Am J Transl Res 2021;13(6):6031-6042
www.ajtr.org /ISSN:1943-8141/AJTR0123205

Original Article

Ulinastatin protects against acetaminophen-induced liver injury by alleviating ferroptosis via the SIRT1/NRF2/HO-1 pathway

Cong Wang[1], Tong Liu[1], Yingmu Tong[1], Ruixia Cui[1], Kai Qu[1], Chang Liu[1,2], Jingyao Zhang[1,2]

Departments of [1]Hepatobiliary Surgery, [2]SICU, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, Shaanxi, People's Republic of China

Received September 27, 2020; Accepted April 11, 2021; Epub June 15, 2021; Published June 30, 2021

Abstract: Acetaminophen (APAP) overdose has been considered responsible for the drug-induced liver injury for many years. Ferroptosis is defined as an iron-dependent form of cell death associated with lipid peroxide accumulation. Ferroptosis is involved in APAP-induced acute liver failure, and UTI is an effective drug treatment for acute liver failure. Thus, we aimed to determine whether UTI protects the liver against APAP-induced acute liver failure by decreasing ferroptosis-induced lipid peroxide accumulation. C57BL/6 mice and LO2 cell line were treated with UTI before and after the exposure to APAP. Liver tissues and LO2 cells were collected for biochemical assessment of molecular parameters. APAP-induced upregulation of ferroptotic events (iron content), lipid hydroperoxides (ROS production, MDA, and 4-HNE), and depletion of GSH were effectively relieved by ferrostatin-1 (Fer-1), a ferroptosis inhibitor, and UTI. UTI blocked ferroptosis-induced lipid peroxide accumulation by promoting nuclear translocation of NRF2 to activate its downstream targets (HO-1). An increased expression or knockdown of of SIRT1 influenced the UTI effect on the NRF2 pathway and had an impact on lipid accumulation. Overall, UTI plays a role in mitigation of APAP-induced acute liver injury by inhibiting ferroptosis-induced lipid peroxide accumulation, and the effect of UT1 was mediated by the NRF2/HO-1 pathway and SIRT1 expression.

Keywords: Ulinastatin, acetaminophen, hepatotoxicity, ferroptosis, NRF2, SIRT1

Introduction

Liver is one of the most important organs of our body which maintains metabolism, detoxification, and secretion. Viruses, drugs, alcohol, toxic chemicals, and nutritional supplements can damage the liver by direct or indirect toxicity. Acetaminophen (APAP) is a frequently used antipyretic and analgesic drug widely available throughout the world. The therapeutic dose of APAP is effective and safe, but overdose and long-term use will cause adverse reactions and severe liver toxicity, including ALF and death. APAP toxicity is responsible for 46% of ALF cases in the United States. Previous mechanistic studies indicated that the formation of a reactive metabolite, NAPQI, is responsible for hepatic glutathione depletion and initiation of the toxicity that requires rapid administration of N-acetylcysteine (NAC) as a clinical antidote. Additionally, APAP-induced NAPQI can lead to the inhibition of mitochondrial respiration resulting in ROS formation [1, 2]. NAC is very effective, especially when administered immediately after the APAP overdose; however, limited efficacy and adverse effects of NAC are a concern in late presenting patients. Thus, investigation of a new therapy for APAP-induced hepatoxicity is necessary.

Ferroptosis is triggered by iron-dependent lipid peroxide accumulation and differs from other cell death programs, such as apoptosis and necrosis, in cytological changes [3] including mitochondrial shrinkage and increased mitochondrial membrane density [4]. System X_c^-, a membrane Na^+-dependent cysteine-glutamate exchange transporter, participates in the process of ferroptosis by facilitating cystine translocation into the cytoplasm during glutathione (GSH) synthesis [5]. Glutathione peroxidase 4 (GPX4), a GSH-dependent enzyme, plays an

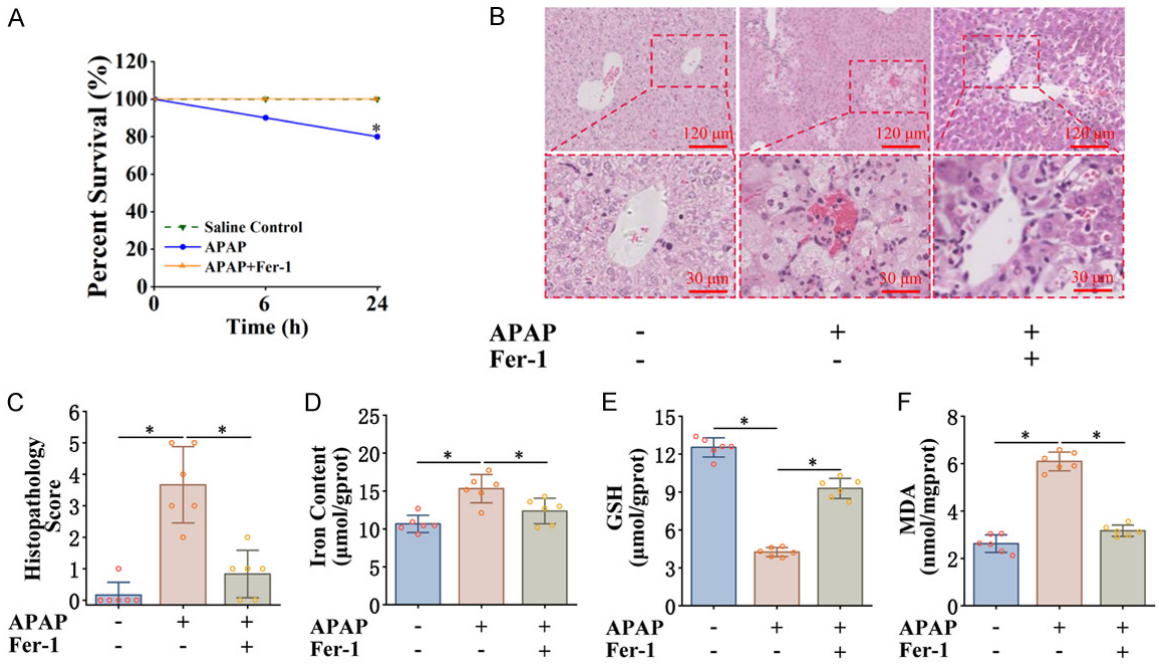

Figure 1. Ferroptosis participates in APAP-induced hepatotoxicity. C57BL/6 mice were exposed to APAP (350 mg/kg) and treated with Fer-1. A. The line chart represents the percentage of survival of mice treated with APAP or APAP + Fer-1. B, C. H&E staining (representative images, magnification ×200 and ×400) of the saline control, APAP, and APAP + Fer-1 groups. The bars represent the score of liver necrosis. The bars in the images of H&E staining correspond to 120 μm or 30 μm. D. Iron content in mice in the saline control, APAP, and APAP + Fer-1 groups. E, F. GSH and MDA levels in mice in the saline control, APAP, and APAP + Fer-1 groups. The data are presented as the mean ± SD. *$P < 0.05$.

important role in ferroptosis by decreasing the clearance of lipid peroxides [6]. Ferroptosis is involved in some human diseases, including acute kidney injury [7] and hepatic ischemia-reperfusion injury [8, 9]. APAP-induced liver injury is associated with GSH depletion; hence, ferroptosis may be a factor that influences ALF.

