Drug Design, Development and Therapy

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ORIGINAL RESEARCH

Sakuranetin Prevents Acetaminophen-Induced Liver Injury via Nrf2-Induced Inhibition of Hepatocyte Ferroptosis

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Introduction: Oxidative stress is an important cause of acetaminophen (APAP)-induced liver injury (AILI). Sakuranetin (Sak) is an antitoxin from the cherry flavonoid plant with good antioxidant effects. However, whether sakuranetine has a protective effect on APAP-induced liver injury is not clear.

Methods: Mouse and HepG2 cell models of APAP injury were used to investigate the effect of sakuranetin on AILI and its mechanism. Serum transaminase levels, histological changes, inflammatory mediators, oxidative stress, ferroptosis-related markers and Nrf2 signaling pathway proteins were analyzed.

Results: Sakuranetin significantly reduced serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), as well as inflammatory factor; increased HepG2 activity and decreased cell death; inhibited ROS production, increased glutathione (GSH) content, expression of Glutathione Peroxidase 4 (GPX4) and Solute Carrier Family 7 Member 11 (SLC7A11), and decreased malondialdehyde and Acyl-CoA Synthetase Long Chain Family Member 4 (ACSL4) expression in mice and HepG2 cells after APAP treatment. Further analysis showed that sakuranetin induced the activation of the NFE2 Like BZIP Transcription Factor 2 (Nrf2) signaling pathway in liver tissue and HepG2 cells and promoted the nuclear translocation of Nrf2. Moreover, the hepatoprotective effect of sakuranetin and its inhibitory effect on ferroptosis were significantly attenuated by the Nrf2 inhibitor ML385.

Conclusion: Sakuranetin alleviates AILI by activating the Nrf2 signaling pathway and inhibiting ferroptosis, and sakuranetin may be a potential therapeutic agent for the treatment of AILI.

Keywords: sakuranetin, AILI, ferroptosis, oxidation, Nrf2

Introduction

Acetaminophen is a commonly used over-the-counter antipyretic analgesic that is widely accepted due to its good efficacy and relatively low incidence of side effects.[1] However, in overdose or under certain conditions, paracetamol can cause drug-related liver damage, which is one of the most serious side effects. In recent years, excessive or inappropriate use of APAP has led to an increase in liver toxicity and damage, making it a leading cause of drug-related liver injury and acute liver failure.[2] Therefore, it is important to explore the mechanism of liver damage caused by paracetamol and find new treatment options.

APAP is metabolized in the liver by cytochrome P450 enzymes (mainly CYP2E1) to produce a highly reactive metabolite, N-acetyl-p-benzoquinoneimine (NAPQI).[3–5] Under normal circumstances, NAPQI and antioxidant glutathione (GSH) form harmless compounds and cleared.[3–5] When APAP intake is too large to be processed by the liver, massive NAPQI production leads to rapid depletion of glutathione stores. After glutathione depletion, unbound NAPQI forms covalent associations with cellular proteins and other molecules, resulting in direct cellular damage.[3–5] This process is accompanied by the massive formation of reactive oxygen species (ROS), which further worsens the

Received: 15 October 2024
Accepted: 7 January 2025
Published: 10 January 2025

oxidative stress state.[6] Oxidative stress plays a central role in AILI, which is not only directly involved in hepatocyte damage, but also promotes the subsequent inflammatory response and cell death.[6] Therefore, it can strengthen the antioxidant capacity of cells and effectively relieve oxidative stress, thereby reducing or preventing the hepatotoxic effect of APAP.

Ferroptosis is a new form of iron and reactive oxygen species (ROS)-dependent regulated cell death characterized by decreased intracellular glutathione (GSH), decreased glutathione peroxidase 4 (GPX4) activities, attenuated GPX4-mediated anti-redox response, iron overload, and a large amount of ROS production. Ferroptosis plays an important regulatory role in a variety of liver diseases, including liver ischemia-reperfusion injury, hepatocellular carcinoma, liver fibrosis, and drug-induced liver injury.[7–9] Studies have shown that the ferroptosis inhibitor ferrostatin-1 could reduce liver damage and improve the survival rate of mice in AILI.[10] Furthermore, promoting ferroptosis may worsen APAP liver injury. For example, overexpression of GAS1 exacerbates APAP liver injury by promoting ferroptosis-induced accumulation of lipid peroxides.[11]

Nrf2 is a key molecule in the antioxidant system and plays a crucial role in maintaining cellular redox homeostasis.[12] Under oxidative stress conditions, ROS accumulation induces the nuclear translocation of Nrf2 and activates the gene expression of a number of antioxidant proteins and detoxification enzymes.[12,13] In particular, taraxasterol exerts a protective effect on AILI by activating the Nrf2 signaling pathway.[14] In a mouse model of AILI, abietic acid exerted a hepatoprotective effect by enhancing Nrf2 expression to alleviate ferroptosis.[15] In addition, oxidative stress and ferroptosis are closely related to AILI. These findings suggest that targeting the Nrf2 signaling pathway could be an effective treatment for AILI.

