can be measured spectrophotometrically at OD=560nm.

2.11. Terminal Deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay

Apoptotic cell death in the liver was assessed by detection of DNA fragmentation using an ApopTag peroxidase *in situ* Apoptosis Detection Kit (Millipore, Billerica, MA). Briefly, liver tissue slides were pretreated with H₂O₂ to inactivate endogenous peroxidase. The liver tissue slides were then incubated with the reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and digoxigenin-conjugated dUTP for 1h at 37 °C. The

labeled DNA was visualized with HRP-conjugated anti-digoxigenin antibody with DAB as the chromagen.

2.12. Statistical analysis

Results are expressed as mean $\pm$ standard deviation (SD). Differences among multiple groups were analyzed by analysis of variance followed by Tukey's test. The significance between groups was defined as $P < 0.05$.

3. Results

3.1. Fisetin supplementation ameliorates alcohol-induced liver injury

There were no significant differences in average daily food intake among the 3 groups, 11.50 ± 0.91 g in ctrl group, 11.04 ± 1.18 g in EtOH group, and 11.42 ± 0.96 g in E/Fisetin group. Light microscopy exam revealed that alcohol exposure caused lipid accumulation in the liver as indicated by arrows, and fisetin supplementation along with alcohol exposure reduced the size and number of lipid droplets compared with the EtOH only group (Fig. 1A). Alcohol exposure also significantly increased plasma ALT and AST activities, which were attenuated by fisetin supplementation (Fig 1B).

3.2. Fisetin supplementation accelerates ethanol and acetaldehyde clearance and increases hepatic ALDH2 activity

To determine whether fisetin affects ethanol and acetaldehyde clearance, the levels of ethanol and acetaldehyde in the plasma and the liver of mice were measured. As shown in Figure 2A, plasma ethanol levels were significantly increased after alcohol exposure, and fisetin supplementation significantly decreased plasma ethanol concentrations compared to ethanol alone group. Plasma acetaldehyde levels were also significantly increased by alcohol exposure, which was not affected by fisetin (Fig 2A). Hepatic ethanol concentrations were significantly increased by alcohol exposure, which were reduced by fisetin supplementation (Fig 2B). Hepatic acetaldehyde concentrations were significantly upregulated by alcohol exposure as well, however, fisetin supplementation did not affect the levels of acetaldehyde in the liver after alcohol exposure (Fig 2B). The protein expression of ethanol oxidation enzymes were evaluated by immunoblot and the result showed that alcohol exposure induced the expression of CYP2E1 but not the other enzymes, such as ADH, catalase, and ALDH2 (Fig 2C). Fisetin supplementation did not affect the protein expression of ethanol oxidation enzymes (Fig 2C). As shown in figure 2D, alcohol exposure increased the activity of ALDH, which was further elevated by fisetin supplementation.

3.3. Fisetin supplementation suppresses alcohol-induced oxidative stress and inhibits the expression of NOX4 in the liver

Oxidative stress was evaluated by measuring plasma hydrogen peroxide (H_2O_2) and hepatic superoxide and 4HNE levels. Alcohol exposure significantly increased plasma levels of H_2O_2 , which was suppressed by fisetin supplementation (Fig 3A). Hepatic superoxide levels was evaluated as shown by DHE staining, suggesting that alcohol exposure induced ROS accumulation in the liver, as indicated by bright red fluorescence formation in the nuclei (arrows) (Fig 3B). Fisetin supplementation attenuated the formation of ROS in the liver, as

indicated by reduced intensity of red fluorescence (Fig 3B). The formation of 4HNE was assessed by immunohistochemical staining. As shown in figure 3B, mice fed alcohol had a stronger staining of 4HNE in the liver, especially around vein area as indicated by arrows, compared to that in controls. Fisetin supplementation reduced alcohol-induced generation of 4HNE in the liver of mice (Fig 3B). The expression of antioxidant enzymes were measured by immunoblot. The results showed that both alcohol exposure and fisetin did not affect the hepatic protein levels of antioxidant enzymes including SOD1, SOD2, and Gpx1 (Fig 3C). The protein levels of NOX4 were also assessed by immunoblot. As shown in figure 3D, alcohol feeding significantly increased hepatic NOX4, which was attenuated by fisetin supplementation.