Sirtuin 1 (SIRT1), a nicotinamide adenine dinucleotide (NAD^+)-dependent histone deacetylase, plays a role in liver injury and metabolic and heart diseases [10-12]. SIRT1 is regarded as a protector of the cells against oxidative stress injury that acts by mediating the expression of Nrf2 and its downstream targets to eliminate the effect of lipid peroxidation and upregulate the resistance to oxidative injury [13]. An increase in the production of ROS may be responsible for APAP-induced hepatotoxicity [6], and the nuclear factor erythroid 2-related factor 2 (Nrf2) activation results in the regulation of the antioxidant defense genes and enzymes, such as heme oxygense-1 (HO-1) and NADPH: quinone oxidoreductase 1 (NQO-1) [8]. Previous studies demonstrated that Nrf2 is activated by redox status via Kelch like ECH-associated protein 1 (Keap1) [1] and is important for the prevention of drug-induced hepatotoxicity [9]. However, it is not clear whether APAP-induced acute liver injury driven by ferroptosis relies on SIRT1-dependent pathways.

Ulinastatin (UTI) was isolated and purified from human urine and is a broad-spectrum serine protease inhibitor with anti-inflammatory and cytoprotective effects in cell and animal models [10]. UTI is used clinically to alleviate acute pancreatitis and sepsis [11]. Additionally, UTI was reported to have antioxidant and cytoprotective functions by attenuating the level of free oxygen radicals and consumption of superoxide dismutase [12]. Antioxidant action of UTI can be partially explained by a reduction in protease release; however, the mechanisms of the effects of UTI on APAP-induced hepatoxicity driven by ferroptosis are unknown. Therefore, in this study, we evaluated the protective effects of UTI on APAP-induced hepatotoxicity driven by ferroptosis and investigated

the molecular mechanism of UTI inhibition of lipid peroxide accumulation induced by ferroptosis *in vitro and vivo*.

Materials and methods

Reagents

Ulinastatin (UTI) was provided by the Techpool Bio-pharma Co, Ltd (Guangzhou, China). APAP and N Acetyl-L-Carnosine (NAC) (Sigma, St. Louis, MO, USA). Primary antibodies: anti-SIRT1, anti-GPX4, anti-GAPDH, anti-Histone-H3 (Proteintech, Wuhan, China), anti-NRF2, anti-HO-1 (Abcam, Cambridge, MA, USA), anti-4-HNE (R&D, America). Peroxidase conjugated secondary antibodies: Anti-mouse and rabbit IgG (Pioneer Biotechnology, China). MDA, GSH test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). ROS Fluorescent Probe-DHE (Vigorous Biotechnology, Beijing, China). Ferrostatin-1 (Fer-1) and Resveratrol (Res) were from MCE (China).

Animals and experimental protocol

Male C57BL/6 mice were from the Experimental Animal Center of Xi'an Jiaotong University. The animal experiment procedures were performed in accordance with the Guide of Laboratory Animal Care and Use from the United States National Institution of Health and were approved by the Laboratory Animal Care Committee (LACC) of Xi'an Jiaotong University, China (No. XJTULAC2017-207). Mice were initially housed for 7 days to adjust to the environment. The experimental design included five groups (n=10 per group): the control group included the saline control (0.9% saline) group, and the test groups included APAP, APAP + UTI (5×10^4 units/kg and 1×10^5 units/kg), APAP + Fer-1 (10 mg/kg), and APAP + Res (50 mg/kg) treatments administered by tail vein or intraperitoneal injection. The experimental design for the investigation of hepatoprotective effect of UTI on ALI mice is summarized in **Figure 2A**. After 24 h APAP treatment, the mice were sacrificed after anesthesia, and serum and liver tissue samples were harvested and used in the subsequent experiments.

Histopathological evaluation and immunohistochemistry

Formalin-fixed liver embedded in paraffin was cut into 4 μm sections stained with hematoxylin and eosin (H&E) for routine histology and examined by using a light microscope. The stained liver sections were scored from 1 to 4 corresponding to no damage, mild damage, moderate damage, and severe damage, respectively. IHC staining using 4-HNE was performed as described previously [14]. At least three fields of view per section were evaluated in histological analysis.

Biochemical assays

Mouse liver and blood were collected for biochemical assays. ALT and AST levels in the serum were detected by a biochemical analyzer. Additionally, the changes in GSH and MDA were assessed using commercial kits according to the manufacturer instructions.

Cell culture conditions and experimental protocol

LO2 cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C and 5% CO_2 in an incubator. Four groups were set up for the experiment: control (PBS), APAP (10 mM dissolved in PBS), UTI (5,000 U/ml) + APAP, Fer-1 (8 μM) + APAP, and Res (50 μM) + APAP groups. LO2 cells were preincubated with UTI, Fer-1, or Res and then stimulated with or without APAP for 24 h to obtain the samples.

Oil Red O

Frozen liver sections and LO2 cell samples were prepared. Oil Red O (Heart Biological, China) staining was performed according to the standard protocol. Staining intensity in the samples was quantified by using a fluorescence microscope and NIH ImageJ software.

siRNA transfection

SIRT1 siRNAs were obtained from Biomics Biotechnologies (China). Cells treated with SIRT1 siRNA and NC siRNA were cultured according to the manufacturer's instructions. The siRNA sequences are listed below. Subsequent procedures were performed 48 h after the transfection.

Reactive oxygen species (ROS) production

The slides with sections of liver tissue and LO2 cells were incubated with 10 μM DHE, a ROS