Sakuranetin is a tricyclic sesquiterpene isolated from Murraya koenigii with antioxidant, anti-inflammatory, anti-apoptotic, and anticancer properties.[16–19] Sakuranetine has been reported to improve D-galactose-induced learning and memory impairment in rats through an antioxidant mechanism.[20] In addition, sakuranetine has been shown to alleviate LPS-induced acute lung injury in part through its antioxidant effects.[21] Oxidative stress was an important cause of AILI, and sakuranetin may also be involved in the regulation of oxidative stress during AILI. However, the role of Sakuranetin in AILI has not been investigated. Therefore, the aim of this study is to investigate the possible effects of sakuranetine on AILI.

Materials and Methods

Animals and Liver APAP Injury Model

WT wild male C57BL/6J mice (6–8 weeks, 20–25 g) used in the experiment were obtained from Jinan Xinbainuo Biotechnology Co, LTD and were maintained under a 12-hour light/dark cycle with free access to food and water. A total of 54 mice were used in this study (n = 6 per group). All experimental protocols followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the ARRIVE guidelines. The experiment was approved by the Ethics Committee of Jingzhou Hospital Affiliated to Yangtze University (approve number: 202401018). Before the experiment, the animals were allowed to get used to the environment for a week. After overnight fasting, mice were injected intraperitoneally with 400 mg/kg APAP (Aladdin, Shanghai, China) to induce liver injury, and the mice were anesthetized with intraperitoneal sodium pentobarbital (60 mg/kg) and sacrificed 24 h after APAP administration, and liver tissue and serum samples were collected for assay analysis. Sakuranetin was diluted in DMSO and 0.9% saline (4:1) (Vehicle), sakuranetin was administered by intraperitoneal injection once daily for 7 days. The dose of SK used in the present study (20 $mg.kg^{-1}$) was based on previous studies.[22–24]

Measurement of Liver Function

24 h after APAP administration, blood was collected from the orbit, centrifuged at 3000 rpm for 10 minutes, the supernatant was collected, and serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using ALT and AST kits (JianCheng Bioengineering Institute, Nanjing, China).

H&E Staining

Liver tissue was fixed in 10% formalin for 48 h, embedded in paraffin, and cut into 5-μm-thick paraffin sections. Sections were roasted at 60°C for 2 h, deparaffinized in xylene, dehydrated in gradient ethanol, and stained according to the hematoxylin and eosin (H&E) staining reagent instructions (Servicebio, Wuhan, China). After staining, the sections were soaked in gradient ethanol and xylene dewaxing, sealed in neutral resin, and dried in a ventilated place. The pathological changes in the liver tissue were observed under a light microscope (Olympus, Japan).

ELISA

24 h after APAP administration, blood was collected from the orbit, centrifuged at 3000 rpm for 10 min, and the supernatant was collected. The samples were added according to the instructions of the IL-1β (Cat No. KE10003, Proteintech, Wuhan, China), IL-6 (Cat No. KE10007, Proteintech, Wuhan, China) and TNF-α (Cat No. KE10002, Proteintech, Wuhan, China) ELISA kit, and then detected by the enzyme marker, the standard curve was plotted according to the OD values of the standard wells and the serum levels of the corresponding inflammatory factors were calculated.

Oxidative Stress Analysis

Mouse liver tissue was homogenised with the extraction solution at a ratio of weight (g): volume (mL) = 1: 9. The supernatant was collected by centrifugation at 4°C, 8000 rpm for 10 min. The protein concentration was determined using the BCA assay kit, and then the levels of MDA and GSH in liver tissue were determined according to the instructions of the MDA (BC0025, Solarbio, Beijing, China) and GSH (BC1175, Solarbio, Beijing, China) kits.

DHE Staining

Liver tissues were embedded in OTC, frozen and cut into 5-μm-thick sections, stained with a 10 μM dihydroethidium (DHE) fluorescent probe (Servicebio, Wuhan, China), incubated in the dark at room temperature for 1 h, then stained with DAPI for 10 min and finally the sections were observed under a fluorescence microscope (IX71, Olympus, Japan).

Western Blot

Protein was extracted from liver tissue and HepG2 cells with different treatments according to the RIPA lysis buffer instructions (Solarbio, Beijing, China), and the protein concentration was determined using the BCA kit (Solarbio, Beijing, China) and adjusted to 5μg/μL. Protein was separated with 10% or 12% SDS-PAGE glue and transferred to PVDF membrane after electrophoresis. After membrane transfer, the membranes were blocked with 5% skimmed milk for 1 h at room temperature and incubated with primary antibodies overnight at 4°C. The PVDF membrane was then incubated with goat anti-rabbit or goat anti-mouse secondary antibodies for 1 hour at room temperature. After the membranes were washed three times with TBST, ECL-Luminescence regent was added uniformly to the membrane and the ImageQuant 800 system (Cytiva, USA) was used for imaging. Antibody information is presented in Table 1.

Table 1 Antibodies Information for Western Blot

Antibody	Company	Catalog Number	Source	Concentration
ACSL4	HUABIO	ET7111-43	Rabbit	1:1000
SLC7A11	Proteintech	26864-1-AP	Rabbit	1:1000
GPX4	CST	52455	Rabbit	1:1000
Nrf2	Proteintech	16396-1-AP	Rabbit	1:1000
HO-1	Proteintech	10701-1-AP	Rabbit	1:1000
GAPDH	Proteintech	60004-1-Ig	Mouse	1:5000

https://doi.org/10.2147/DDDT.S497817

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent was used to extract total RNA from liver tissue and cells, and a NanoDrop 2000 spectrophotometer was used to measure the amount of RNA extracted. Using a reverse transcription kit, 1 μg of total RNA was converted to cDNA. On a qPCR apparatus, cDNA was amplified using SYBR Green qPCR Mix. GAPDH served as the internal benchmark. GAPDH served as the internal benchmark. The $2^{-\Delta\Delta}$CT approach was applied to the analysis. The amplification primer sequences are displayed in Table 2.