3.4. Fisetin supplementation inhibits alcohol-induced activation of apoptosis in the liver

To determine whether fisetin supplementation inhibits alcohol-induced apoptosis in the liver of mice, TUNEL assay was performed. As shown in figure 4A, the number of apoptotic cells (arrows) was increased by alcohol exposure, which was reduced by fisetin supplementation. The protein levels of Fas, FasL, cleaved caspase-3, and caspase 3 were assessed by immunoblot. The levels of Fas, FasL and cleaved caspase-3 were significantly increased after alcohol exposure, which was normalized by fisetin treatment (Fig 4B).

3.5. Fisetin supplementation ameliorates alcohol-induced lipid accumulation through upregulating adiponectin levels and increasing protein expression of ACOX1, CYP4A and MTP in the liver

To define the effect of fisetin on lipid metabolism, hepatic TG and FFA levels, plasma adiponectin levels, and hepatic lipid metabolism-related proteins/enzymes were measured. Alcohol exposure significantly increased the levels of TG (Fig 5A) and FFA (Fig 5B) in the liver, which were attenuated by fisetin supplementation. As shown in figure 5C, chronic alcohol feeding did not affect the levels of plasma adiponectin, however, fisetin supplementation significantly increased the levels of plasma adiponectin. The protein levels of p-AMPK was significantly decreased by alcohol feeding, which was prevented by fisetin supplementation (Fig 5D). Chronic alcohol feeding significantly decreased the protein levels of ACOX1, which was prevented by fisetin (Fig 5E). The protein levels of CYP4A (Fig 5E) and MTP (Fig 5F) were not affected by alcohol exposure, but both were significantly increased by fisetin supplementation.

4. Discussion

The present study demonstrates that dietary fisetin supplementation ameliorates alcohol-induced liver injury through accelerating alcohol clearance, inhibiting oxidative stress, and correcting lipid metabolism disorder (Figure 6). Enhanced activity of ALDH represents one mechanism for observed effect of fisetin on decrease of ethanol levels in the plasma and liver after alcohol exposure. In addition, increase of antioxidant ability and decrease of ROS production through reducing NOX4 levels account for the protective effect of alcohol-induced oxidative stress. Suppressed oxidative stress by fisetin prevented activation of hepatic apoptotic signaling. Furthermore, fisetin modulated hepatic lipid metabolism toward reduction of lipid accumulation via activating adiponectin-AMPK signaling.

Accelerating of ethanol clearance plays an important role in preventing the development of ALD. ALDH2 knockout mice exhibited accumulation of methoxyacetaldehyde (Kitagawa et al., 2000), N²-ethylidene-2'-deoxyguanosine DNA adducts (Matsuda et al., 2007) as well as malondialdehyde adducts (Kwon et al., 2014) after alcohol exposure. On the contrary, ALDH2 overexpression mice showed attenuated liver injury after alcohol exposure (Guo et al., 2009). In the present study, we showed that fisetin supplementation decreased the ethanol levels in plasma and liver but did not affect acetaldehyde levels. Because ethanol is metabolized to acetaldehyde, reduction of ethanol levels should be accompanied with an increase in acetaldehyde levels. Based on the fact that fisetin reduced ethanol levels without increasing acetaldehyde levels, acetaldehyde clearance should be accelerated by fisetin. Consistent with our finding, previous study reported that high levels of dietary flavonoids accelerated serum acetaldehyde clearance in human subjects (Wickramasinghe et al., 1996). In addition, we found that fisetin increased ALDH activity, which may represent one mechanism of fisetin in accelerating clearance of ethanol and acetaldehyde. Fisetin mediated elevation of ALDH activities might be resulted from suppressed oxidative stress after alcohol exposure. Previous study from our laboratory have demonstrated that inhibition of oxidative stress by zinc supplementation leads to an increase of ALDH activities in the colon (Zhong et al., 2015a). However, we do not rule out the possibility that fisetin might react with ethanol to decrease the concentration of ethanol after alcohol exposure. The precise mechanism need to be further explored in the future studies. To our knowledge, the present study is the first to report that fisetin accelerates ethanol clearance through elevating ALDH activity.