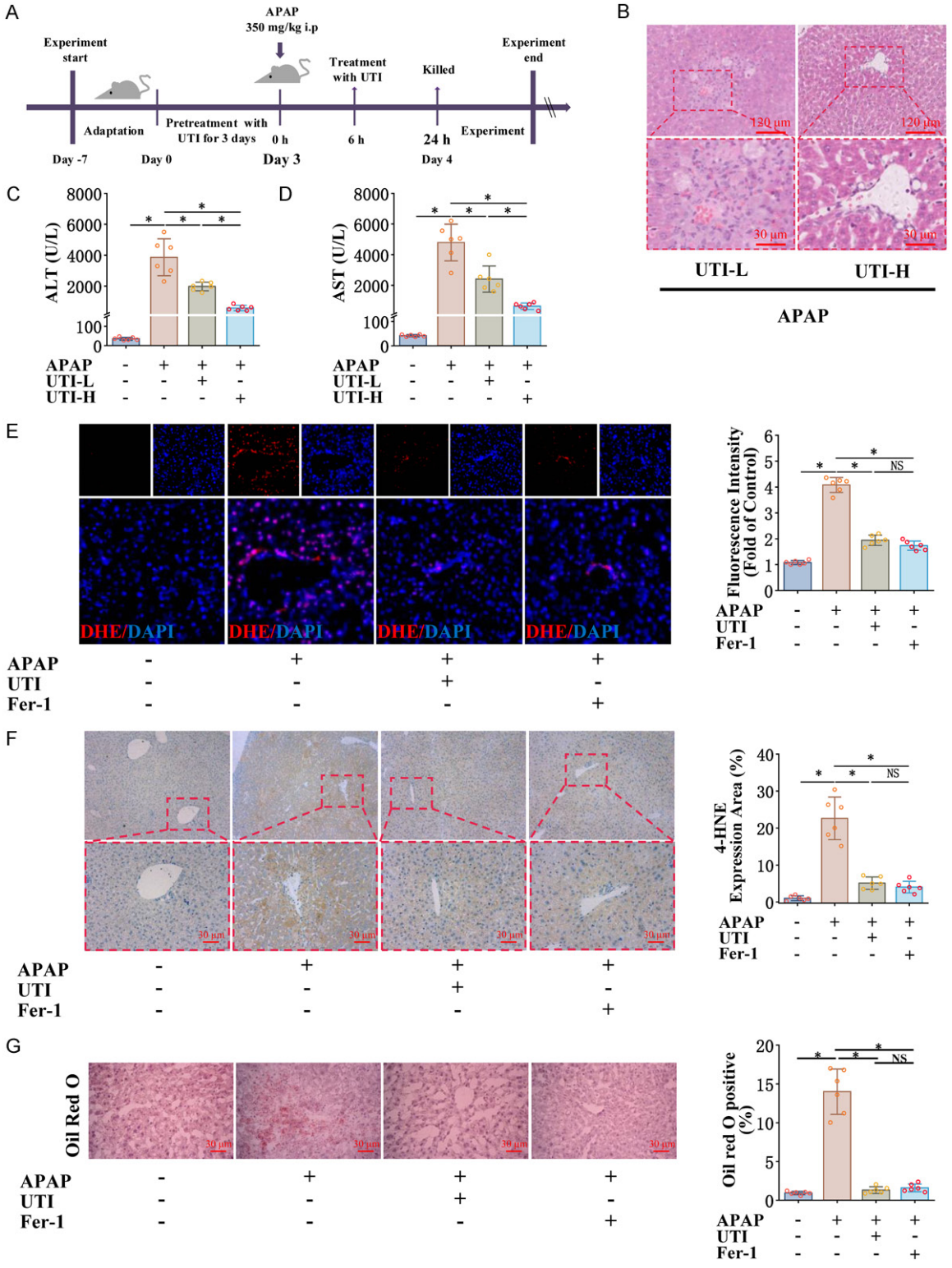

Figure 2. Mice treated with UTI were more resistant to APAP-triggered hepatotoxicity. C57BL/6 mice were treated with APAP (350 mg/kg) with or without the treatment with UTI. A. Experimental design for evaluation of the hepatoprotective effect of UTI on APAP-induced ALI in mice. B. H&E staining (representative images, magnification ×200 and ×400) of samples of mice treated with various doses (5×10^4 U/kg and 1×10^5 U/kg) of UTI. The bars correspond to 120 μm or 30 μm. C, D. The effect of high and low doses of UTI on the serum AST and ALT levels in mice injected with APAP after 24 h. E. ROS (red fluorescent signal) were detected using DHE staining (representative images,

magnification ×400) in the saline control, APAP, APAP + UTI, and APAP + Fer-1 groups. The fluorescence signal was immediately detected by a fluorescence microscope. F. The expression of 4-HNE was examined by immunohistochemistry (representative images, magnification ×200 and ×400) in the saline control, APAP, APAP + UTI, and APAP + Fer-1 groups. G. Oil Red O staining (representative images, magnification ×400) in the saline control, APAP, APAP + UTI, and APAP + Fer-1 groups. The percentage and expression area were calculated by ImageJ. The data are presented as the mean ± SD. *$P < 0.05$.

fluorescent probe, for 30 min, and the intracellular ROS generation was detected. The signals in the liver and cell samples were quantified by fluorescence microscopy and NIH ImageJ software.

Western blot analysis

Proteins were extracted from the mouse liver tissue or LO2 cells using radioimmunoprecipitation assay (RIPA) buffer, and protein concentration was measured using a BCA protein assay (Hat Biotechnology. China). Western blot analysis was performed as described previously [15]. The primary antibodies included anti-SIRT1, anti-GPX4, anti-NRF2, anti-HO-1, anti-GAPDH, and anti-histone H3. Secondary antibodies included anti-mouse and -rabbit IgGs that were used to detect the target bands.

Statistical analysis

The data are presented as the mean ± SD. Results were acquired by three independent experiments. Statistical significance between multiple groups was evaluated by one-way or two-way analysis of variance followed by Dunnett's post-hoc test using GraphPad Prism 5 software. *P* values < 0.05 were considered to be statistically significant.

Results

Ferroptosis participates in APAP-induced hepatotoxicity

To determine whether ferroptosis participates in APAP-induced ALI in mice, Fer-1, a ferroptosis-specific inhibitor, was used as a control. As shown in **Figure 1A**, Fer-1 reduced death of APAP-intoxicated mice and increased the survival rate. Hematoxylin-eosin (H&E) staining of the liver samples of the APAP group was used to demonstrate considerable disruption of liver tissue architecture compared to that in the Fer-1 group indicating that Fer-1 attenuated APAP-induced liver damage (**Figure 1B**, **1C**). APAP increased the level of iron in the liver, and this increase was significantly reduced by the treatment with Fer-1 (**Figure 1D**). APAP treatment induced rapid GSH depletion and excessive accumulation of MDA leading to oxidative insult in the mouse liver (**Figure 1E**, **1F**), and these effects were reduced in the Fer-1 group. These results indicate that ferroptosis appears to be involved in APAP-induced ALI.

Mice treated with UTI were more resistant to APAP-induced hepatotoxicity

Two doses of UTI were used in mice after acute APAP treatment. As shown in **Figure 2B**, the data of hematoxylin-eosin (H&E) staining indicated that mice in the UTI group demonstrated a considerable decrease in disruption of liver tissue architecture compared with that in the APAP group, and a stronger effect was detected in the UTI group (1×10^5 U/kg) indicating efficient attenuation of APAP-induced liver injury by UTI. Saline control group had no changes in the liver architecture. ALT and AST levels in the serum inversely correlate with hepatic function; therefore, these enzymes were assayed in the serum. APAP exposure led to the pronounced upregulation of ALT and AST compared with those in mice in the saline control group. The detoxification effect of UTI induced a decrease in the changes in ALT and AST concomitant to an increase in the concentrations of UTI, and the dose of 1×10^5 U/kg had the best effect (**Figure 2C**, **2D**). According to these results, the dose of 1×10^5 U/kg was selected for subsequent experiments. Lipid peroxidation plays a central role in ferroptosis [3]; hence, we detected the UTI effect on lipid peroxidation markers. ROS levels were determined using DHE staining, and a pronounced increase in the ROS level was detected in the APAP-treated group in contrast with the data in the saline control group (**Figure 2E**). UTI efficiently reversed an increase in the ROS level, and a similar effect was detected in the Fer-1 group. Then, we assayed the level of 4-HNE (a lipid peroxidation byproduct) using IHC (**Figure 2F**). The signal was strong in the APAP group but was decreased in the UTI and Fer-1 groups. The results of Oil Red O staining (**Figure 2G**) showed that UTI and Fer-1 decreased the lipid accumulation in the liver of APAP-treated

