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# NRF2 mitigates acute alcohol-induced hepatic and pancreatic injury in mice

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ABSTRACT

Binge alcohol drinking is an important health concern and well-known risk factor for the development of numerous disorders. Oxidative stress plays a critical role in the pathogenesis of acute alcoholism. Nuclear factor erythroid 2 like 2 (NRF2) is a master regulator of cellular adaptive response to oxidative insults. However, the role of NRF2 in acute alcoholism and associated pathologies remains unclear. We found that *Nrf2*-knockout (*Nrf2*-KO) mice had exaggerated hypoglycemia and hypothermia and increased mortality compared to wildtype mice after binge ethanol exposure. This phenotype was partially rescued by providing warm environment and/or glucose administration. Acute high dose of alcohol exposure resulted in substantially worsened liver and pancreatic injuries in *Nrf2*-KO mice. Importantly, deficiency of *Nrf2* allowed severe pancreatitis and pancreatic β-cell injury with increased insulin secretion and/or leaking during binge ethanol exposure, which contributed to hypoglycemia. In contrast, a clinically used NRF2 activator dimethyl fumarate (DMF) protected against hypoglycemia and lethality induced by acute ethanol exposure. Furthermore, *Nrf2*-KO mice likely had defective hepatic acetaldehyde metabolism. Taken together, NRF2 plays an important protective role against acute binge alcohol-induced hepatic and pancreatic damage, which may be partially attributable to its primary regulating role in antioxidant response and impact on ethanol metabolism.

## 1. Introduction

Alcohol is a psychoactive drug with dependence-producing potential, which has been widely used in beverages as a recreational substance worldwide for centuries (Prom-Wormley et al., 2017). Recently, the number of alcoholics and binge (high dose, short-term) drinkers has dramatically increased around the world. According to the WHO, alcohol abuse caused 3.3 million deaths worldwide in 2012 (Wang et al., 2014). Thus, the health and associated economic impacts of excessive ethanol consumption on society, families and individuals is an important challenge to the public health system.

Long-term or excessive alcohol consumption often induces multi-organ pathology, including neurologic, hepatic, gastrointestinal and cardiovascular disease, even cancer (Klatsky, 2007; Rehm et al., 2003). In chronic alcohol abuse, the liver is primarily affected, as it is the primary organ responsible for metabolizing ethanol (Cederbaum,

*Abbreviations:* ADH1, alcohol dehydrogenase 1; ALD, alcoholic liver disease; ALDH, aldehyde dehydrogenase; AMS, amylase; ALT, alanine transaminase; ARE, antioxidant response element; AST, aspartate aminotransferase; CAT, catalase; CYP2E1, cytochrome P450 2E1; DMF, dimethyl fumarate; EtOH, ethanol; EtOH-WT, *Nrf2*-WT ethanol group; EtOH-KO, *Nrf2*-KO ethanol group; GC, gas chromatography; Gclc, glutamate-cysteine ligase; H&E, hematoxylin and eosin staining; Ho-1, Heme oxygenase-1; KO, knock out; LPS, lipase; Nqo1, NAD(P)H:quinone oxidoreductase 1; NRF2, nuclear factor erythroid 2 like 2; ROS, reactive oxygen species; Veh-WT, *Nrf2*-WT vehicle control group; Veh-KO, *Nrf2*-KO vehicle control group; WT, wild type

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2012). The increased levels of reactive oxygen species (ROS), production of acetaldehyde, induction of cytochrome P450 2E1 (CYP2E1) and direct damage to mitochondria, together contribute to alcoholic liver disease (ALD) (Louvet and Mathurin, 2015). ALD is an important component of liver diseases induced by alcohol abuse, whose spectrum involves simple hepatic steatosis, fibrosis, cirrhosis, and even liver cancer (O'Shea et al., 2010).

It has become apparent that the pancreas is another major affected organ of acute high dose alcohol exposure (Setiawan et al., 2017). The pancreas is a key digestive organ responsible for digestion of energy substances and glucose homeostasis. When inflamed, the pancreas releases digestive enzymes that attack pancreas itself as well as surrounding tissues. This self-digestion process enhances the progression of pancreatitis (Ji et al., 2009). Indeed, alcoholic pancreatitis accounts for 36% of all the cases of acute pancreatitis (Schneider et al., 2010), and often trigger worsening of pancreatitis. Although the pathogenesis of acute alcoholic pancreatitis is still unclear, the toxicity of alcohol and the metabolites, including acetaldehyde and ROS are potentially toxic. In the process of alcohol metabolism, ROS generated from various sources. Acetaldehyde formation (Niemela, 2001), activation of Kupffer cells, mitochondrial injury (Hoek et al., 2002), and induction of CYP2E1 have been proposed to promote alcohol-induced oxidative stress (Luedde et al., 2014). It appears that oxidative stress is a primary contributor to acute alcohol-induced pancreatitis, as ROS causes direct oxidative damage to lipids and proteins and distortion of redox-sensitive signal transduction pathways (Dong et al., 2016). Although the liver is the main organ oxidizing alcohol, there is a small amount of alcohol metabolized in the pancreas (Gukovskaya et al., 2002; Haber et al., 2004). In the pancreas, ethanol is metabolized through both oxidation and non-oxidation pathways and their metabolic products may contribute to many deleterious effects in this organ. The change of the intracellular redox balance brought about by diminishing the $NAD^+$/NADH ratio caused by pancreatic alcohol metabolism clearly speeds up local metabolic alterations and acinar cell damage (Perez et al., 2015).

Nuclear factor erythroid 2 like 2 (NRF2), a member of the cap 'n' collar basic-region leucine zipper transcription factor family, plays critical roles in regulating adaptive antioxidant response. Under normal conditions, NRF2 activity is suppressed by interaction with the KEAP1-Cul3 ubiquitin ligase complex. With cellular oxidative stress, KEAP1 becomes inactivated which induces accumulation of NRF2 in the nucleus. NRF2 forms nuclear heterodimers with small Maf proteins and binds to antioxidant response elements (AREs), activating expression of ARE-dependent genes. In addition to mitigating oxidative stress, NRF2 plays important roles in the pathogenesis of various disease processes (Canning et al., 2015; Suzuki and Yamamoto, 2015). Prior work showed that NRF2 is activated by ethanol exposure (Ma, 2013; Shin et al., 2013). Mice with global knockout of *Nrf2* chronically given the Lieber-deCarli liquid alcohol diet have increased mortality compared to wild-type (Lamle et al., 2008). An increased level of acetaldehyde, rapid accumulation of lipids and aggravated Kupffer cells-mediated inflammation are also observed in alcohol-exposed *Nrf2*-knockout (*Nrf2*-KO) mice (Lamle et al., 2008), indicating a much more rapid onset and progression of alcoholic liver disease. Indeed, it appears that NRF2 is a critical factor in alcoholic liver disease. Many compounds and plant extracts can protect liver cells from alcohol exposure by activating the NRF2-ARE signaling pathway (Lu et al., 2015; Choi et al., 2015). In addition, the administration of NRF2 activators may attenuate acute pancreatitis (Dong et al., 2016). However, the precise role of NRF2 in acute or chronic alcoholic pancreatitis remains unclear.

