Original Research

Febuxostat ameliorates APAP-induced acute liver injury by activating Keap1/Nrf2 and inhibiting TLR4/NF-κB p65 pathways

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Impact Statement

Overdose of acetaminophen (APAP) is responsible for most cases of acute liver failure worldwide. Currently, N-acetyl-cysteine (NAC) is the only available drug for APAP overdose, but it has several limitations. For example, NAC should be used in the early stages of APAP intoxication, and the narrow therapeutic window of NAC still requires more studies. It is therefore urgent to discover new candidates for the treatment of APAP-induced hepatotoxicity. Febuxostat (Feb), an inhibitor of xanthine oxidase, was used to lower uric acid in patients with hyperuricemia or gout in the clinic. Our current study demonstrates that Feb posttreatment efficiently protects against APAP-induced liver damage by inducing GPX4 expression, activating the Keap1/Nrf2 pathway, and inhibiting the TLR4/NF-κB p65 pathway *in vivo* and *in vitro*. Feb also inhibited GSH depletion and JNK activation in the early injury phase of APAP-induced liver injury. In addition, pretreatment with Feb also ameliorates APAP-induced hepatotoxicity in mice. The results provide a candidate for the development of novel strategies to treat APAP overdose-induced hepatotoxicity, especially for those with hyperuricemia or gout.

Abstract

Excessive acetaminophen (APAP) application is a major cause of drug-induced liver injury (DILI). Febuxostat (Feb), a drug for reducing uric acid (UA) levels, was demonstrated to relieve hepatic inflammation and reverse organ functions. However, the effect of Feb on APAP-induced DILI and its mechanisms have not been fully explored. In this study, Feb (10 mg/kg) was given to mice by gavage 1 h after APAP (300 mg/kg, i.g.) induction. Serum and liver samples were collected 12 or 3 h after APAP challenge. Feb treatment was found to remarkably improve APAP-induced DILI, as evidenced by reduced serum ALT, AST and UA levels, pathomorphology, inflammatory, and oxidative responses. Consistently, treatment with Feb also reduced the cell injury induced by APAP in LO2 cells. Mechanistically, Feb induced GPX4 expression, activated the Keap1/Nrf2 pathway, and inhibited the TLR4/NF-κB p65 pathway. Feb also inhibited glutathione (GSH) depletion and Jun N-terminal kinase (JNK) activation in the early injury phase. Notably, pretreatment with Feb for 3 days also revealed preventive effects against APAP-induced DILI in mice. Overall, our data revealed a potential health impact of Feb on APAP-mediated DILI *in vivo* and *in vitro*, suggesting that Feb might be a potential candidate for treating DILI.

Keywords: Febuxostat, acetaminophen, liver injury, Keap1/Nrf2 pathway, TLR4/NF-κB p65 pathway, oxidative stress

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Introduction

Drug-induced liver injury (DILI) may lead to fatal acute liver injury.[1] Excessive acetaminophen (APAP) application is a leading cause of DILI worldwide.[2] Currently, N-acetyl-cysteine (NAC) is the only available drug for APAP overdose, but it has several limitations. For example, NAC should be used in the early stages of APAP intoxication,[3] and the narrow therapeutic window of NAC still requires more studies.[4,5]

APAP-induced liver injury is caused by a series of events. Excess APAP often leads to the formation of N-acetyl-p-benzoquinone (NAPQI), which is rapidly conjugated with glutathione (GSH) to induce toxic reactions.[6] Once the GSH is exhausted, excessive NAPQI will lead to severe hepatotoxicity and hepatocellular necrosis by reacting with various targets.[7] During this process, APAP overdose also induces strong inflammatory responses accompanied by the formation of excessive reactive oxygen species (ROS), which might further induce oxidative stress if not properly handled.[8,9] Therefore, the inhibition of oxidative stress has become an important approach to combat APAP-induced liver injury.

Nuclear factor-erythroid-related factor 2 (Nrf2), known as an important regulator of the antioxidant defense system, is required to maintain the balance of cell survival and is responsible for the regulation of oxidative stress by binding to antioxidant response elements (AREs).[10,11] After binding to ARE, Nrf2 then activates the downstream target genes encoding detoxification and antioxidant enzymes, including nicotinamide adenine dinucleotide phosphate (NAD(P)H)-quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO-1), and others.[12] Nrf2 is therefore widely considered as a critical target for drug development for the treatment of various liver diseases. Moreover, accumulating evidence shows that Nrf2 plays a crucial role in ameliorating APAP-induced hepatotoxicity, and many drugs alleviated APAP-induced DILI via Nrf2 activation.[13,14] Thus, activating the Nrf2 pathway to defend against oxidative stress might be an attractive option to prevent APAP-induced hepatotoxicity.[15,16]

Febuxostat (Feb), an inhibitor of xanthine oxidase (XO), was used to lower elevated uric acid (UA) in patients with hyperuricemia or gout in the clinic.[17] Feb exerted excellent roles in several experimental models via its antioxidant and anti-inflammatory effects,[18,19] such as doxorubicin-induced cardiotoxicity, cisplatin-induced renal injury, and injury-induced liver and lung damage.[20–22] Specifically, the role of Feb in APAP-induced liver injury from APAP overdose has not been well investigated. Here, the interesting role of Feb in APAP-induced DILI was revealed in mice and LO2 cells.

Materials and methods

Drugs and reagents

APAP and NAC with purity of up to 99.0% were obtained from Macklin (Shanghai, China). Feb was purchased from Bidepharm (GuangZhou, China). Corn oil was selected from Aladdin (Shanghai, China). Fetal bovine serum (FBS) was provided by Gibco BRL (Gaithersburg, MD, USA). Roswell Park Memorial Institute (RPMI) 1640 medium was provided by Pricella (Wuhan, China).

Cell culture and MTT assay

Human liver LO2 cells were obtained from Pricella (Wuhan, China). LO2 cells were cultured in 1640 medium supplemented with 10% FBS and 100 U/mL penicillin and streptomycin at 37°C with 5% CO_2. Cells were treated with APAP (0, 5, 10, 15, 20 mM) with or without Feb (10 μM) for 24 h in 96-well plates. At least six duplicate wells were set up for each group. After the indicated treatments, MTT (5 mg/mL) reagent (Solarbio, Beijing, China) was added to the cells and incubated for 4 h at 37°C, and then 100 μL/well DMSO was added to each well to dissolve the formazan. The optical density (OD) value at 570 nm was then detected using an 800TS microplate reader after shaking for 10 min (BioTek Instruments, Inc., Beijing, China).

Animal experiments

Six- to eight-week-old male C57BL/6 mice weighing 18–22 g were purchased from Guangdong ZhiYuan Biopharmaceutical Co, Ltd. (Certificate SCXK (YUE) 2021-0167; GuangZhou, China). The animals were fed for 7 days under SPF conditions. The whole study protocol was approved by the Committee of Animal Research of Southern Medical University and performed following their regulations. Besides, all the animal experiments in the present study were conducted according to the international guidelines.

APAP-induced DILI was induced by APAP gavage. Mice were randomly divided into four groups in this study: the control, Feb (10 mg/kg), APAP (300 mg/kg), and Feb + APAP groups, with 6 mice in each group.

To investigate the therapeutic effect of Feb on APAP-induced DILI, mice were given 300 mg/kg APAP after 12 h fasting, and Feb (10 mg/kg) was then administered orally 1 h after APAP treatment. Feb was diluted with corn oil, and NAC and APAP were prepared with PBS solution.

The animals were then euthanized by pentobarbital sodium (30 mg/kg) via intraperitoneal injection, and the serum samples and liver tissues were collected 12 or 3 h after APAP administration (illustrated by Figures 1(A) and 7(A)).