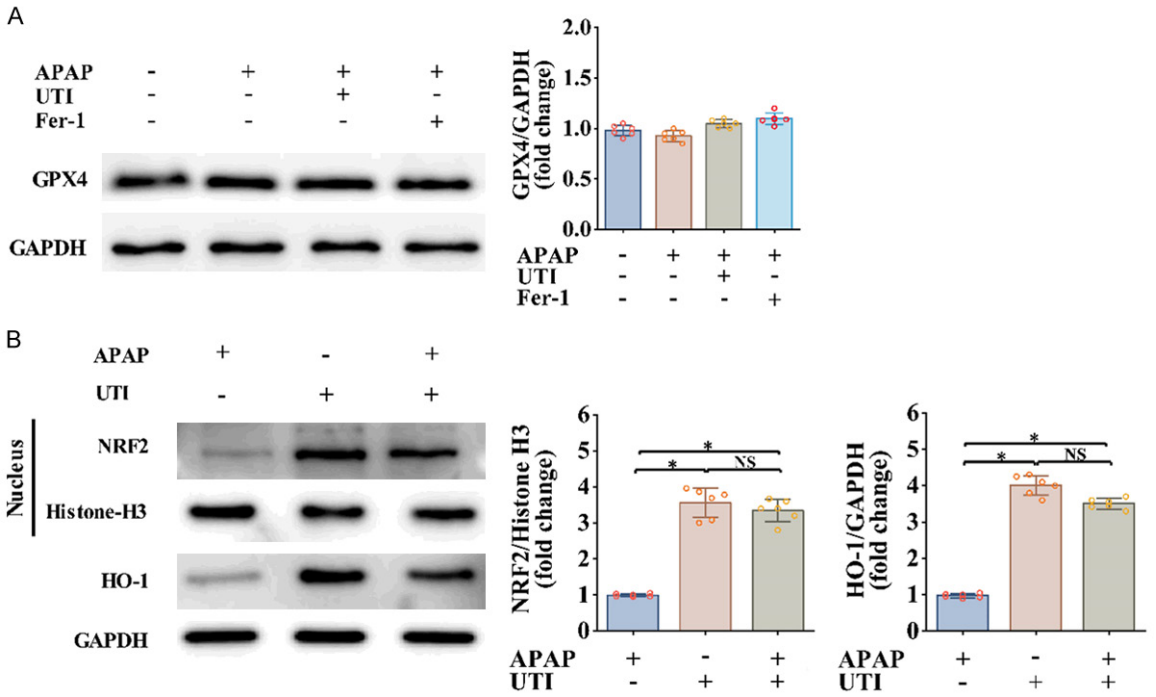

Figure 3. The effects of UTI on APAP-induced hepatotoxicity were mediated by the NRF2/HO-1 signaling pathway. Twenty-four hours after APAP treatment, the tissue samples were harvested and assayed. A. GPX4 expression was detected by western blot. The bars represent the quantitation of the GPX4 protein levels in the saline control, APAP, APAP + UTI, and APAP + Fer-1 groups. The data of immunoblots were normalized to GAPDH. B. Influence of UTI on the NRF2 and HO-1 expression was detected in the APAP, UTI, and APAP + UTI groups. GAPDH or histone H3 were used as the loading controls. NRF2 and HO-1 expression was quantified by densitometry. The data are presented as the mean ± SD. $*P < 0.05$.

mice. These results indicate that UTI and Fer-1 decrease ROS and 4-HNE levels suggesting that UTI reduces oxidative stress and ferroptosis in APAP-treated mice.

The effect of UTI on APAP-induced hepatotoxicity is mediated by the NRF2/HO-1 signaling pathway

Inhibition of GPX4 is one of the effective mechanisms of ferroptosis [4]. An increase in the expression of GPX4 demonstrated that GPX4 levels were only marginally different between the groups (**Figure 3A**). Therefore, we determined whether UTI alleviated liver damage by inhibiting lipid peroxidation without direct inhibition of ferroptosis. A previous study demonstrated that NRF2-regulated signaling pathway diminished APAP-induced hepatic injury by inhibiting oxidative stress [16]. Thus, we determined whether the effect of UTI on APAP-induced hepatotoxicity is mediated by the NRF2 and HO-1 pathway (**Figure 3B**). The results suggested that UTI significantly increased the nuclear translocation of NRF2 compared with that in the APAP group. Additionally, HO-1, the downstream component of the NRF2 pathway, was upregulated by treatment with UTI. Thus, we suggest that UTI mitigates the acetaminophen-induced ALI independently of the GPX4 pathway via the NRF2/HO-1 pathway.

UTI alleviated APAP-induced hepatotoxicity in a SIRT1-dependent manner

SIRT1 is considered an effective protector of the cells against oxidative insult acting by targeting NRF2 to regulate the expression of HO-1 [17]. The data of **Figure 4A** indicate that APAP induced a decrease in the expression of SIRT1 compared to that in the saline control. We used resveratrol (Res), a SIRT1 activator [10], to determine the role of SIRT1 in the mechanism of the effect of UTI against an overdose of APAP. The data of **Figure 4B** indicate that UTI restored a decrease in the SIRT1 level induced by APAP similar to the effect of Res, indicating that the effect of UTI is mediated by regulation of SIRT1. Additionally, the target of SIRT1, NRF2, was translocated into the nucleus to protect the cells against lipid peroxidation in