Therefore, we carried out this study using *Nrf2*-KO mice with acute high-dose (i.e. 'binge') ethanol exposure. We found that deficiency of *Nrf2* substantially affected the expression of ethanol metabolism enzymes caused by binge ethanol exposure, and also precipitated acute alcoholic liver injury and pancreatitis. A clinically used drug dimethyl fumarate (DMF), an NRF2 activator, protected against alcohol-related intoxication and improved survival of mice after binge exposure. These findings provide new scientific insight for prevention and potential remedial actions in dealing with the impact of binge drinking.

## 2. Materials and methods

### 2.1. Animals

*Nrf2*-KO mice on a C57BL/6 background were developed by Dr. Masayuki Yamamoto (Tohoku University, Japan) (Itoh et al., 1997). *Nrf2*-WT mice and *Nrf2*-KO littermates were generated from a breeding colony at the animal facility of China Medical University. Mice were housed in a specific pathogen free room, maintained at a controlled temperature (23 ± 1 °C) and on successive 12-h light/dark cycles (lights on: 06:00–18:00). Water and mouse chow diet (Shukebeita Specific Pathogen Free Mouse Maintenance Diet, Xietong Organism, Jiangsu, China) were provided *ad libitum*. Genotyping was performed by PCR using genomic DNA that was isolated from tail snips, as described previously (Xue et al., 2013). Female mice were used in the present study. All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of China Medical University (Shenyang, China) following all current guidelines for animal care and welfare.

### 2.2. Experimental design and tissue collection

To assess survival rate after binge exposure, female mice (8–12 wks old) were randomly sorted to form two groups: *Nrf2*-WT ethanol group (EtOH-WT) and *Nrf2*-KO ethanol group (EtOH-KO). Following 4 h fasting, mice were administrated a single intragastric dose of ethanol (6.0 g/kg BW), and then closely monitored for 36 h. Blood glucose levels and core temperature were measured at the indicated time points (see Fig. 1 and Fig. 3) using the methods previously described (Zheng et al., 2015). In order to rescue the hypoglycemia and hypothermia induced by binge ethanol administration, some mice were treated with 5% glucose injection intraperitoneally and/or a warm pad (37 °C). For further analysis of the impact of ethanol, a new set of mice were randomly divided into four groups: *Nrf2*-WT vehicle group (Veh-WT), *Nrf2*-KO vehicle group (Veh-KO), EtOH-WT and EtOH-KO. The mice received a lower dose of ethanol (4.8 g/kg BW) at time 0 then received another identical dose 12 h later. Control mice received an equivalent volume of distilled water (1.2 ml/kg BW) intragastrically at the same time with ethanol groups. The mice were euthanized 12 h after the last dose of ethanol by carbon dioxide asphyxia. Another set of experiments was designed to see the effect of DMF on mice exposed to binge ethanol. The mice were divided into vehicle control (Veh) and DMF-treated groups. Mice were either pretreated with 25 mg/kg of DMF intragastrically or an equivalent volume of carboxymethyl cellulose twice daily for five days. Then, they received two high doses of 6.0 g/kg BW of ethanol intragastrically 12 h apart. Survival rate, blood glucose, and core temperature were assessed, and various relevant tissues were collected and processed for transcripts or histopathological analyses. The blood and tissue samples were collected and stored at −80 °C until subsequent determination. Parts of liver and pancreas tissues were collected and fixed in 4% of paraformaldehyde buffer for histopathology.

### 2.3. Plasma biochemistry

Fresh blood samples were centrifuged at 5000 × g for 5 min at 4 °C. The levels of aspartate aminotransferase (AST), alanine transaminase (ALT), amylase (AMS) and lipase (LPS) activity in the plasma samples were measured using specific kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions (Wu et al., 2017; He et al., 2017). Plasma insulin level was determined following the standard protocol of a mouse enzyme linked immunosorbent assay kit from Elabscience Biotechnology

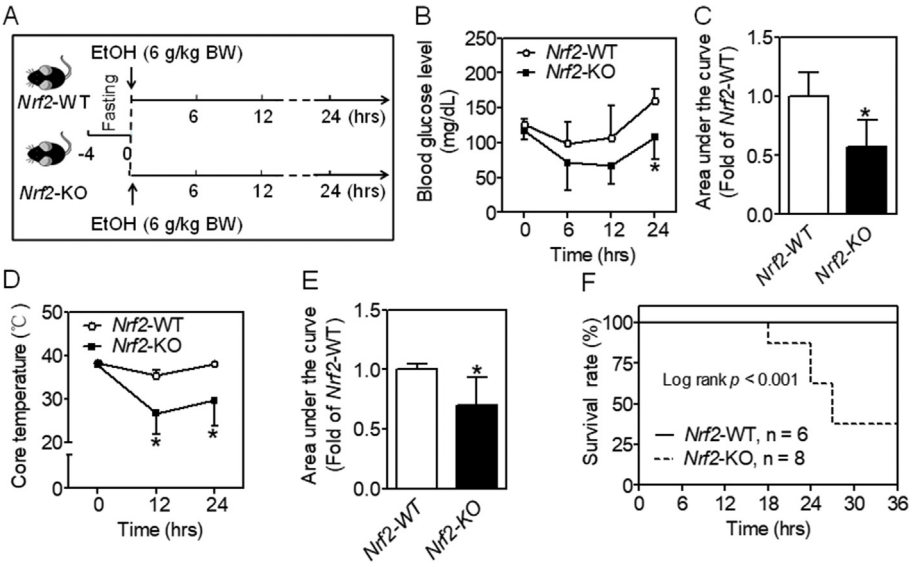


**Fig. 1.** *Nrf2*-KO mice showed systemic toxic effects and increased mortality after binge ethanol exposure. (A) Schematic of how acute 'binge' alcohol intoxication was induced in *Nrf2*-KO and *Nrf2*-WT mice by a single high dose ethanol exposure (6 g/kg BW; intragastrically; time = 0). All mice were initiated with 4 h of fasting before ethanol exposure. Blood glucose (B), core temperature (D) were assessed at various times thereafter. Quantification of the areas under the curves for the glycemic excursions (B) and temperature changes (D) shown in panels (C) and (E). (F) Survival curve showed reduced survival in ethanol exposed *Nrf2*-KO mice. Values were expressed as mean ± SD. *, $p < 0.05$ compared with *Nrf2*-WT mice. n = 5–8 in (B–F).