Cell Culture and APAP Injury Model

HepG2 cells were purchased from Procell Biotechnology Co., Ltd. and cultured in DMEM medium containing 10% FBS, 1×10^5 U/mL penicillin and 100 mg/mL streptomycin. The culture was maintained at 37 °C in a 5% CO_2 constant temperature incubator. APAP was dissolved in DMEM culture medium at 37 °C and added to the cells after filtration through a 0.22 μm filter and assayed after 24 hours of APAP treatment.

Cell Counting Kit-8 Assay

HepG2 cells were inoculated into 96-well plates at a density of 5000 cells/well and APAP was added 12 h later. After 24 h of APAP administration, 10 μL of CCK8 solution (Solarbio, Beijing, China) was added to each well. After incubation at 37°C for 1 hour, absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc).

Cellular ROS Detection

HepG2 cells were inoculated into 6-well plates, APAP was added after 12 h, and DCFH-DA fluorescence probe (Beyotime, Shanghai, China) was performed after 24 h according to the reagent instructions. After incubation at 37°C for 20 min, the nuclei of the cells were stained with DAPI for 10 min and washed three times with serum-free DMEM medium, then photographed and analysed by fluorescence microscopy (IX71, Olympus, Japan).

Molecular Docking

The 3D structure of sakuranetin was downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov) and saved in mol2 format after energy minimisation of the 3D structure of sakuranetin using ChemBio3D Ultra 14.0. AutodockTools was then used to hydrogenate the mol2 format, calculate the charge, assign the charge, set the rotate key and save as "pdbqt" format. In addition, the protein structure of Nrf2 (AF-Q60795-F1) was downloaded from the AlphaFold protein structure database (https://alphafold.ebi.ac.uk/) and the protein structure was imported into AutoDocktools (v1.5.6) for hydrogenation, charge calculation, charge assignment, atom type designation and saving in "pdbqt" format after removing protein crystal water and original ligand using Pymol 2.3.0. POCASA 1.1 was used to predict protein binding sites, and AutoDock Vina 1.1.2 was used for docking. Finally, the docking results were analysed for interaction patterns using PyMOL 2.3.0.

Table 2 Primers for Real-Time PCR Detection

Primer	Primer Sequence
GPX4-F	GCCAAAGTCCTAGGAAACGC
GPX4-R	AAGGTTCAGGAATGGGCTCC
SLC7A11-F	AAATACGGAGCCTTCCACGAG
SLC7A11-R	CAGCCTTCGCTGGCTCTATAA
ACSL4-F	CTCACCATTATATTGCTGCCTGT
ACSL4-R	TCTCTTTGCCATAGCGTTTTTCT
GAPDH-F	AGGTCGGTGTGAACGGATTTG
GAPDH-R	TGTAGACCATGTAGTTGAGGTCA

Immunofluorescence (IF) Staining

HepG2 cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton for 5 min, closed with 5% BSA for 30 min at room temperature, and incubated with anti-Nrf2 primary antibody at 4°C overnight. After being washed three times with PBS, the cells were incubated with goat anti-rabbit fluorescent secondary antibody for 1 h at 37°C, and then the nuclei were stained with DAPI for 10 min. Images were obtained under a fluorescence microscope (IX71, Olympus, Japan).

Statistical Analysis

Statistical analysis was performed using SPSS 22.0 software. Data were expressed as mean ± SD. Comparisons between two groups were made using Student's *t*-test. Comparisons between multiple groups were made using one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Results

Sakuranetin Alleviates APAP-Induced Liver Injury

We established a mouse model of APAP-induced liver injury and evaluated the degree of liver injury by alanine aminotransferase (ALT), aspartate aminotransferase (AST) and necrotic area. The structure of sakuranetin was shown in Figure 1A. Compared with the vehicle+NS group, serum ALT, AST levels and necrotic area of liver tissue were significantly increased after APAP administration (Figure 1B–E). Sakuranetin pretreatment significantly reduced ALT