To investigate the preventive effect of Feb, mice in the Feb and Feb + APAP groups were treated with Feb for 3 days before APAP administration. Corn oil was given to the control and APAP groups from day 3 to day 1. On day 0, mice in the APAP and Feb + APAP groups were treated with APAP after 12 h of fasting. Then, Feb was applied by oral administration 1 h after APAP administration. Finally, the animals were sacrificed and the samples were collected as described above (illustrated by Figure 8(A)).

Histopathological analysis

Fresh liver tissues were kept in 10% neutral buffered formalin and fixed for 24 h (Servicebio, Wuhan, China). The fixed tissues were then embedded in paraffin and cut into 5-μm sections to be stained with hematoxylin-eosin (H&E) for histopathological analysis using light microscopy (Olympus/CX43, Japan).

Biochemical determinations

The blood samples were kept at 37°C for 30 min and subjected to centrifugation at 3000 rpm/min for 10 min to obtain the serum samples. Homogenates of the liver tissues were prepared by smashing and centrifugation at 3000 rpm/min for 10 min. The serum and hepatic ALT and AST levels, and the TC and TG contents in the liver were examined using a Bio-Chemical Analyzer (Mindary BS-330E, ShenZhen, China). The serum UA level was determined by Sinocare EA-12 (Changsha, China).

Enzyme-linked immunosorbent assay

The levels of IL-1β, TNF-α, ROS, MDA, and GSH in the serum and hepatic homogenates were detected by using enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Enzyme-linked Biotechnology, Shanghai, China) following the manufacturer's instructions.

Quantitative real-time polymerase chain reaction

Total RNA in liver tissue was extracted using an Animal Total RNA Isolation Kit by following the manufacturer's

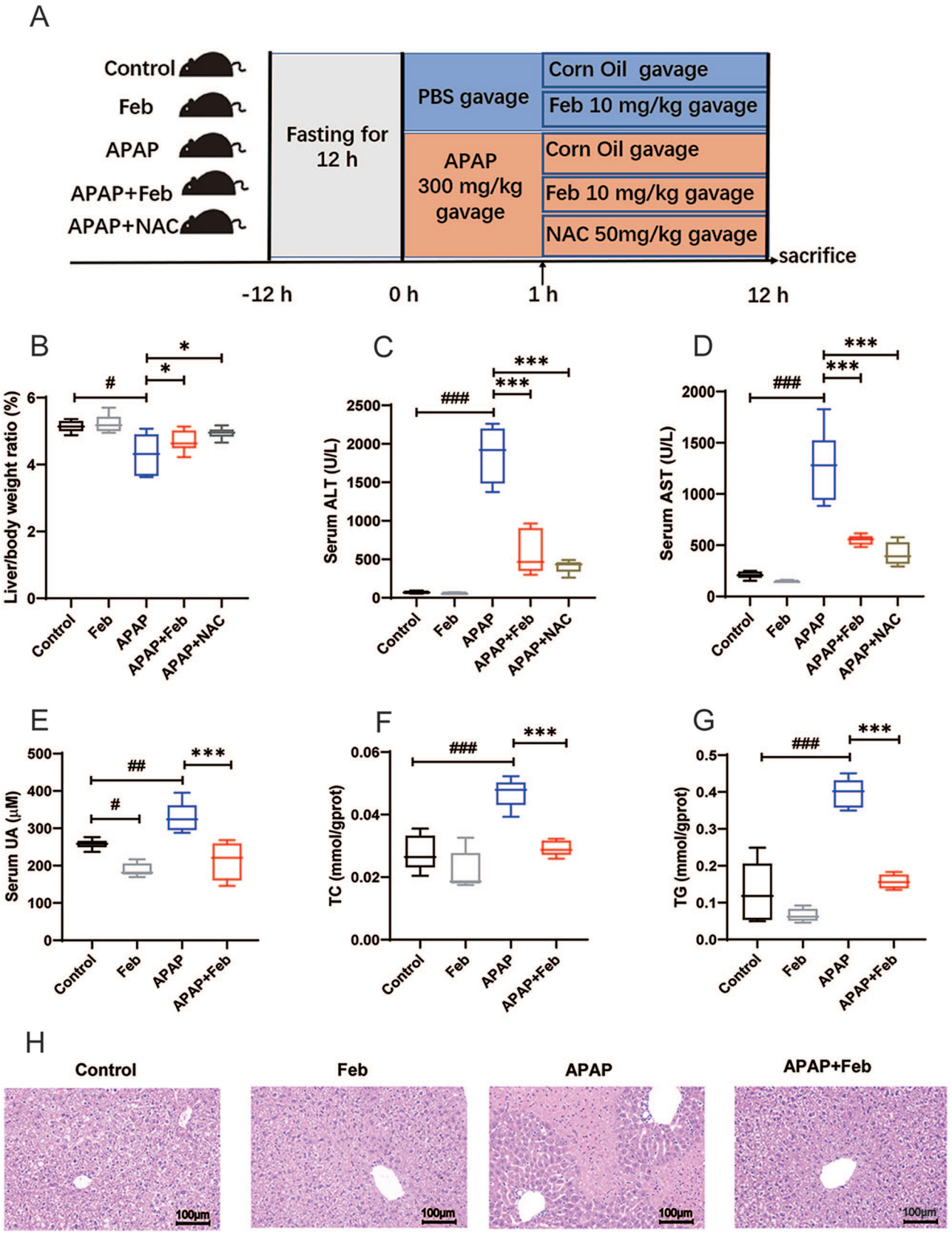

Figure 1. Therapeutic effect of Feb on APAP overdose-induced DILI. (A) Experimental design. (B) The ratio of liver weight/body weight. (C) Serum ALT. (D) AST. (E) UA levels. (F) Hepatic TC and (G) TG contents. (H) H&E staining of livers (200×).
Data are means ± SD; $n = 6$/group.
$^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus the Control group; $^{*}P < 0.05$, $^{***}P < 0.001$ versus the APAP group.