Flavonols possess the strong antioxidant property as manifested by scavenging of superoxide anions, singlet oxygen, and lipid peroxy radicals (Piao et al., 2013). Fisetin supplementation partially reversed ALT and AST activities, decreased hepatic TBARS and H₂O₂ levels, and increased hepatic GSH levels in streptozotocin-induced diabetes in rats (Prasath and Subramanian, 2013). Consistent with previous study, we found that fisetin decreased the plasma H₂O₂ levels as well as hepatic superoxide and 4HNE levels after alcohol exposure. In addition, we surprisingly found that alcohol-induced elevation of NOX4, a component of NADPH oxidases, was partially reversed by fisetin supplementation. NADPH oxidases generates free radicals, superoxide and hydrogen peroxide, from molecular oxygen using NADPH as an electron donor (Paik et al., 2014). Human study reported a 12.7 fold elevation of NOX4 genes in the liver of alcoholic hepatitis patients compared to normal subjects (Colmenero et al., 2007). NADPH oxidase deficiency mice (p47^{phox}^{-/-}) were protected against alcohol-induced hepatic lipid accumulation and inflammation (Kono et al., 2000). Knockout of NOX4 decreased bile duct ligation-induced apoptosis and fibrosis in the liver of mice (Jiang et al., 2012). Our unpublished data suggests that administration of NOX4 inhibitor, GKT137831, to mice ameliorated oxidative stress and protected mice against hepatic steatosis and apoptosis. To our knowledge, it is the first time that fisetin has been reported to affect the protein expression of NOX4 after alcohol exposure. The present study suggests that fisetin suppresses oxidative stress through enhancing antioxidant ability and inhibition of ROS production after alcohol exposure.

Disturbed lipid homeostasis is a character of ALD. Chronic alcohol feeding downregulates the expression of p-AMPK, which is associated with decrease of lipolysis and increase of de

Acknowledgments

Sources of support: This research was supported by the National Institutes of Health (R01AA020212 and 2R01AA018844).

References

- Ajmo JM, Liang X, Rogers CQ, Pennock B, You M. Resveratrol alleviates alcoholic fatty liver in mice. *American journal of physiology. Gastrointestinal and liver physiology*. 2008; 295:G833–G842. [PubMed: 18755807]
- Berg AH, Combs TP, Scherer PE. ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism. *Trends in endocrinology and metabolism*. 2002; 13:84–89. [PubMed: 11854024]
- Cederbaum AI. Alcohol metabolism. *Clinics in liver disease*. 2012; 16:667–685. [PubMed: 23101976]
- Colmenero J, Bataller R, Sancho-Bru P, Bellot P, Miquel R, Moreno M, Jares P, Bosch J, Arroyo V, Caballeria J, Gines P. Hepatic expression of candidate genes in patients with alcoholic hepatitis: correlation with disease severity. *Gastroenterology*. 2007; 132:687–697. [PubMed: 17258719]
- Dey A, Cederbaum AI. Alcohol and oxidative liver injury. *Hepatology*. 2006; 43:S63–S74. [PubMed: 16447273]
- Guo R, Zhong L, Ren J. Overexpression of aldehyde dehydrogenase-2 attenuates chronic alcohol exposure-induced apoptosis, change in Akt and Pim signalling in liver. *Clinical and experimental pharmacology & physiology*. 2009; 36:463–468. [PubMed: 19215238]
- Jeon TI, Park JW, Ahn J, Jung CH, Ha TY. Fisetin protects against hepatosteatosis in mice by inhibiting miR-378. *Molecular nutrition & food research*. 2013; 57:1931–1937. [PubMed: 23818290]
- Jiang JX, Chen X, Serizawa N, Szyndralewicz C, Page P, Schroder K, Brandes RP, Devaraj S, Torok NJ. Liver fibrosis and hepatocyte apoptosis are attenuated by GKT137831, a novel NOX4/NOX1 inhibitor in vivo. *Free radical biology & medicine*. 2012; 53:289–296. [PubMed: 22618020]
- Jin T, Kim OY, Shin MJ, Choi EY, Lee SS, Han YS, Chung JH. Fisetin up-regulates the expression of adiponectin in 3T3-L1 adipocytes via the activation of silent mating type information regulation 2 homologue 1 (SIRT1)-deacetylase and peroxisome proliferator-activated receptors (PPARs). *Journal of agricultural and food chemistry*. 2014; 62:10468–10474. [PubMed: 25286082]
- Kang X, Zhong W, Liu J, Song Z, McClain CJ, Kang YJ, Zhou Z. Zinc supplementation reverses alcohol-induced steatosis in mice through reactivating hepatocyte nuclear factor-4alpha and peroxisome proliferator-activated receptor-alpha. *Hepatology*. 2009; 50:1241–1250. [PubMed: 19637192]
- Kessova IG, Ho YS, Thung S, Cederbaum AI. Alcohol-induced liver injury in mice lacking Cu, Zn-superoxide dismutase. *Hepatology*. 2003; 38:1136–1145. [PubMed: 14578852]
- Khan N, Afaq F, Khusro FH, Mustafa Adhami V, Suh Y, Mukhtar H. Dual inhibition of phosphatidylinositol 3-kinase/Akt and mammalian target of rapamycin signaling in human nonsmall cell lung cancer cells by a dietary flavonoid fisetin. *International journal of cancer. Journal international du cancer*. 2012; 130:1695–1705. [PubMed: 21618507]
- Khan N, Syed DN, Ahmad N, Mukhtar H. Fisetin: a dietary antioxidant for health promotion. *Antioxidants & redox signaling*. 2013; 19:151–162. [PubMed: 23121441]
- Kitagawa K, Kawamoto T, Kunugita N, Tsukiyama T, Okamoto K, Yoshida A, Nakayama K, Nakayama K. Aldehyde dehydrogenase (ALDH) 2 associates with oxidation of methoxyacetaldehyde; in vitro analysis with liver subcellular fraction derived from human and Aldh2 gene targeting mouse. *FEBS letters*. 2000; 476:306–311. [PubMed: 10913633]
- Kono H, Rusyn I, Yin M, Gabele E, Yamashina S, Dikalova A, Kadiiska MB, Connor HD, Mason RP, Segal BH, Bradford BU, Holland SM, Thurman RG. NADPH oxidase-derived free radicals are key oxidants in alcohol-induced liver disease. *The Journal of clinical investigation*. 2000; 106:867–872. [PubMed: 11018074]
- Kwon HJ, Won YS, Park O, Chang B, Duryee MJ, Thiele GE, Matsumoto A, Singh S, Abdelmegeed MA, Song BJ, Kawamoto T, Vasiliou V, Thiele GM, Gao B. Aldehyde dehydrogenase 2 deficiency