Co., Ltd (Wuhan, China). All biochemical assessments in the plasma were completed within one week of sample collection.

### 2.4. Histological and immunohistochemical analysis

The samples of liver and pancreas were fixed in 4% paraformaldehyde buffer overnight and then transferred to 70% ethanol. Tissues were embedded in paraffin, sectioned at 3 μm and stained with hematoxylin and eosin (H&E).

For immunohistochemistry, the sections were mounted on poly-L-lysine slides. The protein levels of insulin and cleaved-Caspase 3 were assessed using primary rabbit anti-insulin polyclonal antibodies (insulin, dilution 1:3000, Santa Cruz Biotechnology, Santa Cruz, CA; cleaved-Caspase 3, dilution 1:200, Cell Signaling Technology, Danvers, MA), following kit instructions (IHC-DAB; Beijing Zhongshan Jinqiao Biological Technology Co., Beijing, China) (Zheng et al., 2015).

### 2.5. RT-qPCR

Total RNA was isolated from tissues using TRIzol reagent (Invitrogen, Carlsbad, USA). cDNA was generated using Prime Script RT reagent Kit (Takara, Dalian, China). Real-time PCR reactions were

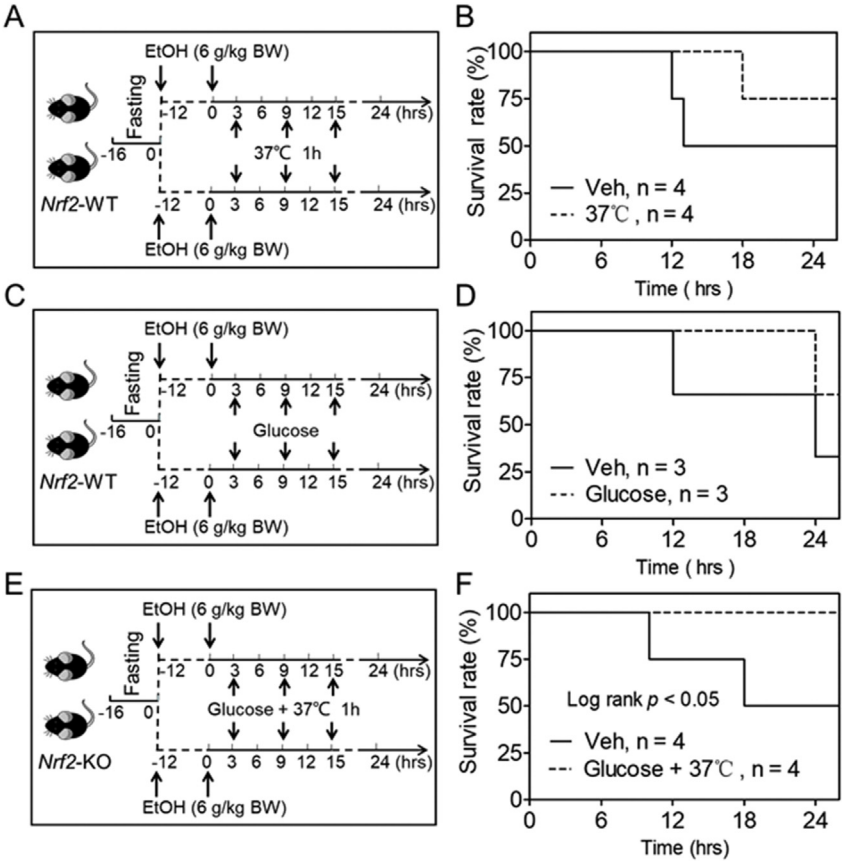


**Fig. 2.** Treatment with glucose administration and warm pad alleviated the lethality caused by acute ethanol exposure in mice. Diagram of how to interfere in acute 'binge' alcohol intoxication of mice by administrated to a warm pad (A), glucose (C) or both (E). All mice were fasted for 4 h before the ethanol exposure. Survival curves of *Nrf2*-WT (B, D) and *Nrf2*-KO (F) or both mice treated with a warm pad (B), and glucose (D). n = 3–4.

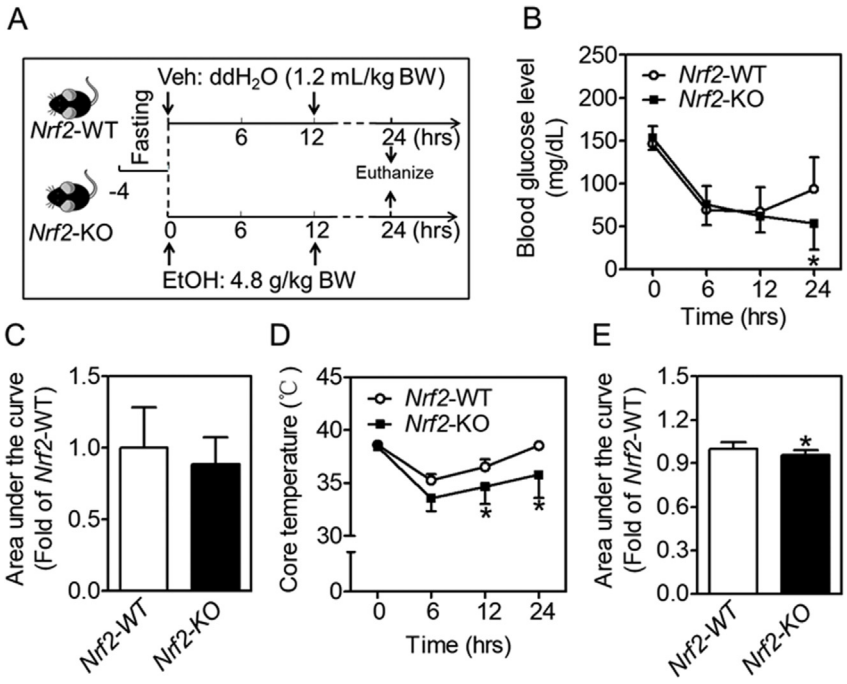


**Fig. 3.** *Nrf2* deficiency caused hypoglycemia and hypothermia after two low-dose acute binge ethanol exposures. (A) Schematic of two dose acute alcohol intoxication model. All mice were initiated with 4 h of fasting before ethanol exposure. Then groups of *Nrf2*-KO and *Nrf2*-WT mice received ethanol (4.8 g/kg BW; intragastrically) or water ($ddH_2O$; 1.2 ml/kg BW; intragastrically) at time 0 and again at 12 h. The mice were euthanized 12 h after the second dosing (24 h into the experiment). Blood glucose levels (B) and core temperatures (D) were measured. Area under the curve analysis in figures (C) and (E) for the glycemic excursions (B) and temperature changes (D). *Nrf2*-KO mice developed marked hypoglycemia and hypothermia. Values were expressed as mean ± SD. *, $p < 0.05$ compared with *Nrf2*-WT mice. n = 5–6.