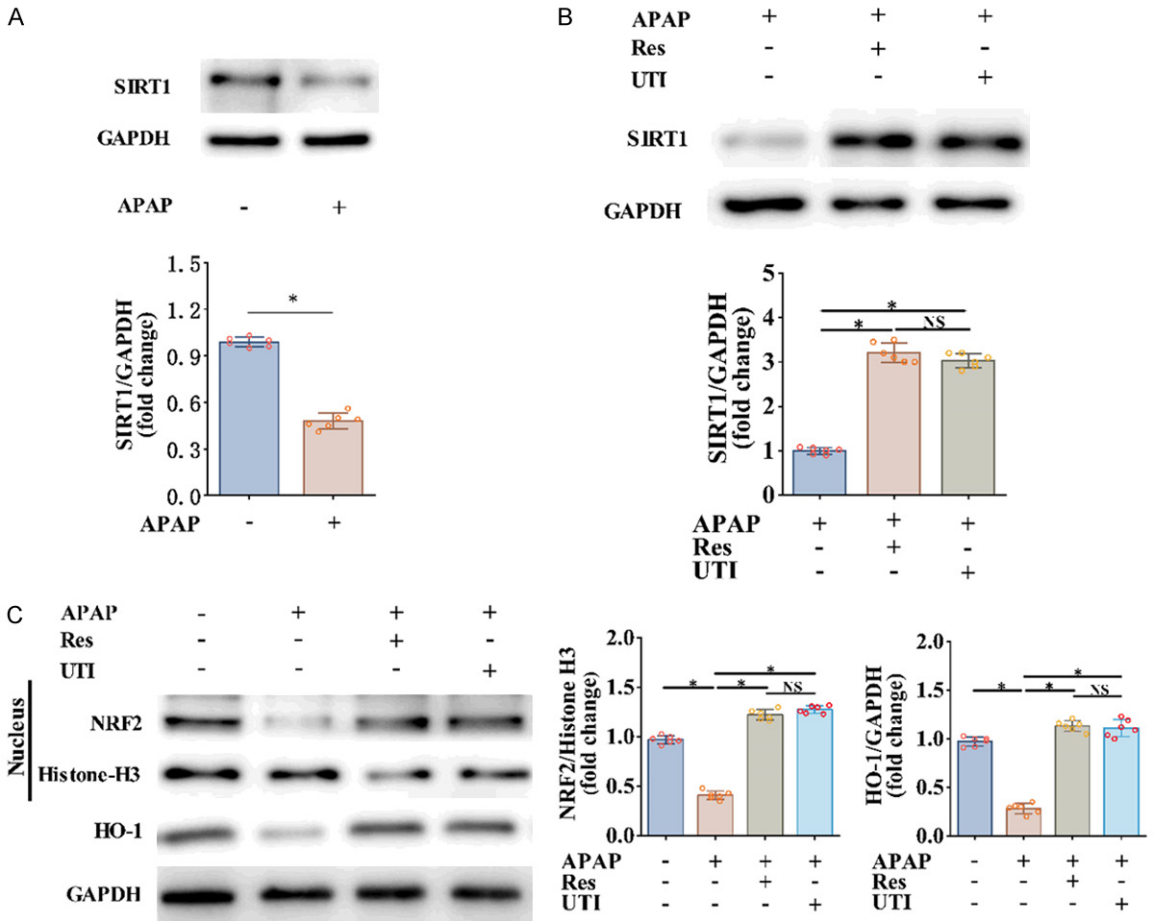

Figure 4. UTI alleviated APAP-induced hepatotoxicity in a SIRT1-dependent manner. C57BL/6 mice were treated with Res and UTI before or after the injection with APAP (350 mg/kg). A-C. Liver samples were assayed by western blot to evaluate the levels of SIRT1, NRF2, and HO-1. GAPDH and histone H3 were used as the loading controls. Expression was quantified by densitometry analysis. The data are presented as the mean ± SD. *$P < 0.05$.

APAP-induced hepatotoxicity. UTI and Res had the same effects on the regulation of NRF2 translocation and an increase in HO-1 expression (**Figure 4C**) compared to those in the APAP group. These data suggest that the NRF2/HO-1 pathway is regulated by SIRT1 to alleviate APAP-induced lipid peroxidation.

UTI ameliorated oxidative insult and ferroptosis induced by APAP in vitro

To determine whether UTI plays an important role in ferroptosis *in vitro*, we challenged hepatocyte cell line (LO2 cells) with UTI and Fer-1. Under these conditions, UTI and Fer-1 mitigated ROS generation induced by APAP in LO2 cells compared with that in the APAP group (**Figure 5A**). UTI and Fer-1 alleviated the lipid deposition induced by APAP in LO2 cells detected by Oil Red O staining (**Figure 5B**). Since lipid peroxidation is a hallmark of ferroptosis, these results indicate that UTI decreases APAP-induced ferroptosis by downregulation of lipid accumulation in hepatocytes.

SIRT1-dependent NRF2/HO-1 pathway is rescued by UTI in vitro

SIRT1 protected mice from APAP-induced liver injury via the NRF2/HO-1 pathway; hence, we determined whether SIRT1 had a similar effect *in vitro*. SIRT1 expression was downregulated by APAP and restored by Res or UTI (**Figre 6A**). UTI upregulated the NRF2 nuclear translocation and HO-1 protein expression in LO2 cells similar to the effect of Res (**Figure 6B**). To validate the effect of UTI on regulation of SIRT1 expression, SIRT1 expression was inhibited with siRNA (**Figure 6C**). Additionally, treatment with APAP or SIRT1 knockdown decreased the NRF2 nuclear translocation and HO-1 protein expression, and the protective effect of UTI was

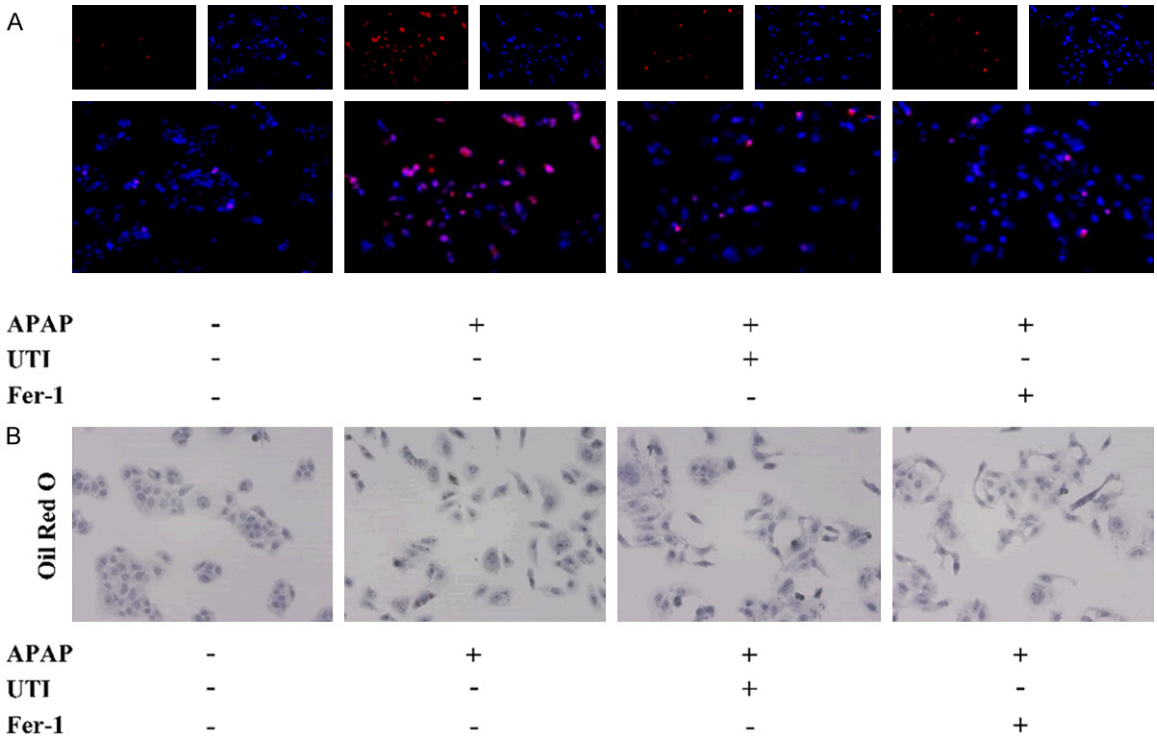

Figure 5. UTI ameliorated oxidative insult and ferroptosis induced by APAP *in vitro*. LO2 cells were treated with UTI or Fer-1 prior to APAP treatment and were used for ROS and Oil Red O staining assays. A. ROS (red fluorescent signal) were detected using DHE staining (representative images, magnification ×200) in the saline control, APAP, APAP + UTI, and APAP + Fer-1 groups. The fluorescence was immediately detected by a fluorescence microscope. B. Oil Red O staining (representative images, magnification ×400) was performed in the saline control, APAP, APAP + UTI, or APAP + Fer-1 groups. The data are presented as the mean ± SD. *$P < 0.05$.

abolished (**Figure 6D**) thus confirming our findings. These data demonstrated that the effects of UTI against lipid accumulation and ferroptosis are mediated by the SIRT1-dependent NRF2/HO-1 pathway.