ameliorates alcoholic fatty liver but worsens liver inflammation and fibrosis in mice. *Hepatology*. 2014; 60:146–157. [PubMed: 24492981]

- Li Q, Zhong W, Qiu Y, Kang X, Sun X, Tan X, Zhao Y, Sun X, Jia W, Zhou Z. Preservation of hepatocyte nuclear factor-4alpha contributes to the beneficial effect of dietary medium chain triglyceride on alcohol-induced hepatic lipid dyshomeostasis in rats. *Alcoholism, clinical and experimental research*. 2013; 37:587–598.
- Lu Y, Cederbaum AI. CYP2E1 and oxidative liver injury by alcohol. *Free radical biology & medicine*. 2008; 44:723–738. [PubMed: 18078827]
- Matsuda T, Matsumoto A, Uchida M, Kanaly RA, Misaki K, Shibutani S, Kawamoto T, Kitagawa K, Nakayama KI, Tomokuni K, Ichiba M. Increased formation of hepatic N2-ethylidene-2'-deoxyguanosine DNA adducts in aldehyde dehydrogenase 2-knockout mice treated with ethanol. *Carcinogenesis*. 2007; 28:2363–2366. [PubMed: 17361010]
- O'Shea RS, Dasarthy S, McCullough AJ. Practice Guideline Committee of the American Association for the Study of Liver D, Practice Parameters Committee of the American College of G. Alcoholic liver disease. *Hepatology*. 2010; 51:307–328. [PubMed: 20034030]
- Paik YH, Kim J, Aoyama T, De Minicis S, Bataller R, Brenner DA. Role of NADPH oxidases in liver fibrosis. *Antioxidants & redox signaling*. 2014; 20:2854–2872. [PubMed: 24040957]
- Piao MJ, Kim KC, Chae S, Keum YS, Kim HS, Hyun JW. Protective Effect of Fisetin (3,7,3',4'-Tetrahydroxyflavone) against gamma-Irradiation-Induced Oxidative Stress and Cell Damage. *Biomolecules & therapeutics*. 2013; 21:210–215. [PubMed: 24265866]
- Prasath GS, Subramanian SP. Fisetin, a tetra hydroxy flavone recuperates antioxidant status and protects hepatocellular ultrastructure from hyperglycemia mediated oxidative stress in streptozotocin induced experimental diabetes in rats. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*. 2013; 59:249–255. [PubMed: 23791753]
- Purohit V, Gao B, Song BJ. Molecular mechanisms of alcoholic fatty liver. *Alcoholism, clinical and experimental research*. 2009; 33:191–205.
- Suh Y, Afaq F, Khan N, Johnson JJ, Khusro FH, Mukhtar H. Fisetin induces autophagic cell death through suppression of mTOR signaling pathway in prostate cancer cells. *Carcinogenesis*. 2010; 31:1424–1433. [PubMed: 20530556]
- Sun Q, Li Q, Zhong W, Zhang J, Sun X, Tan X, Yin X, Sun X, Zhang X, Zhou Z. Dysregulation of hepatic zinc transporters in a mouse model of alcoholic liver disease. *Am J Physiol Gastrointest Liver Physiol*. 2014; 307:G313–G322. [PubMed: 24924749]
- Sun Q, Zhong W, Zhang W, Li Q, Sun X, Tan X, Sun X, Dong D, Zhou Z. Zinc deficiency mediates alcohol-induced apoptotic cell death in the liver of rats through activating ER and mitochondrial cell death pathways. *American journal of physiology. Gastrointestinal and liver physiology*. 2015; 308:G757–G766. [PubMed: 25767260]