performed using SYBR Premix EX Taq Kit (Takara, Dalian, China) and QuantStudio 6 Flex real-time PCR system (Applied Biosystems) (Cui et al., 2017). mRNA of *Cyp2e1*, *catalase* (*Cat*), *alcohol dehydrogenase* (*Adh*), *acetaldehyde dehydrogenase* (*Aldh2*), *Aldh1a1*, *glutamate-cysteine ligase* (*Gclc*), *NAD(P)H:quinone oxidoreductase 1 (Nqo1)*, *heme oxygenase-1* (*Ho-1*) *and Gadph* were amplified using the following PCR conditions: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Data were analyzed using the $2^{-\Delta\Delta CT}$ method. Primers were synthesized by Takara Co., Ltd. (Takara, Dalian, China). The PCR primer sequences are listed in https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3131439/-SD1, supplementary Table 1. All mRNA levels are presented relative to *Gapdh* of *Nrf2*-WT vehicle samples.

### 2.6. ADH and ALDH enzyme activities

Liver samples were carefully excised, weighed, and made into 10% homogenates using ice cold phosphate buffered saline buffer with a tissue homogenizer. The samples were centrifuged at 2500 × g for 10 min at 4 °C. The activities of ADH and ALDH in the liver were determined using the kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's directions (Luo et al., 2017).

### 2.7. Measurement of ethanol concentration

To determine ethanol concentrations in mouse liver and blood, a headspace gas chromatography (GC) was used in Laboratory of Chemistry, School of Fundamental Sciences, China Medical University (Molina et al., 1992; Jamal et al., 2003). Blood samples were taken from the venae angularis immediately after sacrifice and anticoagulated with heparin. For the preparation of liver samples, fifty μg liver was homogenized in PBS to make a 10% homogenate, and then placed in glass vials with the same volume of internal standard, n-butyl alcohol. The vials were sealed with an aluminum cap sealing clamp and were heated for 30 min. Afterward, samples were analyzed in GC with reporting integrator (Shimadzu, Kyoto, Japan), using an ultra-micro method of direct sample injection.

### 2.8. Statistical analysis

All data are expressed as mean ± standard deviation. Statistical analysis was performed using GraphPad Prism 5 software (Graphpad Software Inc., San Diego, CA), with a $p < 0.05$ considered as significant. Statistical differences were determined by an unpaired Student's *t*-test or by two-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test. In Kaplan-Meier analysis, the comparison between two survival curves is made by the log-rank (Mantel-Cox) test. Differences between two unpaired groups were tested with the two-tailed Mann-Whitney *U* test.

## 3. Results

### 3.1. Nrf2-KO mice exhibit systemic effects and increased lethality after a binge of ethanol ingestion

After administration of high dose of ethanol (see Fig. 1A and S1), the vital signs of the *Nrf2*-KO and *Nrf2*-WT mice were closely monitored. Blood glucose levels after ethanol ingestion decreased at 6–12 h, then started to increase in both genotypes. However, throughout the observation period, the blood glucose levels were always lower in *Nrf2*-KO mice following ethanol exposure compared with *Nrf2*-WT mice. At 24 h, blood glucose was 1.5-fold less in *Nrf2*-KO mice than WT (Fig. 1B). Similarly, core temperature declined significantly in *Nrf2*-KO mice down to about 26 °C by 12 h after ethanol exposure, and stayed below 30 °C even at 24 h (Fig. 1D). In contrast, the core temperature in binge ethanol-treated *Nrf2*-WT mice was essentially normal. Areas under the blood glucose and core temperature curves were significant reduced in *Nrf2*-KO mice compared to *Nrf2*-WT mice (Fig. 1C and E). Most importantly, this binge dose of ethanol caused about a 70% mortality rate in *Nrf2*-KO mice over 36 h, while no deaths were observed in the *Nrf2*-WT mice, Kaplan-Meier survival curve showing increased mortality in *Nrf2*-KO mice (Fig. 1F). These results indicate that *Nrf2*-KO mice are highly susceptible to hypoglycemia, hypothermia and mortality induced by binge ethanol ingestion.

### 3.2. Treatment with warming and glucose alleviates the lethality induced by a binge of ethanol ingestion

To assess the contribution of hypoglycemia and hypothermia in the lethality induced by ethanol administration, we administrated the *Nrf2*-WT mice with a warm pad (37 °C) (Fig. 2A) or 5% glucose injection intraperitoneally (Fig. 2C) alone to see whether a single factor can effectively rescue lethality induced by binge ethanol exposure. As shown in Fig. 2B and D, the death induced by acute ethanol exposure were rescued when *Nrf2*-WT mice were given a warm pad (37 °C) or 5% glucose, indicating that the hypoglycemia and hypothermia are the causes of death in acute alcoholism. To ascertain whether the elevated lethality caused by acute ethanol exposure in *Nrf2*-KO mice is attributed to hypoglycemia and hypothermia, the effect of warm pad & glucose administration was evaluated. As shown in Fig. 2E and S2, a binge dose of ethanol caused about a 50% mortality rate in *Nrf2*-KO mice with Veh over 26 h, consistent with the results in Fig. 1F. Interestingly, there was no death observed in the intervention group, which was treated with warming pad and glucose administration (Fig. 2F and S2).

### 3.3. Deficiency of Nrf2 causes hypoglycemia and hypothermia after two low doses of ethanol exposure

To analyze the effects of *Nrf2* deficiency on multiple binge-like ethanol exposures, the two genotypic mice were given two lower doses of ethanol intragastrically that were non-lethal and allowed tissue collection particularly in the *Nrf2*-KO mice (Fig. 3A). Mice of both genotypes received either water (Veh-group) or ethanol, intragastrically, at 0 and 12 h and were euthanized at 24 h. While the levels of blood glucose decreased in all the ethanol-treated mice between 6 and 12 h, *Nrf2*-WT mice returned to normal by 24 h (Fig. 3B). In contrast, the blood glucose levels were significantly lower in *Nrf2*-KO mice than *Nrf2*-WT mice at 24 h. Notably, the areas under the curves of glycemic excursions marginally but significantly decreased in *Nrf2*-KO mice compared to *Nrf2*-WT (Fig. 3C). The core body temperature of both *Nrf2*-WT and *Nrf2*-KO mice declined to the lowest at 6 h and then gradually recovered (Fig. 3D) thereafter. *Nrf2*-WT mice showed higher body temperatures and gradually returned to normal levels at 24 h after the first ethanol administration. However, *Nrf2*-KO mice had lower body temperatures at 12 and 24 h than *Nrf2*-WT mice post ethanol treatment. As shown in Fig. 3E, the areas under the curves showed that core temperature levels remained significant lower in *Nrf2*-KO mice.