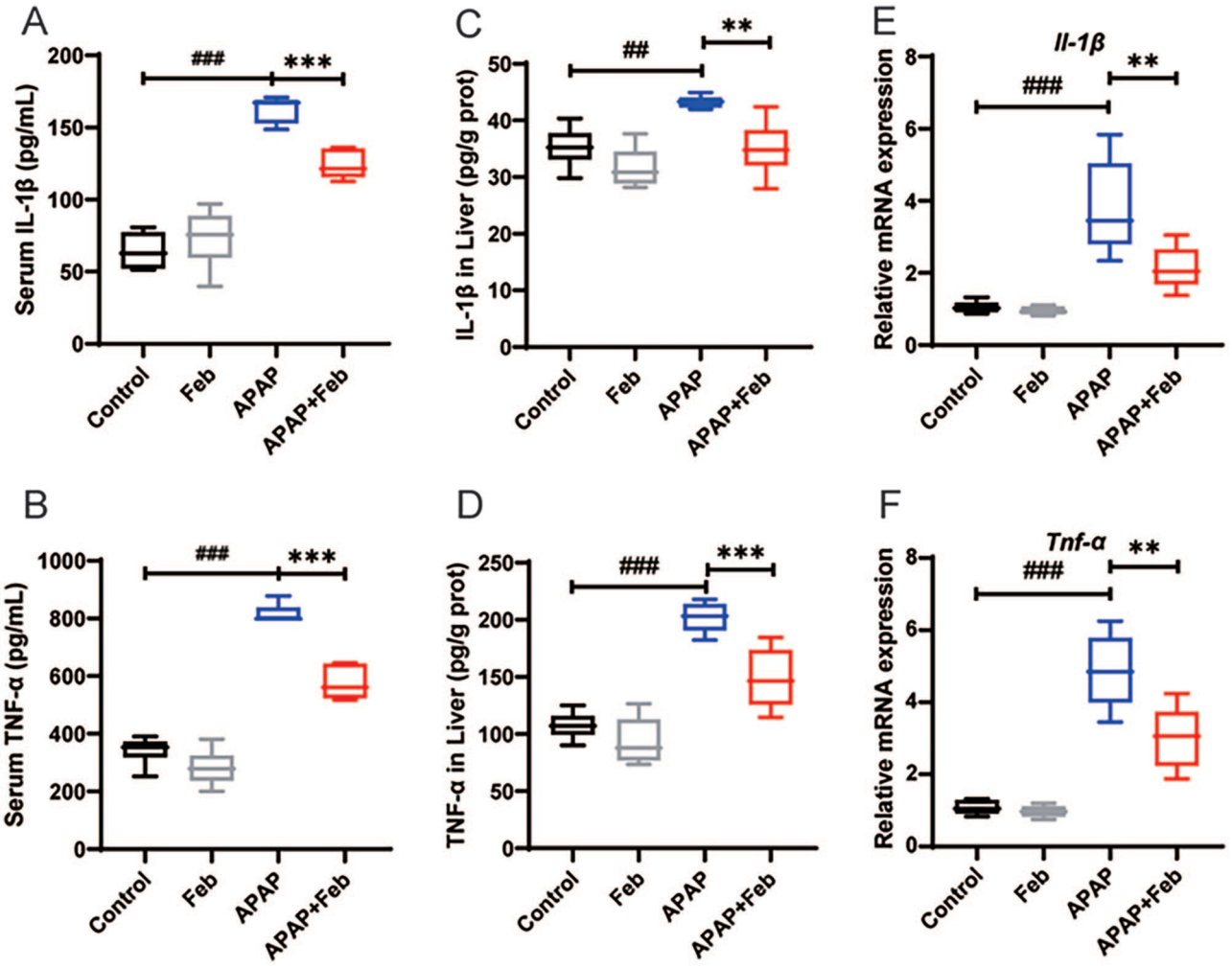

Figure 2. Feb posttreatment alleviated hepatic inflammation in APAP-induced DILI in mice. (A) Serum IL-1β level. (B) Serum TNF-α level. (C) Hepatic IL-1β level. (D) Hepatic TNF-α level. (E and F) Expression of mRNA levels of inflammation genes (*Il-1*β and *Tnf*-α).
Data are means ± SD; $n = 5$/group.
$^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus the Control group; $^{**}P < 0.01$, $^{***}P < 0.001$ versus the APAP group.

Table 1. Sequences of primers used for real-time PCR.

Primers	Sequence (5′-3′)
*Il-1*β Forward	GAAATGCCACCTTTTGACAGTG
*Il-1*β Reverse	TGGATGCTCTCATCAGGACAG
Tnf-α Forward	GACGTGGAACTGGCAGAAGAG
Tnf-α Reverse	TTGGTGGTTTGTGAGTGTGAG
Gapdh Forward	AATGGTGAAGGTCGGTGTGAACG
Gapdh Reverse	TCGCTCCTGGAAGATGGTGATGG

protocol (Foregene Co., Ltd., Chengdu, China). Then, RNA was reverse-transcribed into cDNA by Hifair® III for real-time PCR (RT-PCR) analysis using the 7500 RT-PCR system. The relative expression level of each gene was normalized to the Ct values of two commonly used housekeeping genes namely, *Gapdh*. The target gene-specific primer sequences are shown in Table 1.

Western blot analysis

The liver lysate was extracted with radioimmunoprecipitation assay (RIPA), and then the protein concentration was determined with a BCA kit (Thermo Fisher Scientific, Waltham, USA). The proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to PVDF membranes. After sealing the membranes, GAPDH antibody (Signalway, Beijing, Antibody, China, dilution 1:5000), Keap1 antibody, Nrf2 antibody, HO-1 antibody, NQO1 antibody (Proteintech, Wuhan, China, dilution 1:1000), GPX4 antibody (Abmart, Shanghai, China, dilution 1:1000), TLR4 antibody (Abmart, Shanghai, China, dilution 1:1000), NF-κB p65 antibody (Abmart, Shanghai, China, dilution 1:1000), and p-NF-κB p65 antibody (Abmart, Shanghai, China, dilution 1:1000) were added, and the membranes were incubated overnight at 4°C, which were subjected to the secondary antibody

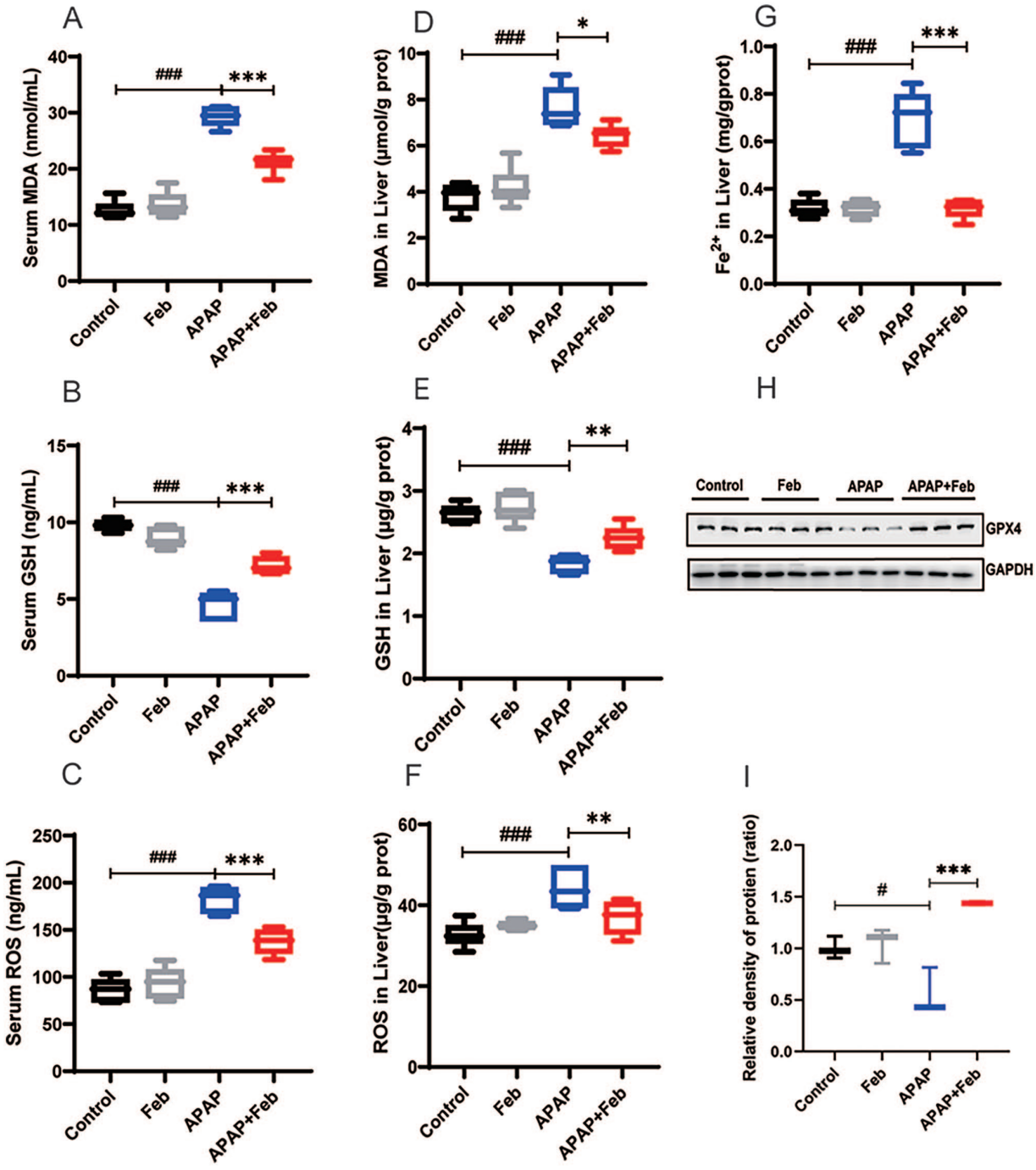

Figure 3. Feb posttreatment inhibited ferroptosis in APAP-induced DILI in mice. (A) Serum MDA level. (B) Serum GSH level. (C) Serum ROS level. (D) Hepatic MDA level. (E) Hepatic GSH levels. (F) Hepatic ROS levels. (G) Hepatic total Fe levels. (H and I) Relative protein expression level of GPX4.
Data are means ± SD; $n = 3$-5/group.
$^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ versus the Control group; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus the APAP group.