- Sun Q, Zhong W, Zhang W, Zhou Z. Defect of mitochondrial respiratory chain is a mechanism of ROS overproduction in a rat model of alcoholic liver disease: role of zinc deficiency. *American journal of physiology. Gastrointestinal and liver physiology*. 2016; 310:G205–G214. [PubMed: 26585415]
- Sun X, Luo W, Tan X, Li Q, Zhao Y, Zhong W, Sun X, Brouwer C, Zhou Z. Increased plasma corticosterone contributes to the development of alcoholic fatty liver in mice. *American journal of physiology. Gastrointestinal and liver physiology*. 2013; 305:G849–G861. [PubMed: 24113770]
- Syed DN, Adhami VM, Khan MI, Mukhtar H. Inhibition of Akt/mTOR signaling by the dietary flavonoid fisetin. *Anti-cancer agents in medicinal chemistry*. 2013; 13:995–1001. [PubMed: 23293889]
- Tan X, Sun X, Li Q, Zhao Y, Zhong W, Sun X, Jia W, McClain CJ, Zhou Z. Leptin deficiency contributes to the pathogenesis of alcoholic fatty liver disease in mice. *The American journal of pathology*. 2012; 181:1279–1286. [PubMed: 22841822]
- Tateya S, Rizzo-De Leon N, Handa P, Cheng AM, Morgan-Stevenson V, Ogimoto K, Kanter JE, Bornfeldt KE, Daum G, Clowes AW, Chait A, Kim F. VASP increases hepatic fatty acid oxidation by activating AMPK in mice. *Diabetes*. 2013; 62:1913–1922. [PubMed: 23349495]

- Wang X, Wu D, Yang L, Gan L, Cederbaum AI. Cytochrome P450 2E1 potentiates ethanol induction of hypoxia and HIF-1alpha in vivo. *Free radical biology & medicine*. 2013; 63:175–186. [PubMed: 23669278]
- Wickramasinghe SN, Hasan R, Khalpey Z. Differences in the serum levels of acetaldehyde and cytotoxic acetaldehyde-albumin complexes after the consumption of red and white wine: in vitro effects of flavonoids, vitamin E, and other dietary antioxidants on cytotoxic complexes. *Alcoholism, clinical and experimental research*. 1996; 20:799–803.
- Zhong W, Li Q, Sun Q, Zhang W, Zhang J, Sun X, Yin X, Zhang X, Zhou Z. Preventing Gut Leakiness and Endotoxemia Contributes to the Protective Effect of Zinc on Alcohol-Induced Steatohepatitis in Rats. *The Journal of nutrition*. 2015a; 145:2690–2698. [PubMed: 26468492]
- Zhong W, Zhang W, Li Q, Xie G, Sun Q, Sun X, Tan X, Sun X, Jia W, Zhou Z. Pharmacological activation of aldehyde dehydrogenase 2 by Alda-1 reverses alcohol-induced hepatic steatosis and cell death in mice. *Journal of hepatology*. 2015b; 62:1375–1381. [PubMed: 25543082]
- Zhu H, Li YR. Oxidative stress and redox signaling mechanisms of inflammatory bowel disease: updated experimental and clinical evidence. *Experimental biology and medicine*. 2012; 237:474–480. [PubMed: 22442342]

Figure 1.