### 3.4. Ethanol ingestion may increase hepatic acetaldehyde levels in Nrf2-KO mice

We hypothesized that the differing impact of ethanol relative to *Nrf2* genotype could be based on differing alcohol metabolism. Thus, we tested whether *Nrf2*-KO mice exhibited different ethanol metabolism activity compared to *Nrf2*-WT mice. Most of ingested ethanol is metabolized by ADH in the liver. Two other enzymes, CYP2E1 and CAT also can oxidize ethanol. Basal expression of *Adh*, *Cyp2e1* and *Cat* were not impacted by *Nrf2* deficiency (Fig. 4A). Hepatic ADH activity also showed no difference between the genotypes (Fig. 4B). Consistent with these results, blood and hepatic ethanol concentrations did not differ based on genotype after ethanol treatment (Fig. 4D and E). After ethanol oxidation, a potentially toxic product acetaldehyde is converted into acetate by ALDH. Thus, we assessed mRNA levels of *Aldh2*, *Aldh1a1* and the activity of ALDH. As shown in Fig. 4A, basal levels of *Aldh1a1* transcript were reduced in *Nrf2*-KO mice compared to *Nrf2*-WT mice. Further, ALDH activity (Fig. 4C) was significantly lower in *Nrf2*-KO mice compared to *Nrf2*-WT mice after ethanol exposure. Thus, deficiency of *Nrf2* may lead to defective hepatic acetaldehyde metabolism.

### 3.5. Nrf2 deficiency aggravates acute ethanol-induced liver damage in mice

Histological evaluation showed swollen hepatocytes, and cytoplasm rarefaction in the liver of mice received a binge ethanol administration. However, there was no pathologically discernible difference between the genotypes (Fig. 5A). Plasma ALT, a circulating marker of liver damage, was significantly elevated in ethanol-treated mice compared to control. Further, the ethanol-induced elevation of plasma ALT increased in *Nrf2*-KO mice compared to *Nrf2*-WT mice (Fig. 5B and C). Similarly, plasma levels of AST were also elevated after ethanol administration. *Nrf2*-KO mice showed higher levels of AST at 12 h than *Nrf2*-WT mice (Fig. 5D), and the areas under the curves were slightly higher in *Nrf2*-KO mice than those in *Nrf2*-WT mice (Fig. 5E). Collectively, these data demonstrated that binge ethanol exposure induced liver injury, and *Nrf2* deficiency aggravated ethanol-induced liver injury in mice.

### 3.6. Ethanol-exposed Nrf2-KO mice exhibit striking pancreas injury and hyperinsulinemia

Acute pancreatitis was clearly observed in mice exposed to binge ethanol treatment (Fig. 6A). Acute pancreatitis developed together with islets damage and acinar cell edema in all mice after ethanol ingestion. Quantitative analysis showed that pancreatic islets were strikingly injured and necrosis with nuclear broken and even disappearance in *Nrf2*-KO mice, but little in *Nrf2*-WT mice (Fig. 6B). To validate whether binge ethanol exposure precipitated pancreatitis, AMS and LPS activities were evaluated. Acute ethanol exposure increased plasma AMS activity in both groups and showed no difference between *Nrf2*-WT and *Nrf2*-KO mice (Fig. 6C). Further, plasma LPS activity in *Nrf2*-KO was significantly higher than that in *Nrf2*-WT mice (Fig. 6D). Nevertheless, the plasma AMS activity is inconsistent with the severity of the mice, and reflects the changes of pancreatic exocrine portion. These pathological findings implied that ethanol-induced islet damage may be a primary driver of hypoglycemia and subsequent death in *Nrf2*-KO mice, which was rescued by glucose administration (Fig. 2D and F). The damage to pancreatic islets can lead to β-cell death and leaking of large amounts of insulin, leading to hypoglycemia. Therefore, we assessed cleaved-Caspase 3 (a marker of apoptosis) and insulin in the pancreas immunohistochemically. Compared with the control group, the pancreas of *Nrf2*-KO mice clearly has more cleaved-Caspase 3 expression (Fig. 6A), which indicated more apoptosis proceeded in endocrine and exocrine portions induced by ethanol or digestive enzymes. Consistent with H&E staining, insulin immunohistochemistry analysis demonstrated that pancreatic islets were broken and insulin was released in large amounts, which even spilled and leaking into the surrounding tissues in *Nrf2*-KO mice (Fig. 6A), even blood system. It was proven by higher plasma insulin levels in *Nrf2*-KO mice after the ethanol ingestion (Fig. 6E). Consistent with these findings, the blood glucose levels were lower in ethanol-treated *Nrf2*-KO compared to *Nrf2*-WT mice. Thus, these results showed that *Nrf2*-KO mice developed severe pancreas damage in both endocrine and exocrine portions after binge ethanol treatment. This process caused massive insulin leaking, which leads to markedly decreased blood glucose levels in ethanol-treated *Nrf2*-KO mice. This hyperinsulinemia was likely a key factor in the lethality of acute high-dose ethanol in *Nrf2*-KO mice.

### 3.7. DMF mitigates the adverse impact of acute alcohol ingestion

In order to test the hypothesis that NRF2 protects against acute ethanol-induced hypoglycemia and death in mice, wildtype mice were treated with DMF as shown in Fig. 7A. The mRNA expression of NRF2 regulated genes *Gclc* and *Nqo1* were induced by DMF treatment in the liver, but not *Ho-1*. After binge alcohol treatment, 86% of DMF-treated mice survived, while nearly 40% of non-treated mice died from binge ethanol (Fig. 7C). As expected, the DMF treatment moderated blood glucose levels after ethanol exposure (Fig. 7D and S3), and areas under