(1:2000, Affinity Biosciences, Melbourne, Australia) for 1 h. The protein bands were detected using electrochemiluminescence (ECL) reagents under Tanon system exposure. Image J was used to quantitatively analyze the intensity of protein bands.

Molecular docking

The molecular structure of Feb for this docking is selected from PubChem database. The structure of the Keap1 protein in combination with Nrf2 protein (PDB code: 2FLU) was obtained from the RCSB Protein Data Bank. The detailed docking methods were conducted as previously described.[23] The docking energies were finally obtained to analyze the docking affinity of Feb to Keap1.

Statistical analysis

Data are shown as means ± standard deviations (SD). The data were processed by GraphPad Prism 7 Software

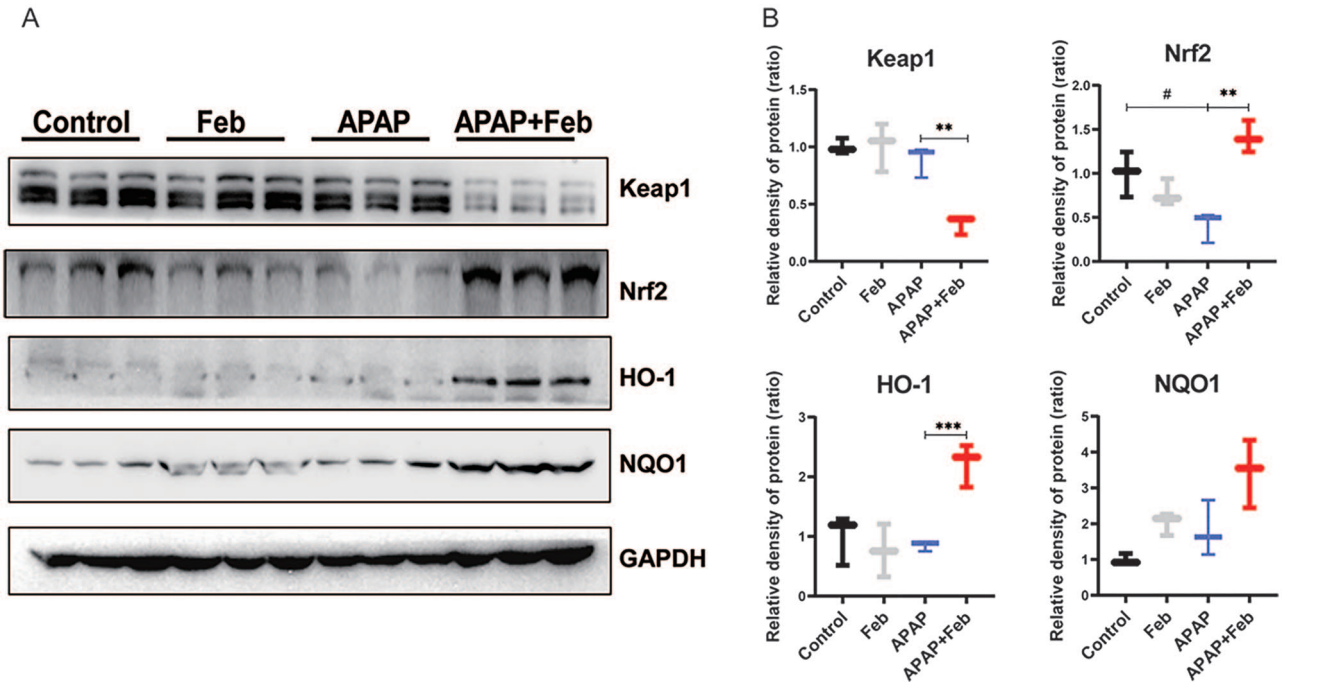

Figure 4. Feb posttreatment improved APAP-induced DILI by activation of Keap1/Nrf2 pathway. (A and B) Relative protein expression levels of Keap1, Nrf2, HO-1 and NQO1 in liver.
Data are means ± SD; $n = 3$/group.
$^{\#}P < 0.05$, versus the Control group; $^{**}P < 0.01$, $^{***}P < 0.001$ versus the APAP group.

to statistically analyze the difference between groups by one-way analysis of variance (ANOVA) followed by with Bonferroni post hoc test. $P < 0.05$ was considered as statistically significant.

Results

Feb posttreatment alleviates APAP-induced liver injury in mice

In order to confirm the contribution of Feb to APAP-induced liver injury *in vivo*, an acute model was employed in which mice were given APAP by gavage after 12 h of fasting (Figure 1(A)). Feb and NAC were applied 1 h after APAP administration. The results showed that Feb alone had no effects on the liver ratio (liver weight/body weight, mg/g) and the serum ALT and AST levels when compared with the control group, indicating that Feb at 10 mg/kg is safe in mice. However, APAP significantly decreased the liver ratio and increased the serum activities of ALT and AST (Figure 1(B) to (D)). Interestingly, the APAP-induced decline in liver ratio and elevation of serum ALT and AST were significantly attenuated by Feb treatment. NAC was used as a positive control and can also significantly reverse the decrease in liver-to-body weight ratio and the increases in serum ALT and AST induced by APAP (Figure 1(B) to (D)). However, we found that the serum UA of APAP group mice was significantly increased, which was also reversed by Feb (Figure 1(E)). Furthermore, we found that the contents of TG and TC in the liver of APAP-treated mice were significantly higher than those of the control group, suggesting that APAP-induced liver injury was accompanied by lipid metabolism imbalance, and treatment with Feb could significantly reduce TG and TC (Figure 1(F) and (G)). As evidenced by H&E staining of liver sections, APAP treatment induced centrilobular necrosis in APAP-treated mice at 12 h. Furthermore, Feb treatment led to much less hepatocellular injury and necrosis compared with the APAP group (Figure 1(H)). These results indicate that Feb posttreatment alleviated APAP-induced liver injury in mice.

Feb posttreatment alleviates APAP-induced inflammatory reactions in mice

Inflammation is often detected in DILI. As depicted in Figure 2, the serum and hepatic IL-1β and TNF-α levels were significantly higher in APAP-treated mice than in mice in the control group. Feb treatment markedly reduced the release of IL-1β and TNF-α, thus diminishing liver inflammatory injury. In addition, Feb inhibited the relative mRNA expression of *IL-1β and Tnf-α* in the liver. These results indicate that the hepatoprotective effect of Feb might be due to its capacity to combat inflammatory reactions.