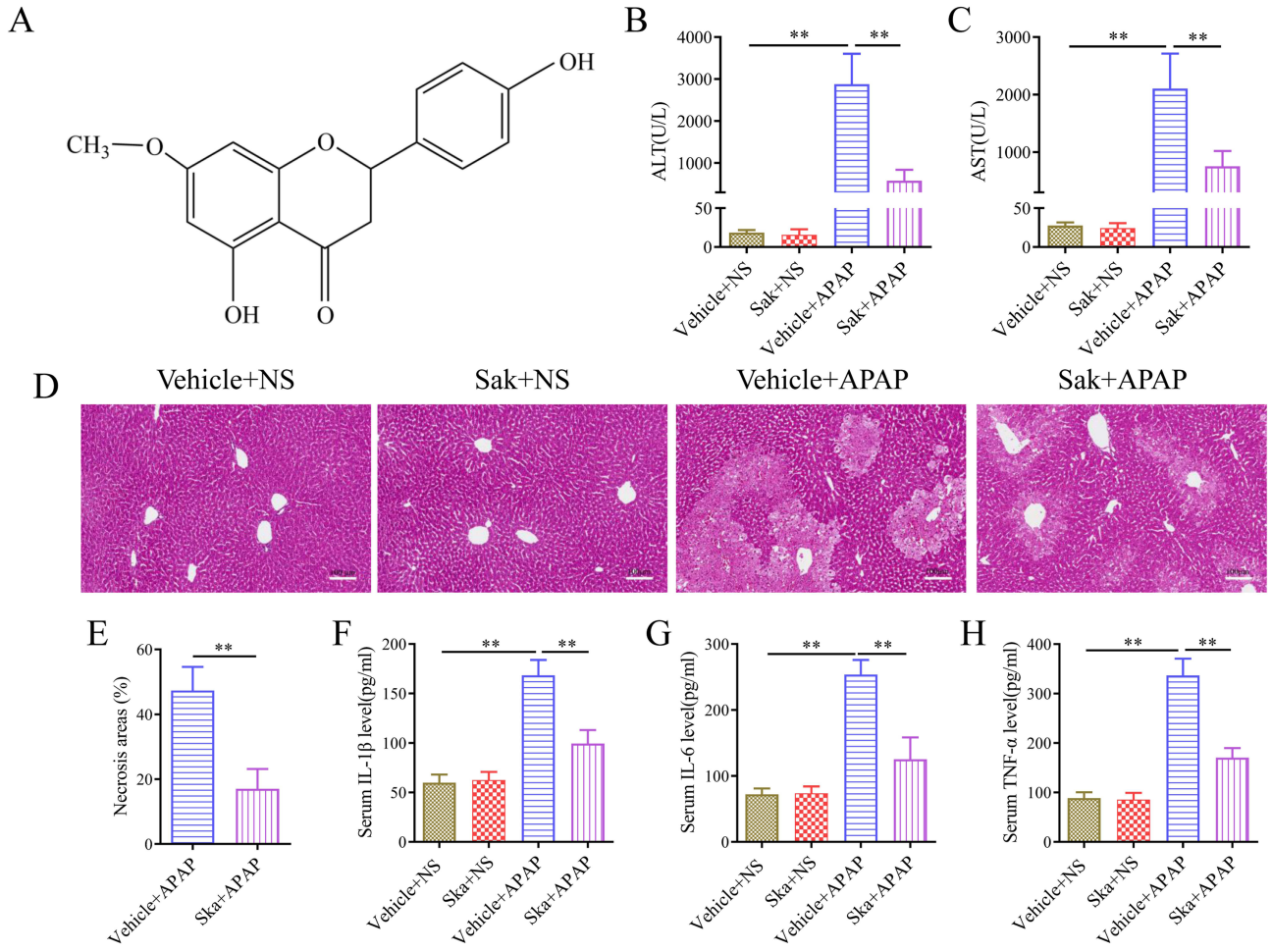

Figure 1 Sakuranetin alleviates APAP-induced liver injury (**A**) The structure of sakuranetin. (**B**) Serum ALT and (**C**) AST levels in each group (n = 6/group); (**D**) H&E staining and (**E**) necrotic area statistics of liver tissue in mice (n = 6/group). (**F–H**) Serum contents of IL-1β, IL-6, and TNF-α were detected by ELISA (n = 6/group). **P < 0.01.

https://doi.org/10.2147/DDDT.S497817

and AST levels and liver necrosis (Figure 1B–E). In addition, serum levels of the inflammatory factors IL-1β, IL-6 and TNF-α were significantly lower in sakuranetin-treated mice than in the APAP group (Figure 1F–H). These results suggest that sakuranetin alleviates AILI.

Sakuranetin Alleviates APAP-Induced HepG2 Cells Injury

HepG2 cells showed no toxic effect when the concentration of sakuranetin was less than 150 μM (Figure 2A). Furthermore, sakuranetin increased the activity of HepG2 cells after APAP administration in a concentration-dependent manner; however, there was no significant difference between the concentrations of 100 and 150 μM (P=0.9001) (Figure 2B). Therefore, 100 μM sakuranetin was chosen for the following experiments. The results of LDH content analysis showed that sakuranetin significantly reduced APAP-induced cell damage (Figure 2C). In addition, PI staining further confirmed that sakuranetin significantly reduced APAP-induced cell death (Figure 2D-E). These cellular results indicate that sakuranetin alleviates APAP-induced damage in HepG2 cells.

Sakuranetin Ameliorates APAP-Induced Liver Oxidative Stress and Ferroptosis in Mice

GSH and MDA are important indices of oxidative stress. Compared with the vehicle+NS group, GSH activities in liver tissue were significantly decreased and MDA levels were significantly increased after APAP administration (Figure 3A and B). However, compared with the APAP group, GSH activities were significantly increased in the APAP+SAK group, while MDA levels were significantly decreased (Figure 3A and B). DHE staining showed that sakuranetin could inhibit the increase in ROS after APAP administration (Figure 3C). The results of ferroptosis indicators showed that APAP treatment increased the mRNA and protein levels of ACSL4 and decreased the mRNA and protein levels of SLC7A11 and GPX4 in liver tissues (Figure 3D–J), whereas sakuranetin treatment reversed the alteration of these molecules to inhibit ferroptosis (Figure 3D–J). These results suggest that sakuranetin reduces APAP-induced liver oxidative stress and ferroptosis in mice.