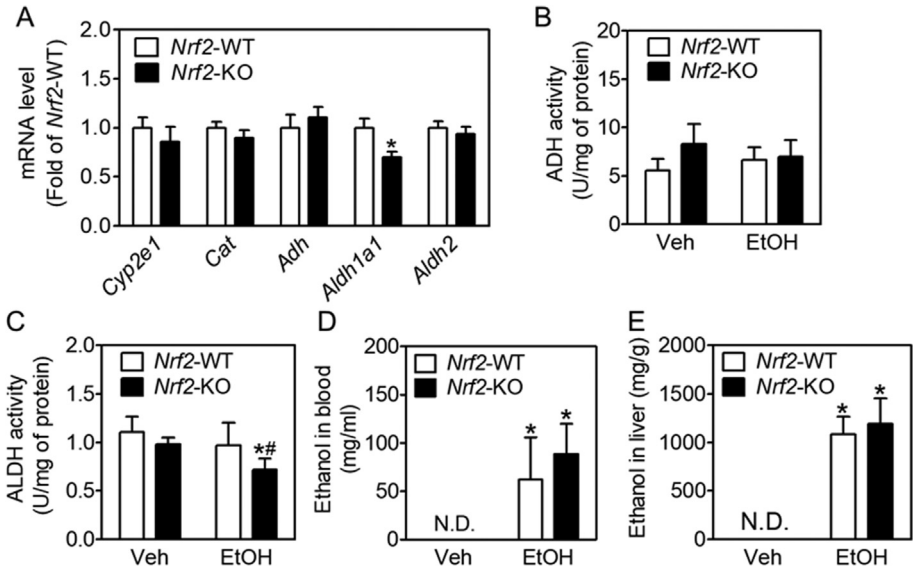


**Fig. 4.** The capacity for ethanol metabolism after acute ethanol exposure in mice. Gene expression levels of *Cyp2e1*, *Cat*, *Adh*, *Aldh1a1* and *Aldh2* (A) were determined by real-time qPCR. Activities of ADH (B) and ALDH (C) were measured in liver. Blood (D) and liver ethanol levels (E) were measured in total blood and liver extracts. Values were expressed as mean ± SD. *, $p < 0.05$ compared with Vehicle of the same genotype. #, $p < 0.05$ compared with *Nrf2*-WT mice ethanol administration. n = 5–6.

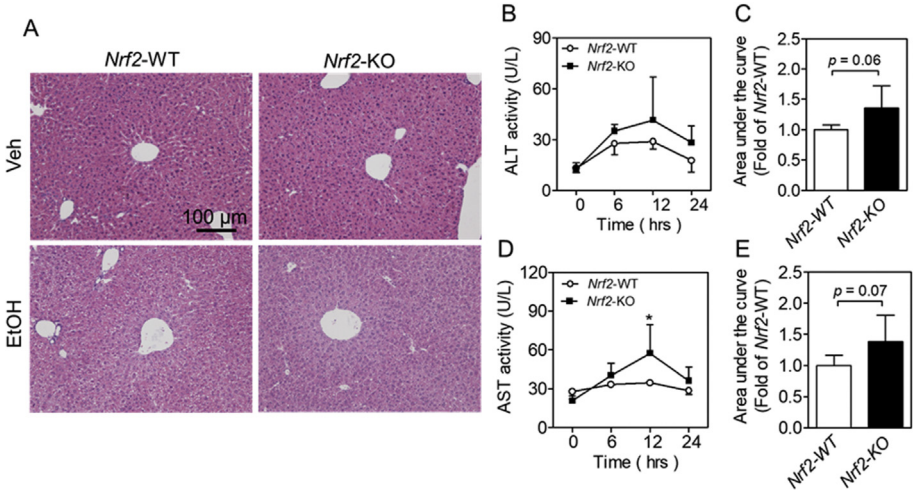


**Fig. 5.** *Nrf2* deficiency aggravated liver damage induced by ethanol exposure in mice. Liver sections stained with H&E (A) from the four experimental groups as indicated (scales bar = 100 μm). Dynamic changes of ALT (B) and AST (D) in the plasma of mice after ethanol exposure. Areas under the curves shown in panels (C) and (E) resulting from Figure (B) and (D). Values were expressed as mean ± SD. *, $p < 0.05$ compared with Veh of the same genotype. n = 5–6.

the curves were significantly increased in the DMF-pretreated group compared with Veh group (Fig. 7E). Core temperature was reduced following ethanol exposure in both groups, but returned to normal at 24 h in DMF-pretreated mice (Fig. 7F and G and S3). These results showed that DMF pretreatment activated NRF2 and protected from acute ethanol-induced hypoglycemia, hypothermia and death in mice.

## 4. Discussion

Alcoholism and binge drinking are dramatically increasing around the world. Alcohol intoxication is the result of ingesting substantial quantities of alcoholic beverages in a short period of time, which induces a cascade of harmful effects including hypothermia, hypoglycemia, seizures, coma and death (Jung and Namkoong, 2014; Vonghia et al., 2008). Beyond symptomatic treatment, there is little or no effective clinical intervention for binge alcohol intoxication. This study was undertaken to determine whether NRF2 participates in ethanol metabolism and may protect against aspects of binge ethanol-mediated pathology. We observed that the loss of *Nrf2* gene in mice caused a higher rate of death after binge ethanol treatment. Furthermore, we found an exacerbated, severe pancreatitis in *Nrf2*-KO mice associated with increased insulin secretion and leaking likely resulting in a lethal hypoglycemia. A clinically utilized NRF2 activating drug DMF prevented acute binge alcohol-induced hypoglycemia and hypothermia, and improved survival in mice. All these data points towards NRF2 having a key role in defining sensitivity to the adverse impact of binge alcohol consumption. Importantly, manipulation of NRF2 may have the potential to modify the clinical outcome of acute binge alcohol overdose.

Ethanol is oxidized to acetaldehyde mainly via ADH with about 10% by CYP2E1 (Ansari et al., 2016; Paton, 2005). Acetaldehyde is then converted into its non-carcinogenic byproduct acetate by ALDH (Shalbueva et al., 2013). Ethanol metabolism is linked to an induced respiratory burst, impairment of oxidative phosphorylation, and activation of NADPH oxidase. Oxidation of alcohol contributes to ROS production, which can activate NRF2 (Jin et al., 2012). Activated NRF2, in turn, can lead to the expression of target genes which maintaining redox homeostasis in the face of oxidative stress. In the present study, the expression of *Adh*, *Cyp2e1* and *Cat* genes were not modified by altered *Nrf2* expression. Consistent with these results, we found that blood and hepatic ethanol concentrations in *Nrf2*-KO mice were not different from *Nrf2*-WT after binge alcohol treatment. ALDH2 is primarily responsible for acetaldehyde metabolism, and ALDH1, which encoded by the *Aldh1a1* gene (Vasiliou and Pappa, 2000; Agarwal, 2001), contributes to acetaldehyde oxidation. The absence of *Nrf2* reduced the activity of acetaldehyde metabolizing enzymes, which likely was the basis for high hepatic acetaldehyde levels seen in the previous study of chronic ethanol-fed *Nrf2*-KO mice (Lamle et al., 2008).