Feb posttreatment inhibits oxidative stress in APAP-induced liver injury in mice

Lipid peroxidation can badly disrupt cellular function and is the driving force of oxidative stress. A previous study discovered lipid peroxidation in APAP-induced liver injury. In the present study, we explored whether oxidative stress is involved in the protective effects of Feb by examining the MDA, GSH, ROS, and total Fe content in the serum and

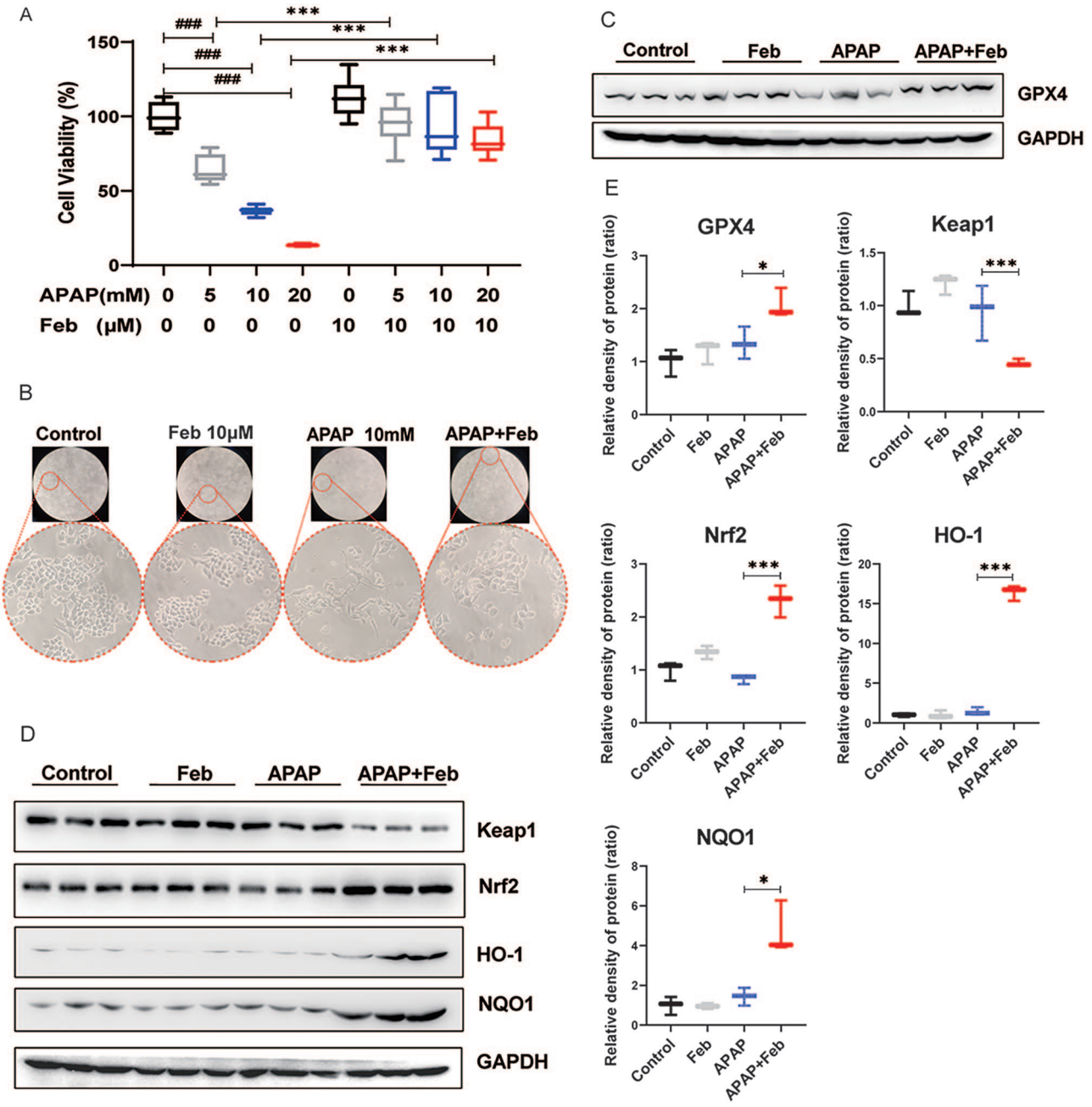

Figure 5. Feb suppressed APAP-induced hepatocellular damage in LO2 cells via increasing GPX4 expression and activating Keap1/Nrf2 pathway. (A) Feb (10 μM) reversed the reduction in cell viability induced by APAP (5–20 mM). (B) The cell appearance of LO2 cells treated with Feb, APAP, or in combination. (C to E) Relative protein expression levels of GPX4 and Keap1/Nrf2 pathway.
Data are means ± SD; $n = 3$-6/group.
###$P < 0.001$ versus the Control group (without APAP treatment); *$P < 0.05$, ***$P < 0.001$ versus the APAP-treated group.

liver. The data showed that Feb decreased the MDA, ROS, and total Fe levels and increased the GSH content compared with the APAP-treated mice (Figure 3(A) to (G)). We then observed an increase in the alteration of GPX4, which is a key regulator in the process of ferroptosis and is accepted to be associated with oxidative stress. Consistent with previous studies, we also found that APAP decreased the protein level of GPX4 in mouse liver. In contrast, Feb inhibited the APAP-induced change in GPX4 (Figure 3(H) and (I)). In addition, these data indicate that Feb regulates the GPX4 expression to ameliorate APAP-induced oxidative stress.

Feb posttreatment reverses the inhibition of the Keap1/Nrf2 pathway in APAP-challenged livers

The activation of the Keap1/Nrf2 signaling pathway is considered to be an important strategy for the treatment of various diseases.[24] To confirm whether the Keap1/Nrf2 pathway is involved in the effect of Feb, the expression of Keap1, an inhibitor of Nrf2 activation, was also detected. As shown in Figure 4, APAP administration induced a significant decrease in Nrf2. Interestingly, Feb inhibited the Keap1 expression and increased Nrf2 expression and its target downstream proteins, such as NQO1 and HO-1. Thus, the

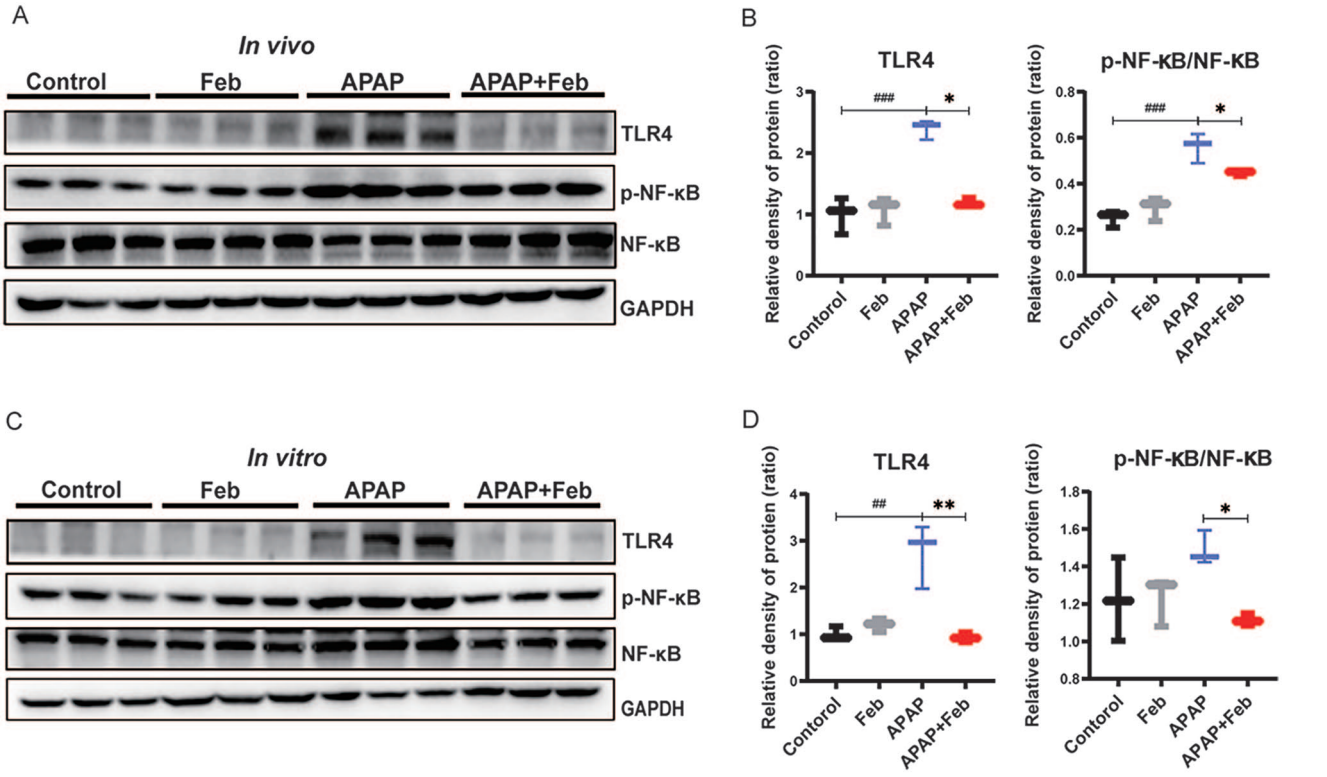

Figure 6. Feb inhibited TLR4/NF-κB p65 pathway in APAP-induced injury *in vivo* and *in vitro*. (A and B) Relative protein expression levels of TLR4/NF-κB p65 pathway *in vivo*. (C and D) Relative protein expression levels of TLR4/NF-κB p65 pathway *in vitro*.
Data are means ± SD; $n = 3$/group.
$^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus the Control group; $^{*}P < 0.05$, $^{**}P < 0.01$ versus the APAP group.

results demonstrate that the beneficial effect of Feb against APAP-induced liver injury might be attributed to its activation of the Keap1/Nrf2 pathway.