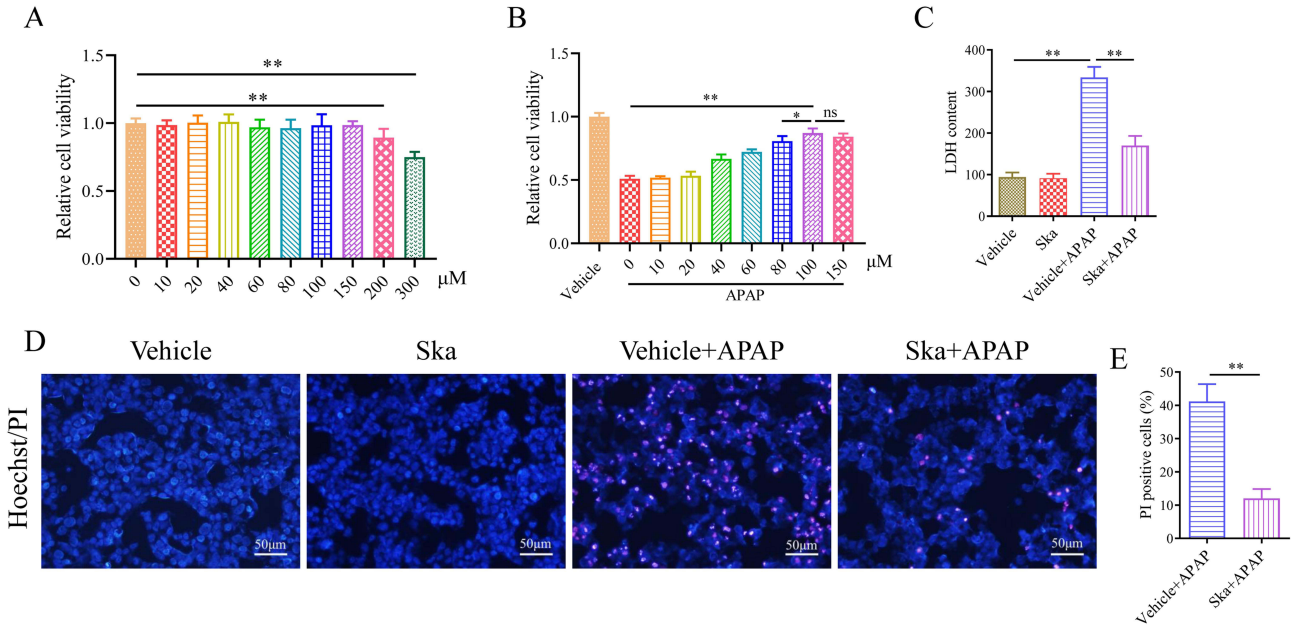

Figure 2 Sakuranetin alleviates APAP-induced HepG2 cells injury (**A**) CCK8 assay was used to detect the effect of Ska on the activity of HepG2 cells (n = 6/group). (**B**) CCK8 assay was used to detect the effect of Ska on the activity of HepG2 cells after APAP injury (n = 6/group). (**C**) LDH content in different group HepG2 cells (n = 6/group). (**D**) PI staining and (**E**) statistical analysis of HepG2 cells (n = 6/group). *P < 0.05 and **P < 0.01.
Abbreviation: ns, none significance.

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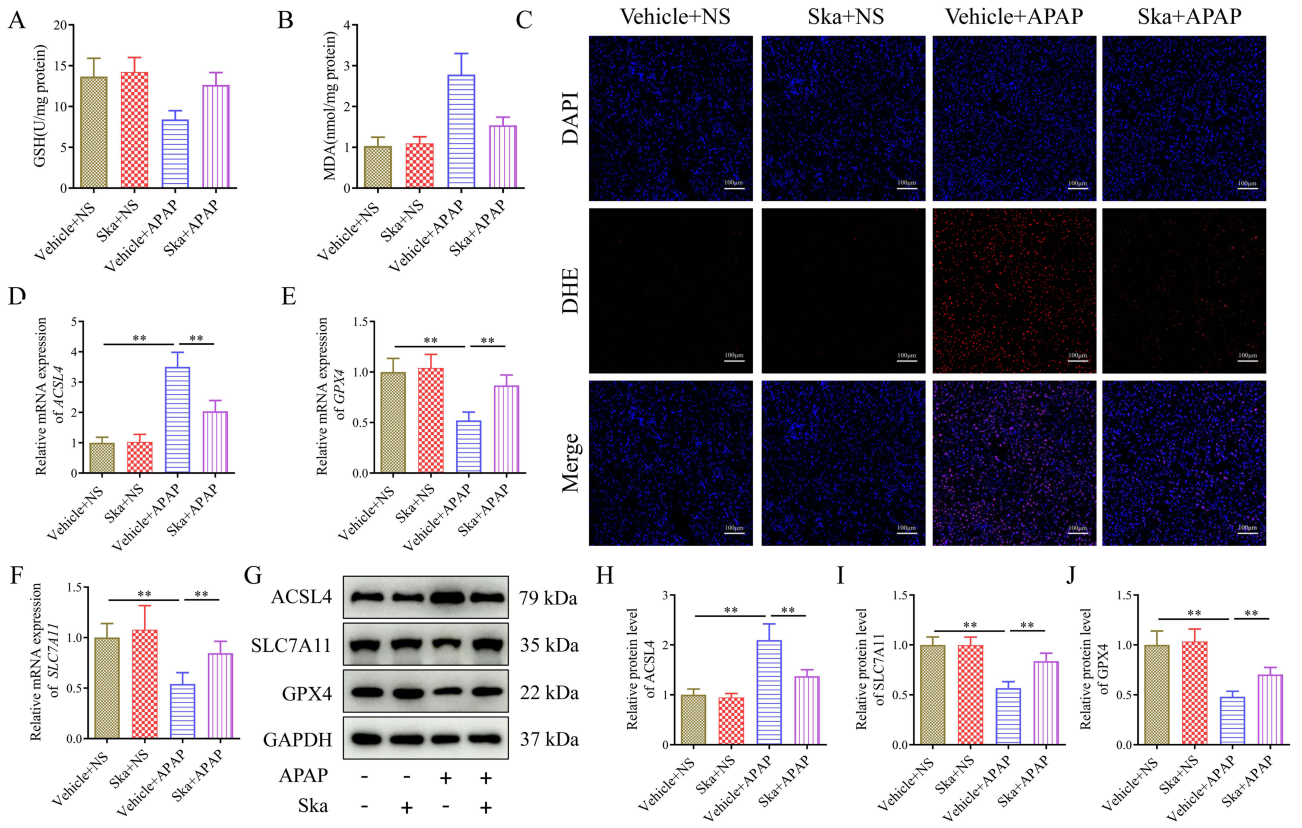