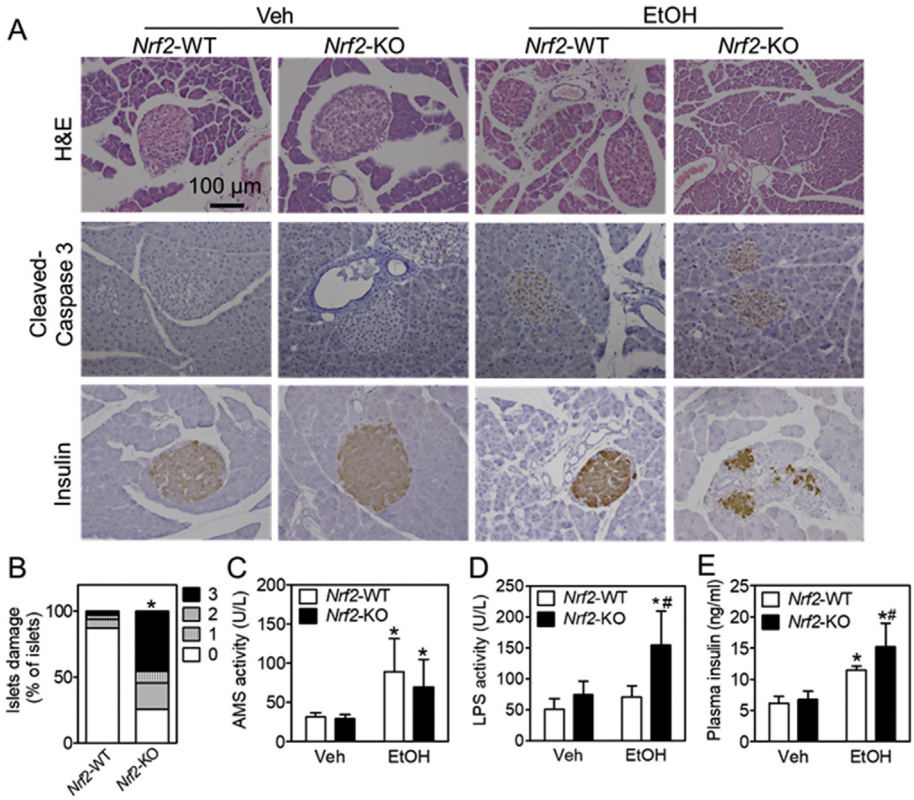


**Fig. 6.** Ethanol-exposed *Nrf2*-KO mice exhibited striking pancreas injury. (A) H&E staining and immunohistochemistry, scale bar = 100 μm. (B) Pancreatic islets were scored using the following scale: grade 0 = 0% nuclear necrosis; grade 1 = 1–10% nuclear necrosis; grade 2 = 11–50% nuclear necrosis; grade 3 > 50% nuclear necrosis. Data was analyzed for statistical significance by Mann-Whitney *U* test, with differences between *Nrf2*-WT and *Nrf2*-KO mice for islets damage being $p < 0.001$ and differences for non-islet damage being $p < 0.001$. Levels of AMS activity (C), LPS (D) and circulating insulin (E) in the plasma after acute ethanol exposure (intragastrically). Values were expressed as mean ± SD. *, $p < 0.05$ compared with Veh of the same. #, $p < 0.05$ compared with *Nrf2*-WT mice after ethanol administration. n = 4–6.

ALD is an important component of liver pathology induced by alcohol abuse. According to the biopsy findings in epidemiological studies, more than 90% of heavy drinkers developed steatosis and approximate 20–30% of alcoholics progress to severe forms of ALD (Ceni et al., 2014). In the prior studies, NRF2 prevented alcohol induced fulminant liver injury *in vivo* due to decreased acetaldehyde metabolism and inflammation mediated by Kupffer cells in *Nrf2*-KO mice (Lamle et al., 2008). *Nrf2* deficient mice showed a decrease in hepatic fat synthesis with inhibited SREBP1 activity and *Scd1* expression by excessive ethanol exposure, confirmed in a '*Nrf2* gene dose-response'

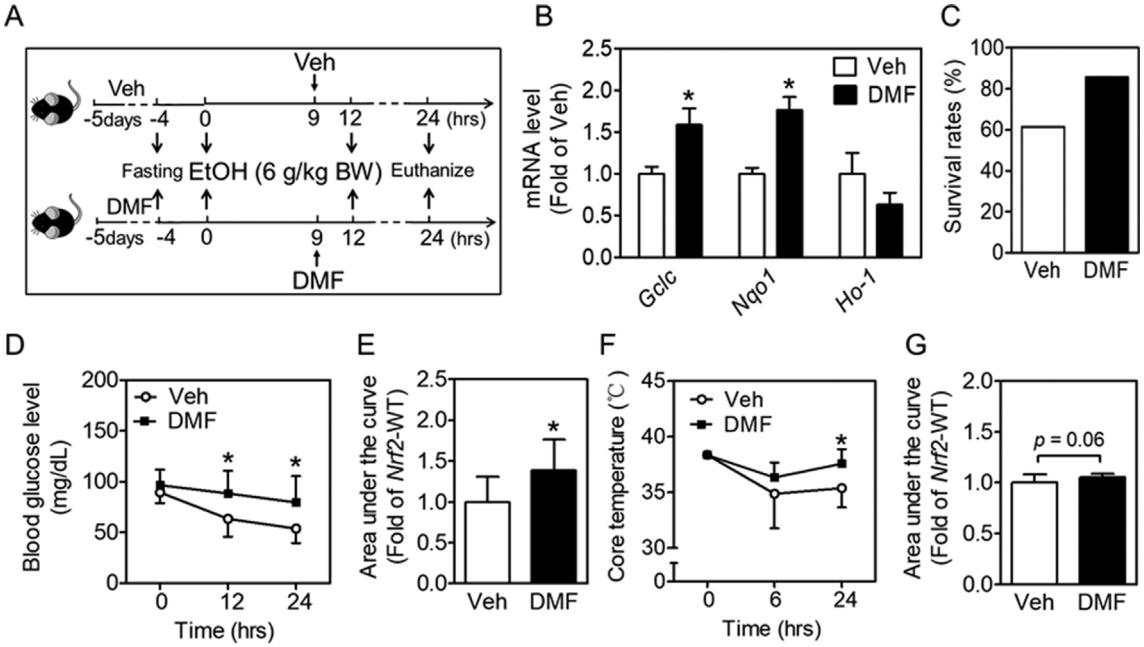


**Fig. 7.** DMF ameliorated acute alcohol exposure-induced lethality in mice. (A) Schematic illustration of DMF pretreatment of acute alcohol intoxication in mice. Two groups of mice initially received either 25 mg/kg BW of DMF or Veh twice daily for 5 days as a pretreatment. All mice were initiated with 4 h fasting before ethanol exposure, followed by two doses of ethanol (6 g/kg BW; intragastrically). (B) Gene expression levels of *Gclc*, *Nqo1* and *Ho-1* in liver were determined by RT-qPCR. (C) DMF-pretreated mice (n = 14) showed increased survival rate compared with control mice (n = 13) after acute ethanol exposure. Blood glucose levels (D) and core temperatures (F) were measured. Panels (E) and (G) illustrated the areas under the curves for glycemic excursions (D) and the change of core temperatures (F). Values were expressed as mean ± SD. *, $p < 0.05$ compared with Veh mice. n = 6–9 in (D–G).