Feb posttreatment attenuates APAP-induced DILI by increasing GPX4 and activating the Keap1/Nrf2 pathway *in vitro*

We next examined the influence of Feb on APAP-treated LO2 cells. The results of the MTT assay showed that cotreatment with Feb (10 μM) and APAP (10 mM) distinctly improved the cell survival ratio and cell appearance compared with APAP treatment alone (Figure 5(A) and (B)). Similarly, Feb increased the relative protein expression of GPX4 in comparison with APAP-treated cells. Furthermore, Feb evidently inhibited Keap1 expression and induced Nrf2 protein expression and its target proteins, such as HO-1 and NQO1 (Figure 5(C) to (E)), when compared with APAP treatment alone, further confirming the beneficial role of Feb in APAP-induced injury.

Feb posttreatment inhibits the TLR4/NF-kB p65 pathway in APAP-induced liver injury *in vivo* and *in vitro*

The (toll-like receptor 4) TLR4/(nuclear factor kappa-light-chain-enhancer of activated B cells) NF-κB p65 pathway has been proved to play significant role in APAP-induced liver injury.[25] Activation of TLR4 triggers downstream signaling pathways and the activation of NF-κB p65, which mediates the release of inflammatory cytokines and promotes oxidative stress, ultimately leading to liver cell death in APAP overdose. Therefore, targeting the TLR4/NF-κB p65 pathway has been proposed as a potential strategy for the prevention or treatment of APAP-induced liver injury.[26] In line with reported results, APAP treatment resulted in the activation of TLR4/NF-κB p65 pathway as indicated by upregulation of TLR4 expression and enhancement of NF-κB p65 phophorylation. In the present study, we found that Feb significantly inhibited TLR4/NF-κB p65 pathway *in vivo* and *in vitro* (as illustrated in Figure 6).

Feb posttreatment inhibits GSH depletion and JNK activation in the early injury phase of APAP-induced liver injury in mice

To evaluate the impact of Feb on the metabolic activation of APAP, we conducted another animal experiment according to the scheme depicted in Figure 7(A). The results indicated that Feb significantly improved the elevation of ALT and AST induced by APAP, inhibited GSH depletion, and markedly suppressed JNK phosphorylation in the early phase of APAP-induced liver injury (as demonstrated in Figure 7(B) to (G)). These findings suggest that Feb may improve APAP-induced liver injury by modulating the metabolic activation of APAP. Moreover, we observed that Feb could activate Nrf2 in the early stage of APAP-induced liver injury, as evidenced by increases in the relative expression level of total and nuclear Nrf2 protein in the liver (as depicted in Figure 7(F) and (G)).

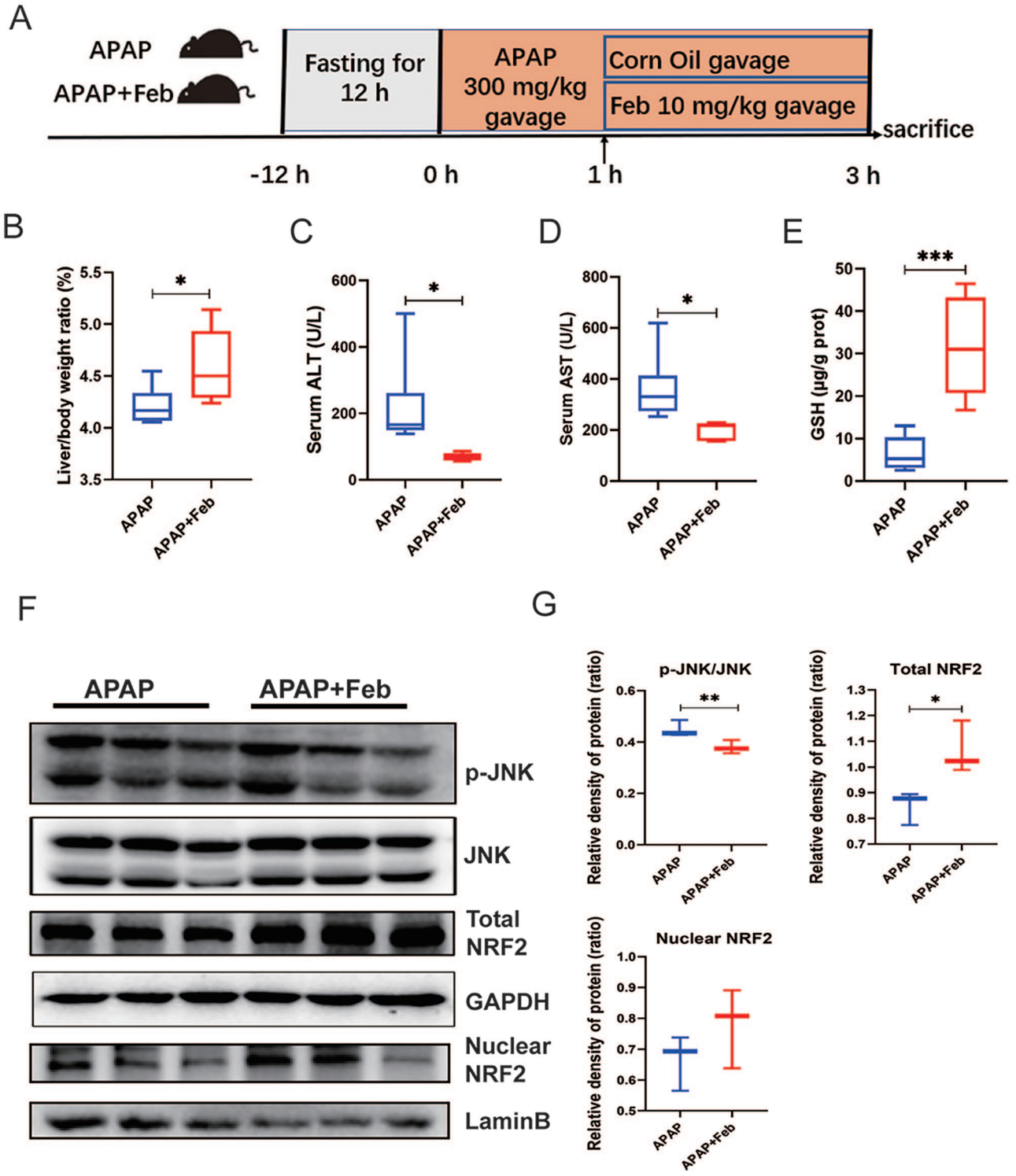

Figure 7. Feb inhibited GSH depletion and JNK activation induced by APAP in mice. (A) Experimental design. (B) The ratio of liver weight/body weight. (C) Serum ALT. (D) Serum AST. (E) Hepatic GSH content. (F and G) Determination of the activation of JNK and NRF2 in the liver after Feb treatment.
Data are means ± SD; $n = 3$-6/group.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus the APAP group.