Figure 3 Sakuranetin ameliorates APAP-induced liver oxidative stress and ferroptosis in mice (**A**) GSH activity in liver tissue (n = 6/group). (**B**) MDA levels in liver tissue (n = 6/group). (**C**) DHE staining (red fluorescence indicates positive DHE staining, n = 6/group). (**D–F**) mRNA expression of ACSL4, SLC7A11 and GPX4 in liver tissues (n = 6/group). (**G**) Protein detection and (**H–J**) statistical analysis of ACSL4, SLC7A11 and GPX4 in mouse liver tissues (n = 6/group). **P < 0.01.

Sakuranetin Ameliorates APAP-Induced Oxidative Stress and Ferroptosis in HepG2 Cells

To further investigate the protective role of sakuranetin in AILI, we examined the changes in oxidative stress and ferroptosis in HepG2 cells after APAP administration.

DCFH-DA staining showed that sakuranetin significantly inhibited APAP-induced ROS production in HepG2 cells (P<0.0001) (Figure 4A and B), and GSH and MDA assays also confirmed the inhibitory effect of sakuranetin on APAP-induced oxidative stress in HepG2 cells (Figure 4C and D). The results of ferroptosis indicators showed that APAP treatment increased the mRNA and protein levels of ACSL4 and decreased the mRNA and protein levels of SLC7A11 and GPX4 in HepG2 cells, whereas sakuranetin treatment reversed the alteration of these molecules to inhibit ferroptosis (Figure 4E–K). Therefore, our in vitro experiments further demonstrate that sakuranetin inhibits APAP-induced oxidative stress and ferroptosis in HepG2 cells.

Sakuranetin Activates Nrf2 Signaling During APAP-Induced Liver Injury

The Nrf2 signaling pathway is a major pathway in the antioxidant defence system and plays a key role in the defence against oxidative stress and ferroptosis. SuperPred (prediction.charite.de) online prediction was used to predict the potential targets of sakuranetin,[25] the results showed that Nrf2 was one of the potential targets of sakuranetin (Supplementary material, Supplementary xlsx), and we further explored the binding relationship between sakuranetin and Nrf2 by molecular docking. The docking results showed that docking binding energy was −8.1 kcal/mol, and sakuranetin formed hydrogen bond interactions with amino acid residues ASP-526, LYS-525 and GLU-518, and the hydrogen bond length was 3.0 Å, 3.1 Å and 2.9 Å, and hydrophobic force with amino acid residues TYR-46, PH-39, GLN-519 and GLY-522 of Nrf2, docking results suggested that sakuranetin can bind Nrf2 more spontaneously