model, including *Nrf2*-null, wild-type, *Keap1*-KD and *Keap1*-HKD mice (Wu et al., 2012). In addition, pretreatment with an NRF2 activator, 3H-1,2 dithiole-3-thione, significantly increased antioxidants as well as diminished ethanol-induced apoptosis in mouse embryos (Dong et al., 2008). Our data showed that acute alcohol administration severely damaged hepatocytes, evidenced by intracellular enzymes (AST and ALT) leaking into blood and corollary pathological changes. Furthermore, *Nrf2* deficiency accelerated liver damage in mice after acute ethanol administration. Thus, ethanol-mediated liver injury likely caused disproportional mortality in the *Nrf2*-KO mice.

We focused additional investigation on the pancreas, another organ commonly disrupted by alcohol abuse. Alcoholism is one of the major etiological factors for pancreatitis. Excessive and binge drinkers tend to experience more serious pancreatitis with clinical complications which result in higher mortality (Lankisch et al., 2015). The pancreas is a complex tissue, which contains both endocrine and exocrine portions. The endocrine pancreas is composed of the islets, amounting to only approximately 1%–2% of the organ. The endocrine pancreas mainly contains α and β-cells, which are responsible for the production and secretion of glucagon and insulin to maintain glucose homeostasis. The exocrine pancreas comprises a large majority of the pancreas and produces multiple digestive enzymes. It is well known that ethanol and acetaldehyde have deleterious effects on both exocrine and endocrine cells of the pancreas (Norton et al., 1998). ROS, causing direct oxidative damage to lipids and proteins, triggers the pathogenesis of alcoholic pancreatitis. Furthermore, pancreatic β-cells appear to have a relatively greater risk of oxidative damage than other tissues owing to lower activity of various antioxidant enzymes, which typically reduce tissue vulnerability to oxidative stress (Tiedge et al., 1997). In our previous studies, we found that the NRF2-mediated antioxidant response mitigated oxidative stress-induced pancreatic β-cell dysfunction, oxidative damage and cell death (Yang et al., 2012; Fu et al., 2015). In addition, some compounds have been reported to protect against pancreatitis by activating the NRF2-ARE signaling pathway. Sulforaphane, an NRF2 activator, protects against pancreatic acinar cell injury by modulating NRF2-mediated oxidative stress and NLRP3/NF-κB inflammatory pathways in rodents (Dong et al., 2016). Treatment with the synthetic triterpenoid RTA dh404 alleviates acute pancreatitis by lowering oxidative stress and reducing inflammatory mediators (Robles et al., 2016).

In the current study, we found that *Nrf2*-KO mice developed severe damage in both endocrine and exocrine portions of the pancreas after ingestion of ethanol. The β-cells injury was aggravated by self-digestion due to pancreatic enzymes released from the damaged exocrine portion, which likely caused massive insulin leaking into plasma. However, insulin hypersecretion was also occurred before acute ethanol-induced severe pancreatitis and pancreatic islets necrosis, which was proven by several groups (Nogueira et al., 2014; Huang and Sjoholm, 2008; Adner and Nygren, 1992). In the first several hours post ethanol ingestion, insulin secretion was evoked by a massive redistribution of pancreatic blood flow and the pancreatic microcirculation changes, leading to hypoglycemia (Nogueira et al., 2014; Huang and Sjoholm, 2008). At the late stage of ethanol-induced severe pancreatitis, pancreatic islets were necrosis and damaged by digestive and lysosomal enzymes. The combination of insulin hypersecretion evoked by acute ethanol exposure and insulin granules leaking from broken pancreatic β-cells most likely contributes to the marked decrease of blood glucose in ethanol-exposed *Nrf2*-KO mice compared to mild changes in that of WT mice. The massive insulin leaking and resultant severe hypoglycemia are major factors contributing to mortality in *Nrf2*-KO mice, since glucose administration (with or without warming) can rescue the death induced by acute alcohol exposure. Of note, the effect of *Nrf2* deficiency on thermo-regulation center hypothamus and neurons should be investigated in future studies.

At present, there are no specific drugs for pancreatitis, and the treatment is mainly supportive. Recently, DMF has been shown to be an NRF2 activator, and used in the clinical treatment of patients with psoriasis, cancer and multiple sclerosis (Linker et al., 2011; Ashrafian et al., 2012; Held et al., 1988; Bomprezzi, 2015; Sheremata et al., 2015). In prior studies, DMF treatment attenuated acute and chronic pancreatitis and islet dysfunction in rats (Robles et al., 2014, 2015). However, any such capacity of DMF has never been specifically studied in alcohol-related diseases. In the current study, DMF was found to prevent alcohol-induced hypoglycemia and hypothermia which, in turn, appeared to reduce the lethal effects of high dose binge alcohol exposure. The protective effect of DMF may be, at least in part, attributed to the activation on antioxidant response. However, considering hypothalamus is the most important thermoregulatory center in the body, and hypothermia is seen in *Nrf2*-KO mice, we cannot exclude that NRF2 in the nervous system plays a pivotal role in relieving alcohol-induced hypothermia by DMF treatment. Therefore, our systematic *Nrf2* deficiency model is limited and a cell-specific *Nrf2* deficiency model may be more informative for future studies.

In summary, our results show that NRF2 is a critical regulator of mortality in a binge ethanol-exposure mouse model. The increased mortality appears likely to be linked to hypoglycemia and hypothermia due to severe hepatic injury, pancreatitis and impaired detoxification of acetaldehyde in binge ethanol-exposed *Nrf2*-KO mice. NRF2 activation by DMF pretreatment protects against death from acute binge alcohol exposure, indicating that NRF2 could be a key clinical target for treatment of severe alcoholic intoxication in the future. However, the role of NRF2 in alcoholism and the exact mechanism of protection is not yet fully clear. A conditional *Nrf2* knockout model is needed to confirm the role of NRF2 in liver or pancreas individually during alcohol intoxication.

## Conflicts of interest

The authors declare that they have no conflict of interest.

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## Appendix A. Supplementary data

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## Transparency document

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