Feb pretreatment reveals a preventive effect on APAP-mediated DILI in mice

Next, we aimed to assess the preventive effect of Feb treatment before APAP overdose in mice (Figure 8(A)). As shown in Figure 8(B) to (F), Feb treatment markedly attenuated APAP-induced hepatotoxicity, as evidenced by reduced ALT, AST, and UA levels as well as H&E analysis of liver sections. These results indicate that Feb could be a potential candidate for preventing APAP-induced DILI.

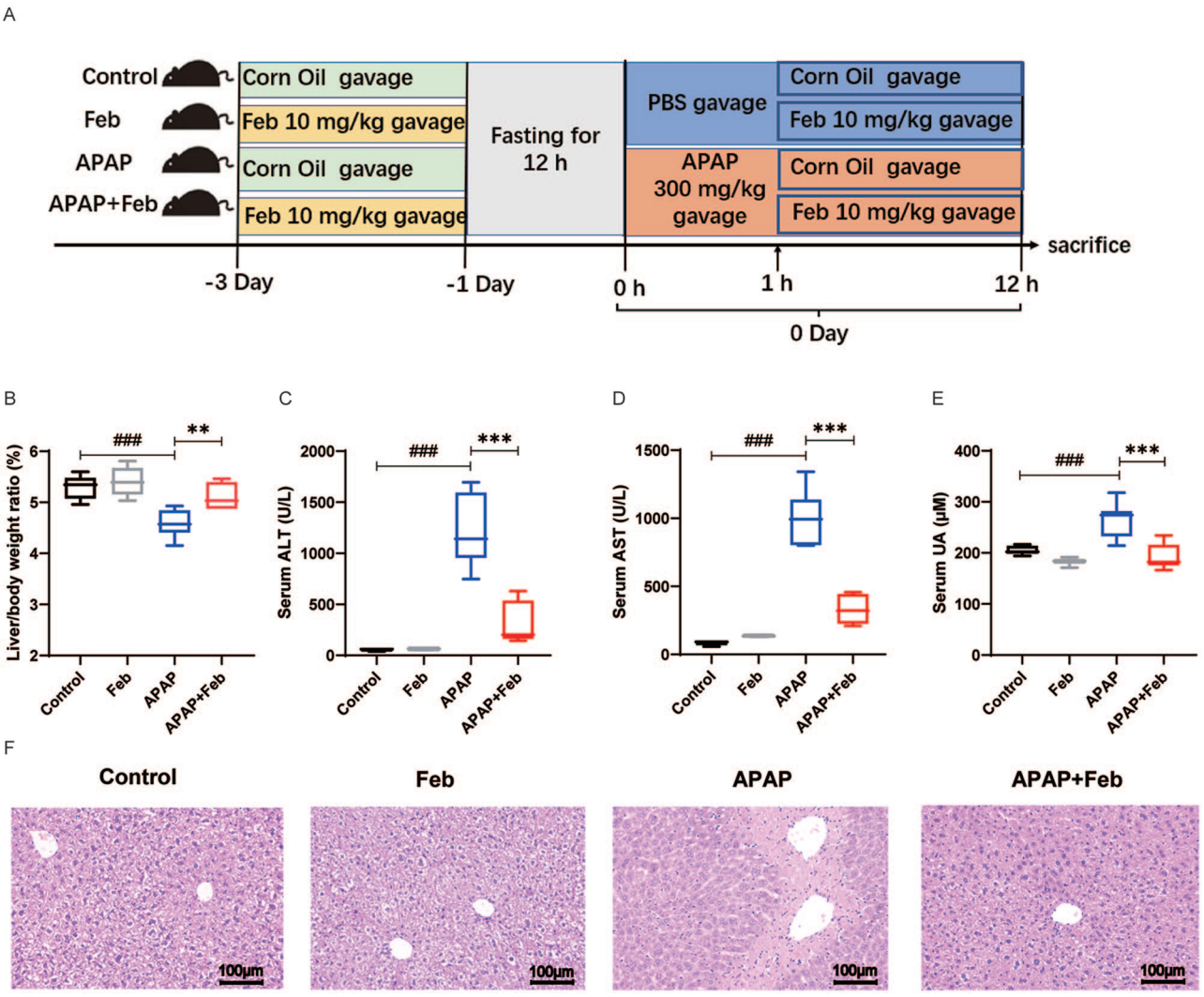

Figure 8. Feb pretreatment for 3 days ameliorates APAP overdose-induced DILI in mice. (A) Experimental design. (B) The ratio of liver weight/body weight; Serum (C) ALT, (D) AST and (E) UA levels. (F) H&E staining of livers (200×).
Data are means ± SD; $n = 3$-6/group.
$^{###}P < 0.001$ versus the Control group; $^{**}P < 0.01$, $^{***}P < 0.001$ versus the APAP group.

Feb binds to Keap-1 to regulate Nrf2 activity

To further investigate whether Feb activates Nrf2 by regulating Keap1, molecular docking was performed. The results of molecular docking showed that Feb had a good binding effect with Keap1 and a high matching degree (the binding energy was −8.05 kcal/mol, less than −5 kcal/mol). According to the binding mode revealed by the Pymol2.1 software, we can clearly find that Feb can bind to the pocket of Keap1 (Figure 9(B)). As exhibited in Figure 9(C), the residues of Arg483 and Ser508 were found to form an attractive charge or hydrogen bond with Feb. Moreover, the residues of Leu365, Val604, Leu557, Gly364, Ser363, Gly462, Phe478, and Tyr525 formed a van der Waals with Feb. Besides, the residues of Ala366, Arg415, and Ala556 formed alkyl or pi-alkyl bond with Feb. Previous studies have indicated that amino residues including Arg415, Arg483, Ser555, Ser508, Tyr525, Ala556, and Phe478 in Keap1 were involved in regulating the activity of Nrf2 or interacting with potent Keap1/Nrf2 regulators.[23,27] Our molecular docking results also revealed that Feb might interact with residues Arg483, Ser508, Leu365, Val604, Leu557, Gly364, Ser363, Gly462, Phe478, Tyr525, Ala366, Arg415, and Ala556 in Keap1 (Figure 9(C)). Altogether, the results in Figure 9 suggest that Feb may competitively bind with Keap1 to promote the dissociation of Nrf2 from Keap1 to enhance the function of Nrf2.

Discussion

APAP overdose is a well-known detrimental factor for DILI.[28] Current therapy for APAP-induced DILI is quite limited. Therefore, it is essential to develop additional drugs for the prevention and treatment of APAP-induced injury. The current study reports that Feb confers hepatoprotection against APAP-induced liver injury by inhibiting oxidative stress. Mechanistically, Feb activates GPX4 expression and the Keap1/Nrf2 pathway.