Discussion

Acute liver injury accounts for life-threatening liver disease. Oral administration of APAP overdose in mice is a common animal model for the investigation of potential therapeutic drugs [18]; this model is associated with elevated levels of oxidative insult in mice [19]. Therefore, ALI can be prevented or treated by using certain drugs that decrease oxidative stress. Our current study assessed the hepatoprotective effect of UTI on APAP-induced ALI triggered by ferroptosis and investigated the relevant pathway. UTI, a urinary trypsin inhibitor, is a widely used antidote drug that is administered clinically to alleviate acute pancreatitis and sepsis; the effects of UTI depend on inflammatory defense and cytoprotection in experimental models [20, 21]. In previous studies, UTI was shown to cure several types of diseases, such as ischemia reperfusion injury [22], myocardial infarction [23], acute pancreatitis [24], and even ovariectomy-induced bone loss [25]. UTI influences many diseases; however, the function of UTI in APAP-induced ALI is unknown. In this study, UTI influenced the expression of the liver enzymes and protected hepatic architecture against damage induced by an overdose of APAP. In a mouse model, UTI attenuated excessive oxidative stress and ferroptosis. These functions of UTI are in agreement with the result obtained in APAP-treated LO2 cells. Thus, we sought to identify the pathway influenced by UTI *in vitro* and *in vivo*. The data of the present study indicate that antiferroptotic and antioxidant effects of UTI protect the liver from APAP-induced hepatotoxicity via the SIRT1/NRF2/HO-1 pathway.

Acute liver injury (ALI) upregulates serum ALT and AST and induces pathological tissue damage in the liver [26]. In the current study, APAP substantially increased the levels of serum ALT and AST and pathological damage in

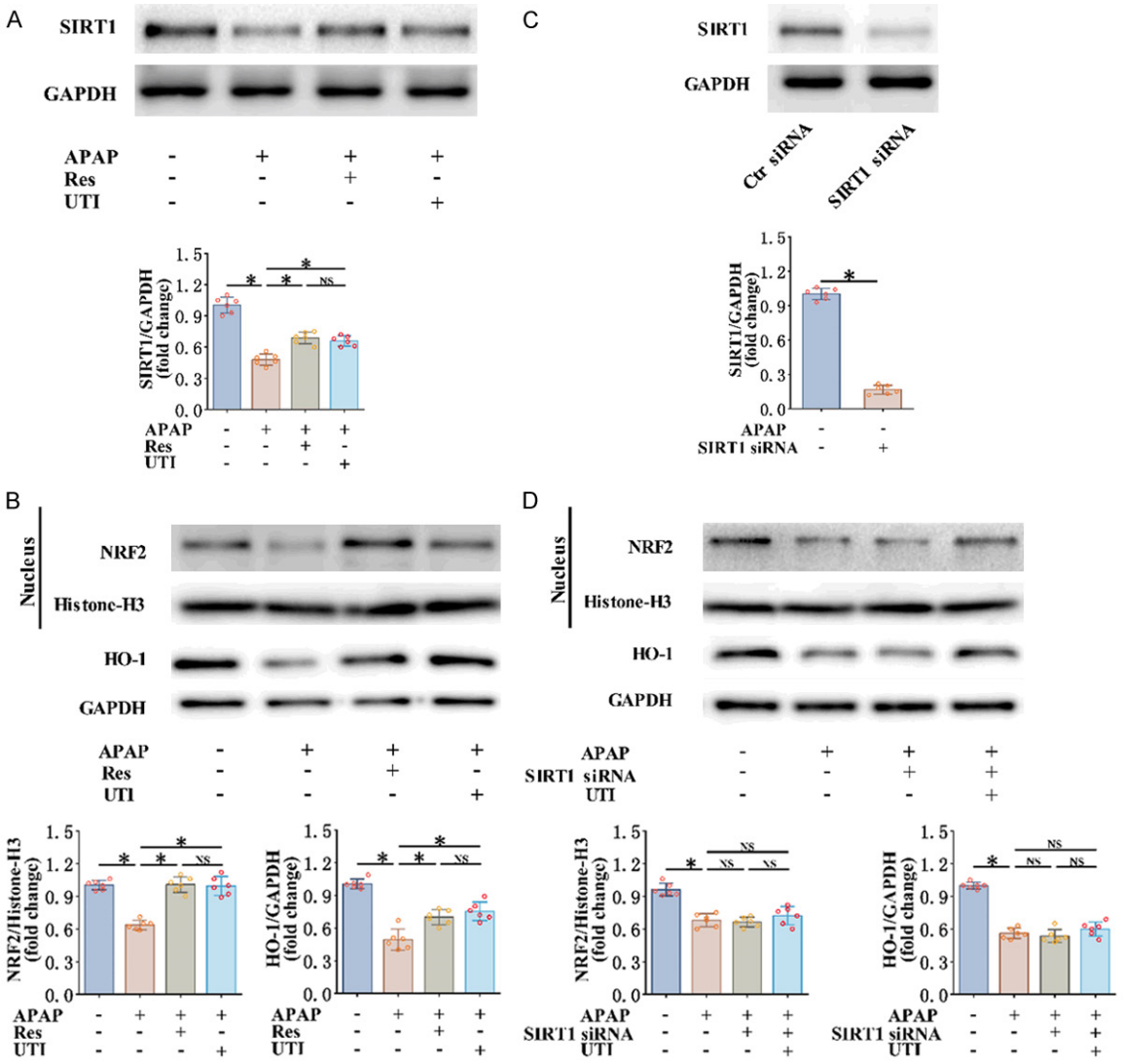

Figure 6. SIRT1-dependent NRF2/HO-1 pathway is rescued by UTI *in vitro*. A, B. LO2 cells were exposed to Res or UTI prior to APAP treatment and were treated with or without APAP for 24 h. The expression of SIRT1, NRF2, and HO-1 was assessed by western blot. GAPDH and histone H3 were used as the loading controls. SIRT1, NRF2, and HO-1 expression was quantified using densitometric analysis. C, D. LO2 cells transfected with SIRT1 siRNA were treated with or without UTI and APAP. The expressions of SIRT1, NRF2, and HO-1 were assessed by western blot. GAPDH and histone H3 were used as the loading controls. SIRT1, NRF2, and HO-1 expression was quantified using densitometric analysis. The data are presented as the mean ± SD. **P* < 0.05.

the liver, while UTI inhibited these effects indicating that UTI protects the liver from hepatotoxicity of APAP. These findings suggest a functional role of UTI in the regulation of tissue injury. Ferroptosis was detected in the APAP-treated group and was decreased in the UTI and Fer-1 groups. The mechanism of ferroptosis was investigated in a number of diseases [27-29]; however, types of cell death in APAP hepatotoxicity are a matter of controversy. One study suggested that APAP triggered necrotic cell death based on morphological parameters, such as cell swelling, organelle disintegration, protein denaturation, and neutrophil infiltration [30]. However, other studies suggested that apoptosis mediated APAP-induced ALI [31, 32]. Ferroptosis is a type of cell death distinguished by lipid peroxide accumulation [33]. Previous studies suggested that ferroptosis plays an important role in liver diseases [34, 35]. Inhibition of glutathione peroxidase 4 (GPX4) leads to the ferroptotic cascade. However, in our study, APAP and UTI had no effect on the expression of GPX4 and APAP-

induced lipid peroxidation, and glutathione (GSH) depletion was observed. Byproducts of lipid peroxidation, such as MDA and 4-HNE, were upregulated by treatment with APAP and downregulated by treatment with UTI and Fer-1. These data demonstrated that ferroptosis promotes APAP-induced hepatotoxicity. Therefore, UTI inhibited ferroptosis and induced cell death in APAP-treated hepatocytes by alleviating the lipid peroxide accumulation.