Liver pathology and serum markers of liver injury in mice fed control (Ctrl), or ethanol (EtOH), or ethanol combined with fisetin (E/Fisetin) diet for four weeks. (A) Light microscopy with hematoxylin & eosin staining shows lipid accumulation in the liver of mice after alcohol exposure, which was alleviated by fisetin administration. (B) Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. Infinity ALT and AST Reagents were used for measuring ALT and AST activities. Scale bar: 50 μ m. Results are mean $\pm$ SD (n=3–6). Results for bars that do not share a letter differed significantly among groups ($P < 0.05$). Significant differences among groups are determined by ANOVA followed by Tukey's test.

Figure 2.

Effect of fisetin on ethanol clearance and the expression/activities of ethanol oxidation-related enzymes in mice chronically fed ethanol for four weeks. (A) Plasma levels of ethanol and acetaldehyde. (B) Hepatic levels of ethanol and acetaldehyde. (C) The immunoblot bands of alcohol oxidation enzymes in the liver. (C) The activities of ALDH in the liver. The ratio to β -actin was calculated by setting the value of ctrl as one. Results are mean $\pm$ SD (n=3–6). Results for bars that do not share a letter differed significantly among groups (*P*

<0.05). Significant differences among groups are determined by ANOVA followed by Tukey's test. Control (Ctrl), ethanol (EtOH), ethanol combined with fisetin (E/Fisetin).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3.

Effect of fisetin on oxidative stress and the expression of oxidative stress-related enzymes in mice chronically fed ethanol for four weeks. (A) Plasma levels of hydrogen peroxide (H₂O₂). (B) ROS accumulation in the liver was detected by dehydroethidium (DHE) and formation of 4-hydroxynonenal (4HNE). Arrows indicate positive staining. (C) The immunoblot bands of hepatic antioxidant enzymes. (D) The immunoblot bands of NOX4. Scale bar: 50μm. The ratio to β-actin was calculated by setting the value of ctrl as one. Results are mean ± SD (n=3–6). Results for bars that do not share a letter differed significantly among groups ($P<0.05$). Significant differences among groups are determined by ANOVA followed by Tukey's test. Control (Ctrl), ethanol (EtOH), ethanol combined with fisetin (E/Fisetin).

Figure 4.

Effect of fisetin on apoptosis in the liver of mice chronically fed ethanol for four weeks. (A) The hepatocellular apoptosis was assessed by TUNEL assay kit. The positive stainings were indicated by arrows. (B) The immunoblot bands of Fas, Fas ligand (FasL), cleaved caspase-3, and caspase-3. Scale bar: 50 μ m. The ratio to β -actin was calculated by setting the value of ctrl as one. Results are mean $\pm$ SD (n=3–6). Results for bars that do not share a letter differed significantly among groups ($P < 0.05$). Significant differences among groups are determined by ANOVA followed by Tukey's test. Control (Ctrl), ethanol (EtOH), ethanol combined with fisetin (E/Fisetin).

Figure 5.

Effect of fisetin on lipid formation and the expression of lipid metabolism-related protein/enzymes in the liver of mice chronically fed ethanol for four weeks. (A) Quantitative measurement of hepatic levels of triglycerides (TG) and (B) free fatty acids (FFA). (C) Plasma levels of adiponectin. (D) Immunoblot bands of AMP-activated protein kinase (AMPK) and p-AMPK. (E) Immunoblot bands of peroxisomal acyl-coenzyme A oxidase 1 (ACOX1) and cytochrome P450 4A (CYP4A). (F) Immunoblot bands of microsomal triglyceride transfer protein (MTTP). The ratio to β -actin was calculated by setting the value of ctrl as one. Results are mean $\pm$ SD ($n=3-6$). Results for bars that do not share a letter differed significantly among groups ($P<0.05$). Significant differences among groups are determined by ANOVA followed by Tukey's test. Control (Ctrl), ethanol (EtOH), ethanol combined with fisetin (E/Fisetin).

Figure 6.
Schematic diagram of the protective effect of fisetin on alcohol-induced liver injury in mice.
Ethanol (EtOH).