High UA has been widely demonstrated to induce inflammation and oxidative stress by inducing excessive ROS.[29] Previous studies found that APAP overdose could result in normal serum UA levels.[30] As a potent inhibitor of XO to

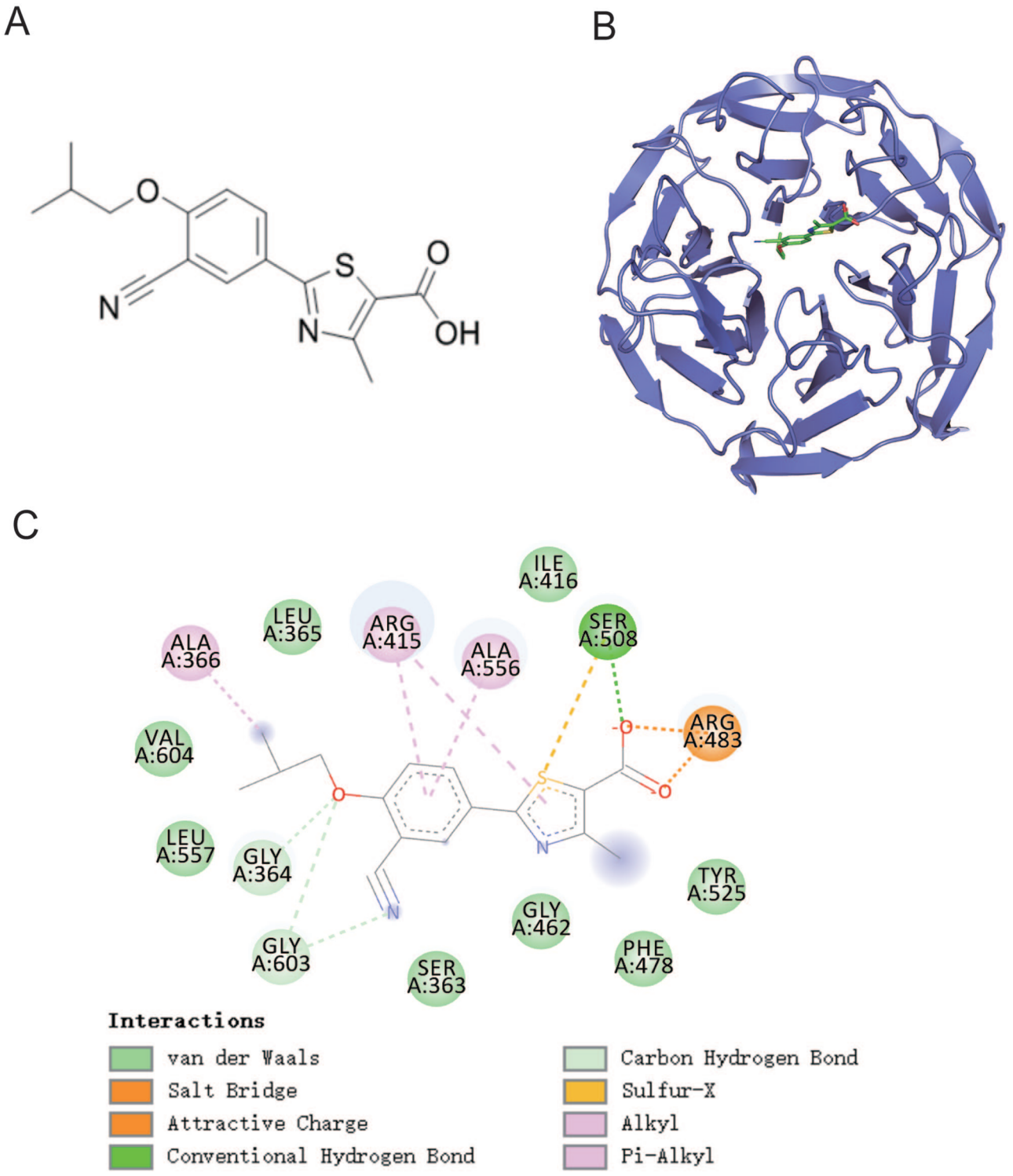

Figure 9. The detail binding mode of Feb with Keap1. (A) The structure of Feb. (B) The 3D structure of the complex of Feb and Keap1. (C) The 2D binding mode of the complex of Feb and Keap1.

reduce UA production, Feb post- or pretreatment was found to potently reduce serum UA in APAP-treated mice in the present study, which might contribute to the desirable effect of Feb on APAP-induced DILI.

Overdose of APAP has been proven to impair lipoprotein metabolism and influence normal cholesterol levels, thus triggering lipid peroxidation. Increases in serum TG and TC levels induced by APAP treatment were found in several studies.[31] In our study, Feb treatment significantly reduced hepatic TG and TC levels, which might prevent the occurrence of lipid peroxidation events and oxidative stress induced by APAP overdose.

A growing amount of evidence has revealed that inflammatory reactions play a critical role in further aggravating APAP-induced DILI.[32] Several studies have shown that Feb inhibits inflammatory responses by reducing the levels of proinflammatory mediators.[20,33] The present study also found that Feb dramatically protected against APAP-induced liver inflammation via reducing the production of inflammatory cytokines such as IL-1β and TNF-α. Besides, We found that Feb can significantly inhibit the increases of toll-like receptor 4 (TLR4) and phosphorylation of NF-κB caused by APAP *in vivo* and *in vitro*, suggesting a potential role of Feb in improving inflammatory responses.

It has been well-established that oxidative stress plays a crucial role in the development of APAP-induced liver injury. The excessive production of ROS and nitrogen species, coupled with the depletion of GSH, results in oxidative stress in the liver,[34] which further leads to lipid peroxidation, oxidative protein modifications, and DNA damage, thereby exacerbating liver injury. To alleviate this injury, researchers have explored the use of antioxidants, inhibitors of oxidative stress pathways, and other therapeutic agents. MDA is a commonly used indicator of lipid peroxidation. Importantly, the downregulation of GPX4 protein expression has been widely accepted to be responsible for APAP-induced liver injury.[35] Our experimental results not only confirm this view but also show that $Fe^{2}+$ is enriched in the liver tissue of APAP-challenged mice. Furthermore, Feb application distinctly reversed the reduction in GSH, the increase in lipid peroxide MDA, the increased $Fe^{2}+$ concentration, and the enhanced ROS level induced by APAP, demonstrating that Feb improves APAP-induced liver injury by partially inhibiting oxidative stress. Further experiments also showed that Feb significantly inhibited GSH depletion and JNK activation (Figure 7), thus alleviating APAP-induced liver injury. As previously reported, allopurinol was found to be effective in improving APAP-induced liver injury, likely due to its impact on JNK phosphorylation.[36] In this study, we observed that Feb posttreatment also significantly inhibited JNK phosphorylation following APAP treatment. However, since we did not examine the phosphorylation of JNK in the pretreatment part, it is currently impossible to determine whether the mechanism of action of Feb differs between pretreatment and posttreatment schemes.

The Keap1/Nrf2 system is crucial in combating APAP-induced DILI by regulating antioxidant enzymes and increasing GSH synthesis. Keap1 promotes the degradation of Nrf2 by the ubiquitination process under unstressed conditions.[37] Here, we showed that treatment with Feb prominently decreased Keap1 expression and increased Nrf2 and its target proteins HO-1 and NQO1 *in vitro* and *in vivo*. In addition to its role in inhibiting oxidative stress, further experiments revealed that Feb significantly enhances early total and nuclear Nrf2 expression (as shown in Figure 7(F) to (G)). These findings provide clear evidence of Nrf2 activation following Feb treatment, further supporting the therapeutic potential of Feb as a treatment for liver injury. In order to specifically illuminate the mechanisms by which Feb promoted Keap1/Nrf2 activation, molecular docking analysis was conducted to find the potential binding sites of Feb and Keap1. Interestingly, the obtained data shown in Figure 9(C) displayed that Feb might bind to Keap1 at Arg483 and Ser508 via attractive charge or conventional hydrogen bond as well as other residues to interpret the binding of Keap1 to Nrf2.

Conclusions

In summary, our current study demonstrates that Feb posttreatment efficiently protects against APAP-induced liver damage by inducing GPX4 expression, activating the Keap1/Nrf2 pathway, and inhibiting the TLR4/NF-κB pathway *in vivo* and *in vitro*. In addition, pretreatment with Feb can also ameliorate APAP-induced DILI in mice. The obtained results provide a candidate for the development of novel strategies to treat APAP overdose-induced DILI, especially for those with hyperuricemia or gout.

AUTHORS' CONTRIBUTIONS

JP, TW, JT, and SZ participated in the study design; JT, SZ, LL, and XL performed the experiments; YY, YL, KZ, FZ, and YC contributed to new reagents and analytic tools; SZ, TW, and JP wrote or contributed to the writing of the manuscript. All authors have read and agreed to the final version of manuscript.

DECLARATION OF CONFLICTING INTERESTS

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