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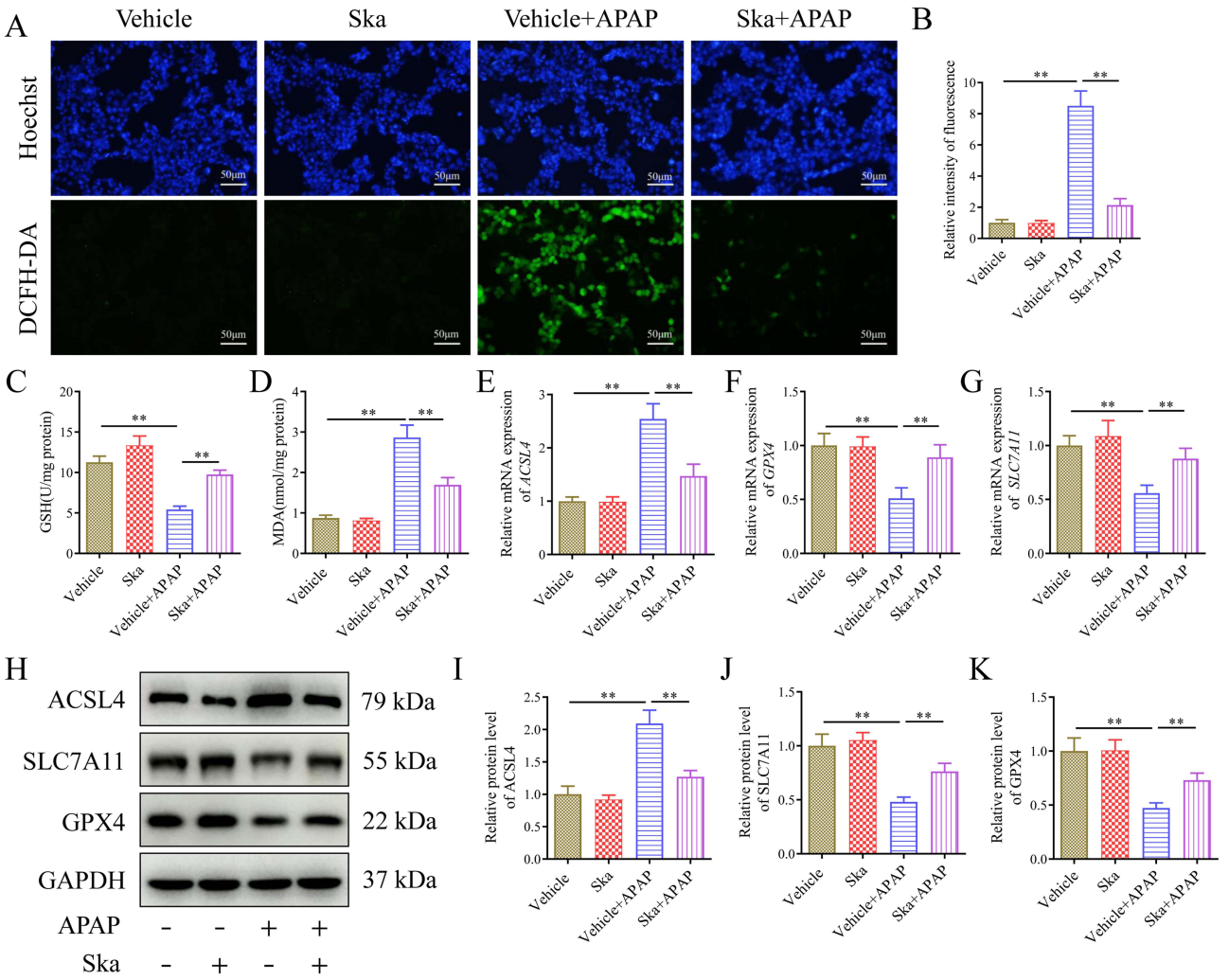

Figure 4 Sakuranetin ameliorates APAP-induced oxidative stress and ferroptosis in HepG2 cells (**A**) DCFH-DA staining and (**B**) fluorescence intensity analysis of HepG2 cells (n = 6/group). (**C**) GSH activity in HepG2 cells (n = 6/group). (**D**) MDA levels in HepG2 cells (n = 6/group). (**E–G**) mRNA expression of ACSL4, SLC7A11 and GPX4 in HepG2 cells (n = 6/group). (**H**) Protein detection and (**I–K**) statistical analysis of ACSL4, SLC7A11 and GPX4 in HepG2 cells (n = 6/group). **P < 0.01.

(Figure 5A). The molecular dynamics simulation results showed that the sakuranetin-Nrf2 complex system was stable and that the complex had good hydrogen bonding (Supplementary material and Supplementary figure S1). In addition, the protein expression of Nrf2 was significantly reduced in the liver tissue (Figure 5B, D–E) and HepG2 cells after APAP administration (Figure 5C, F-G). However, pretreatment with sakuranetin effectively reversed this decrease in Nrf2 expression. Immunofluorescence experiments were then performed to analyze the location of Nrf2 in HepG2 cells. The results showed that nuclear levels of Nrf2 were significantly increased in the Ska+APAP group compared to those in the vehicle+APAP group (Figure 5H). These results suggest that sakuranetin pretreatment had a positive effect on the expression of Nrf2, counteracting the downregulation induced by APAP administration.

The Protection of Sakuranetin Against APAP Hepatotoxicity Is Dependent on the Nrf2 Signaling Pathway

To further investigate whether sakuranetin exerts a protective effect against APAP hepatotoxicity via the Nrf2 pathway. We used ML385, an inhibitor of Nrf2, to treat mice by intraperitoneal injection. The results showed that after Nrf2 inhibition, the Nrf2 signaling pathway was significantly inhibited, and APAP-induced liver injury was significantly aggravated, as indicated by increased ALT and AST levels (Figure 6A and B), liver necrosis area (Figure 6C and D), serum inflammatory factors (Figure 6E–G), oxidative stress (Figure 6H and I) and ferroptosis (Figure 6J–O). Compared