SIRT1 is a known stress response protein deacetylase that deacetylates proteins [36]. Lipid peroxidation is one of the main responses in APAP-induced ALI [37, 38]. NRF2 regulates the antioxidant response elements (ARE) and coordinates the antioxidant system [39]. SIRT1 was shown to deacetylate NRF2 [40]. Activated NRF2 enhances the function of HO-1 by translocating into the nucleus [41]. HO-1 functions by reducing intracellular ROS production [42]. A previous study demonstrated that NRF2 contributes to mitigation of lipid peroxidation and ferroptosis [10]. SIRT1 was downregulated in APAP-treated mice and cells to induce cell death and lipid peroxide accumulation. SIRT1 downregulation was restored by UTI to alleviate ferroptosis. The effect of UTI on the upregulation and translocation of NRF2 and an increase in the protein expression of HO-1 were demonstrated. These findings indicated that UTI promoted NRF2 translocation to the nucleus and increased the expression of HO-1 in mice and LO2 cells in APAP-induced liver injury. Knockdown of SIRT1 downregulated the expression of NRF2 and HO-1 in APAP-induced ALI. Thus, our study demonstrated that UTI influences the SIRT1/NRF2/HO-1 pathway and contributes to amelioration of lipid peroxide accumulation and ferroptosis.

This study mainly illustrated that UTI effectively protected the liver from ferroptosis triggered by APAP-induced ALI by regulating oxidative insult *in vivo* and *in vitro*. The protective effect of UTI on APAP-induced hepatotoxicity was investigated in a mouse model and LO2 cells. UTI is an effective drug with potential hepatoprotective activity mediated by activation of the SIRT1/NRF2/HO-1 signaling pathway. Thus, the results suggest a potential mechanism of the effect of UTI on the prevention of hepatic ferroptotic responses and lipid peroxide accumulation in ALI. Therefore, UTI can be considered a possible therapeutic drug for the inhibition of APAP-induced ALI.

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Kai Qu, Chang Liu and Jingyao Zhang, Department of Hepatobiliary Surgery, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an 710061, Shaanxi, People's Republic of China. Tel: +86-29-85323900; Fax: +86-29-85324642; E-mail: joanne8601@163.com (KQ); liuchangdoctor@163.com (CL); you12ouy@163.com (JYZ)

References

[1] Meyers LL, Beierschmitt WP, Khairallah EA and Cohen SD. Acetaminophen-induced inhibition of hepatic mitochondrial respiration in mice. Toxicol Appl Pharmacol 1988; 93: 378-387.

[2] Hanawa N, Shinohara M, Saberi B, Gaarde WA, Han D and Kaplowitz N. Role of JNK translocation to mitochondria leading to inhibition of mitochondria bioenergetics in acetaminophen-induced liver injury. J Biol Chem 2008; 283: 13565-13577.

[3] Li J, Cao F, Yin HL, Huang ZJ, Lin ZT, Mao N, Sun B and Wang G. Ferroptosis: past, present and future. Cell Death Dis 2020; 11: 88.

[4] Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, Patel DN, Bauer AJ, Cantley AM, Yang WS, Morrison B 3rd and Stockwell BR. Ferroptosis: an iron-dependent form of non-apoptotic cell death. Cell 2012; 149: 1060-1072.

[5] Bridges RJ, Natale NR and Patel SA. System xc-cystine/glutamate antiporter: an update on molecular pharmacology and roles within the CNS. Br J Pharmacol 2012; 165: 20-34.

[6] Yang WS, SriRamaratnam R, Welsch ME, Shimada K, Skouta R, Viswanathan VS, Cheah JH, Clemons PA, Shamji AF, Clish CB, Brown LM, Girotti AW, Cornish VW, Schreiber SL and Stockwell BR. Regulation of ferroptotic cancer cell death by GPX4. Cell 2014; 156: 317-331.

[7] Hu Z, Zhang H, Yang S, Wu X, He D, Cao K and Zhang W. Emerging role of ferroptosis in acute kidney injury. Oxid Med Cell Longev 2019; 2019: 8010614.

[8] Hu Z, Zhang H, Yi B, Yang S, Liu J, Hu J, Wang J, Cao K and Zhang W. VDR activation attenuate cisplatin induced AKI by inhibiting ferroptosis. Cell Death Dis 2020; 11: 73.

[9] Wang H, An P, Xie E, Wu Q, Fang X, Gao H, Zhang Z, Li Y, Wang X, Zhang J, Li G, Yang L, Liu W, Min J and Wang F. Characterization of ferroptosis in murine models of hemochromatosis. Hepatology 2017; 66: 449-465.

[10] Zheng X, Xu F, Liang H, Cao H, Cai M, Xu W and Weng J. SIRT1/HSF1/HSP pathway is essen-

tial for exenatide-alleviated, lipid-induced hepatic endoplasmic reticulum stress. Hepatology 2017; 66: 809-824.
[11] Han Y, Sun W, Ren D, Zhang J, He Z, Fedorova J, Sun X, Han F and Li J. SIRT1 agonism modulates cardiac NLRP3 inflammasome through pyruvate dehydrogenase during ischemia and reperfusion. Redox Biol 2020; 34: 101538.
[12] Hsu CP, Zhai P, Yamamoto T, Maejima Y, Matsushima S, Hariharan N, Shao D, Takagi H, Oka S and Sadoshima J. Silent information regulator 1 protects the heart from ischemia/reperfusion. Circulation 2010; 122: 2170-2182.
[13] Li Y, Xu W, McBurney MW and Longo VD. SirT1 inhibition reduces IGF-I/IRS-2/ras/ERK1/2 signaling and protects neurons. Cell Metabolism 2008; 8: 38-48.
[14] Kim HG, Jang SS, Lee JS, Kim HS and Son CG. Panax ginseng meyer prevents radiation-induced liver injury via modulation of oxidative stress and apoptosis. J Ginseng Res 2017; 41: 159-168.
[15] Sun J, Wen Y, Zhou Y, Jiang Y, Chen Y, Zhang H, Guan L, Yao X, Huang M and Bi H. p53 attenuates acetaminophen-induced hepatotoxicity by regulating drug-metabolizing enzymes and transporter expression. Cell Death Dis 2018; 9: 536.
[16] Li D, Du Y, Yuan X, Han X, Dong Z, Chen X, Wu H, Zhang J, Xu L, Han C, Zhang M and Xia Q. Hepatic hypoxia-inducible factors inhibit PPARα expression to exacerbate acetaminophen induced oxidative stress and hepatotoxicity. Free Radic Biol Med 2017; 110: 102-116.
[17] Li S, Jiang X, Luo Y, Zhou B, Shi M, Liu F and Sha A. Sodium/calcium overload and Sirt1/Nrf2/OH-1 pathway are critical events in mercuric chloride-induced nephrotoxicity. Chemosphere 2019; 234: 579-588.
[18] Larson AM. Acetaminophen hepatotoxicity. Clin Liver Dis 2007; 11: 525-548.
[19] Nam EJ, Hayashida K, Aquino RS, Couchman JR, Kozar RA, Liu J and Park PW. Syndecan-1 limits the progression of liver injury and promotes liver repair in acetaminophen-induced liver injury in mice. Hepatology 2017; 66: 1601-1615.
[20] Inoue K and Takano H. Urinary trypsin inhibitor as a therapeutic option for endotoxin-related inflammatory disorders. Expert Opin Investig Drugs 2010; 19: 513-520.
[21] Linder A and Russell JA. An exciting candidate therapy for sepsis: ulinastatin, a urinary protease inhibitor. Intensive Care Med 2014; 40: 1164-1167.
[22] Zhao Y, Cai H, Zhou P, Lin S, Pan Y and Liang X. Protective effect of ulinastatin on hepatic ischemia reperfusion injury through autophagy activation in Chang liver cells. J Cell Biochem 2019; 120: 14960-14970.
[23] Wang S, Cheng ZY, Chen XJ and Xue HZ. Ulinastatin protects rats with myocardial infarction by activating Nrf2/NOS pathway. Eur Rev Med Pharmacol Sci 2018; 22: 8990-8998.
[24] Zhai Y, Gan L, Huang S, Xing Q, Zhou X, Wang L, Feng C, Chen L and Li T. Therapeutic effect of ultrasound interventional perirenal catheter-assisted early peripancreatic lavage of protease inhibitor on severe acute pancreatitis in miniature pigs. Pancreatology 2019; 19: 158-162.
[25] Huang J, Ren R, Bao Y, Guo J, Xiang W, Jing X, Shi J, Zhang G, Li L, Tian Y, Kang H and Guo F. Corrigendum: ulinastatin inhibits osteoclastogenesis and suppresses ovariectomy-induced bone loss by downregulating uPAR. Front Pharmacol 2018; 9: 1016.
[26] Lancaster EM, Hiatt JR and Zarrinpar A. Acetaminophen hepatotoxicity: an updated review. Arch Toxicol 2015; 89: 193-199.
[27] Zhao J, Dar HH, Deng Y, St Croix CM, Li Z, Minami Y, Shrivastava IH, Tyurina YY, Etling E, Rosenbaum JC, Nagasaki T, Trudeau JB, Watkins SC, Bahar I, Bayır H, VanDemark AP, Kagan VE and Wenzel SE. PEBP1 acts as a rheostat between prosurvival autophagy and ferroptotic death in asthmatic epithelial cells. Proc Natl Acad Sci U S A 2020; 117: 14376-14385.
[28] Chen P, Li X, Zhang R, Liu S, Xiang Y, Zhang M, Chen X, Pan T, Yan L, Feng J, Duan T, Wang D, Chen B, Jin T, Wang W, Chen L, Huang X, Zhang W, Sun Y, Li G, Kong L, Chen X, Li Y, Yang Z, Zhang Q, Zhuo L, Sui X and Xie T. Combinative treatment of β-elemene and cetuximab is sensitive to KRAS mutant colorectal cancer cells by inducing ferroptosis and inhibiting epithelial-mesenchymal transformation. Theranostics 2020; 10: 5107-5119.
[29] Li Y, Cao Y, Xiao J, Shang J, Tan Q, Ping F, Huang W, Wu F, Zhang H and Zhang X. Inhibitor of apoptosis-stimulating protein of p53 inhibits ferroptosis and alleviates intestinal ischemia/reperfusion-induced acute lung injury. Cell Death Differ 2020; 27: 2635-2650.
[30] Hinson JA, Roberts DW and James LP. Mechanisms of acetaminophen-induced liver necrosis. Handb Exp Pharmacol 2010; 369-405.
[31] Chen D, Ni HM, Wang L, Ma X, Yu J, Ding WX and Zhang L. p53 up-regulated modulator of apoptosis induction mediates acetaminophen-induced necrosis and liver injury in mice. Hepatology 2019; 69: 2164-2179.
[32] Ding WX, Ni HM, DiFrancesca D, Stolz DB and Yin XM. Bid-dependent generation of oxygen radicals promotes death receptor activation-induced apoptosis in murine hepatocytes. Hepatology 2004; 40: 403-413.
[33] Angelova PR, Choi ML, Berezhnov AV, Horrocks MH, Hughes CD, De S, Rodrigues M, Yapom R,

Little D, Dolt KS, Kunath T, Devine MJ, Gissen P, Shchepinov MS, Sylantyev S, Pavlov EV, Klenerman D, Abramov AY and Gandhi S. Correction: alpha synuclein aggregation drives ferroptosis: an interplay of iron, calcium and lipid peroxidation. Cell Death Differ 2020; 27: 2747.

[34] Wei S, Qiu T, Wang N, Yao X, Jiang L, Jia X, Tao Y, Zhang J, Zhu Y, Yang G, Liu X, Liu S and Sun X. Ferroptosis mediated by the interaction between Mfn2 and IREα promotes arsenic-induced nonalcoholic steatohepatitis. Environ Res 2020; 188: 109824.

[35] Yu Y, Jiang L, Wang H, Shen Z, Cheng Q, Zhang P, Wang J, Wu Q, Fang X, Duan L, Wang S, Wang K, An P, Shao T, Chung RT, Zheng S, Min J and Wang F. Hepatic transferrin plays a role in systemic iron homeostasis and liver ferroptosis. Blood 2020; 136: 726-739.

[36] Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, Tran H, Ross SE, Mostoslavsky R, Cohen HY, Hu LS, Cheng HL, Jedrychowski MP, Gygi SP, Sinclair DA, Alt FW and Greenberg ME. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science 2004; 303: 2011-2015.

[37] Han D, Canali R, Rettori D and Kaplowitz N. Effect of glutathione depletion on sites and topology of superoxide and hydrogen peroxide production in mitochondria. Mol Pharmacol 2003; 64: 1136-1144.

[38] Shuhendler AJ, Pu K, Cui L, Uetrecht JP and Rao J. Real-time imaging of oxidative and nitrosative stress in the liver of live animals for drug-toxicity testing. Nat Biotechnol 2014; 32: 373-380.

[39] Kaspar JW, Niture SK and Jaiswal AK. Nrf2: INrf2 (Keap1) signaling in oxidative stress. Free Radic Biol Med 2009; 47: 1304-1309.

[40] Fan Z, Wirth AK, Chen D, Wruck CJ, Rauh M, Buchfelder M and Savaskan N. Nrf2-Keap1 pathway promotes cell proliferation and diminishes ferroptosis. Oncogenesis 2017; 6: e371-e371.

[41] Jaiswal AK. Nrf2 signaling in coordinated activation of antioxidant gene expression. Free Radic Biol Med 2004; 36: 1199-1207.

[42] Kobayashi M and Yamamoto M. Molecular mechanisms activating the nrf2-keap1 pathway of antioxidant gene regulation. Antioxid Redox Signal 2005; 7: 385-394.