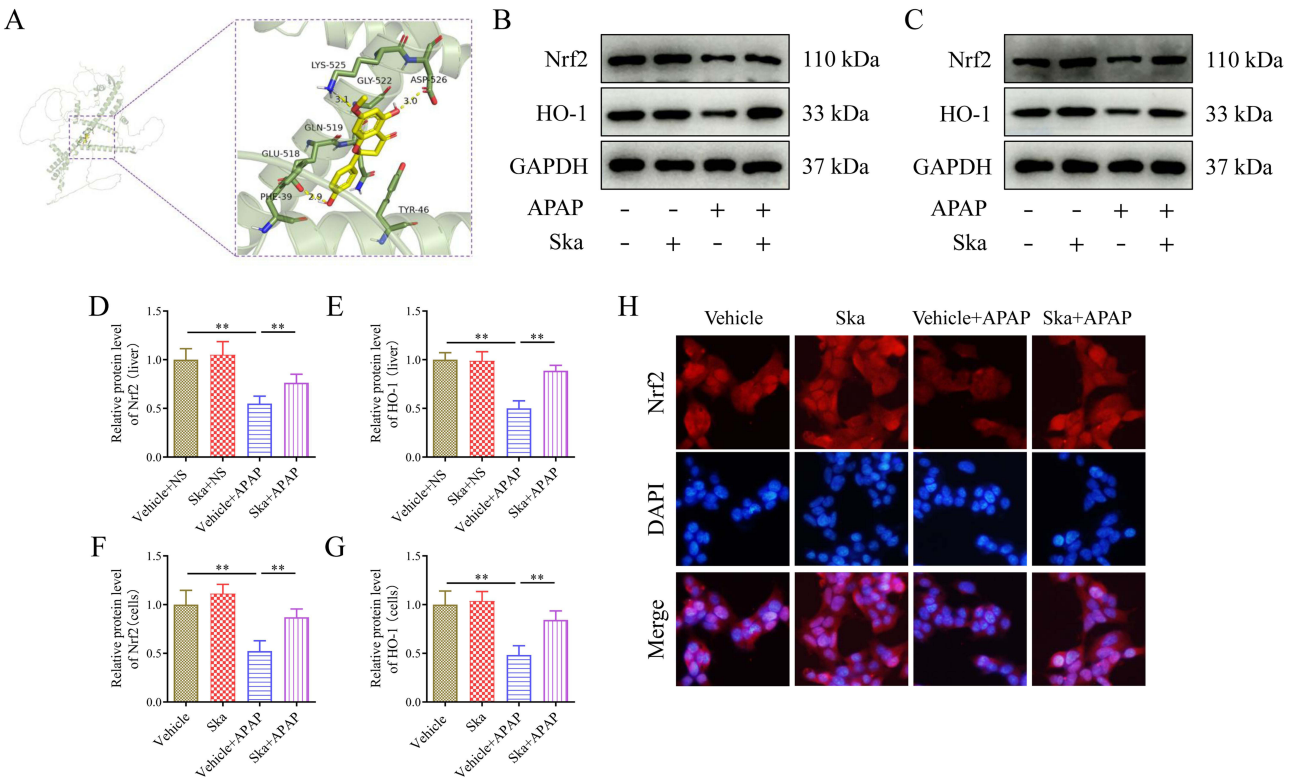

Figure 5 Sakuranetin activates Nrf2 signaling during APAP-induced liver injury **(A)** Molecular docking of Sakuranetin and Nrf2. (**B**) Protein detection of Nrf2 and HO-1 in mouse liver tissue (n = 6/group). (**C**) Protein detection of Nrf2 and HO-1 expression in HepG2 cells (n = 6/group). (**D-E**) Protein statistical analysis of Nrf2 and HO-1 in mouse liver tissue (n = 6/group). (**F-G**) Protein statistical analysis of Nrf2 and HO-1 expression in HepG2 cells (n = 6/group). (**H**) immunofluorescence staining of Nrf2 in HepG2 cells (n = 6/group). **P < 0.01.

with the APAP group, treatment with sakuranetin significantly reduced liver injury, oxidative stress and ferroptosis following APAP injury (Figure 6A–O). These results suggest that the hepatoprotective effects of sakuranetin are associated with the promotion of Nrf2 expression.

Discussion

In the present study, the potential role of sakuranetin in APAP-induced liver injury was investigated using mice and HepG2 cells. The results showed that sakuranetin could inhibit liver injury, oxidative stress and ferroptosis. Furthermore, the protective effect of sakuranetin is associated with the promotion of the Nrf2 signaling pathway, and the hepatoprotective effect of sakuranetin is significantly attenuated after inhibition of Nrf2. Therefore, our results suggest that sakuranetin modulates the Nrf2 signaling pathway and thereby ameliorates APAP-induced liver injury by inhibiting oxidative stress and ferroptosis.

Sakuranetin plays a protective role in various disease models through anti-inflammatory and antioxidant effects.[17,26] Considering the important role of inflammation and oxidative stress in APAP-induced liver injury, we hypothesized that sakuranetin might play a protective role in APAP-induced liver injury. In this study, we established an APAP damage model in mice and HepG2 cells and found that sakuranetin reduced serum transaminase, inflammatory factor levels, liver tissue necrosis area, increased HepG2 cell activity and decreased cell death. This is the first time demonstrated that sakuranetin has been shown to protect against AILI.

Oxidative stress is the key initial link of APAP-induced liver injury, which interacts with mitochondrial dysfunction to form a negative feedback to accelerate APAP-induced liver injury. GSH is an antioxidant enzyme in the body, MDA is the end product of lipid peroxidation, and its level indirectly reflects the degree of tissue peroxidation damage.[27] Previous study showed that sakuranetine ameliorated D-galactose-induced memory impairment in rats and LPS-induced acute lung injury in mice through an antioxidant effects.[20,21] Consistent with previous research, the results of this study showed that

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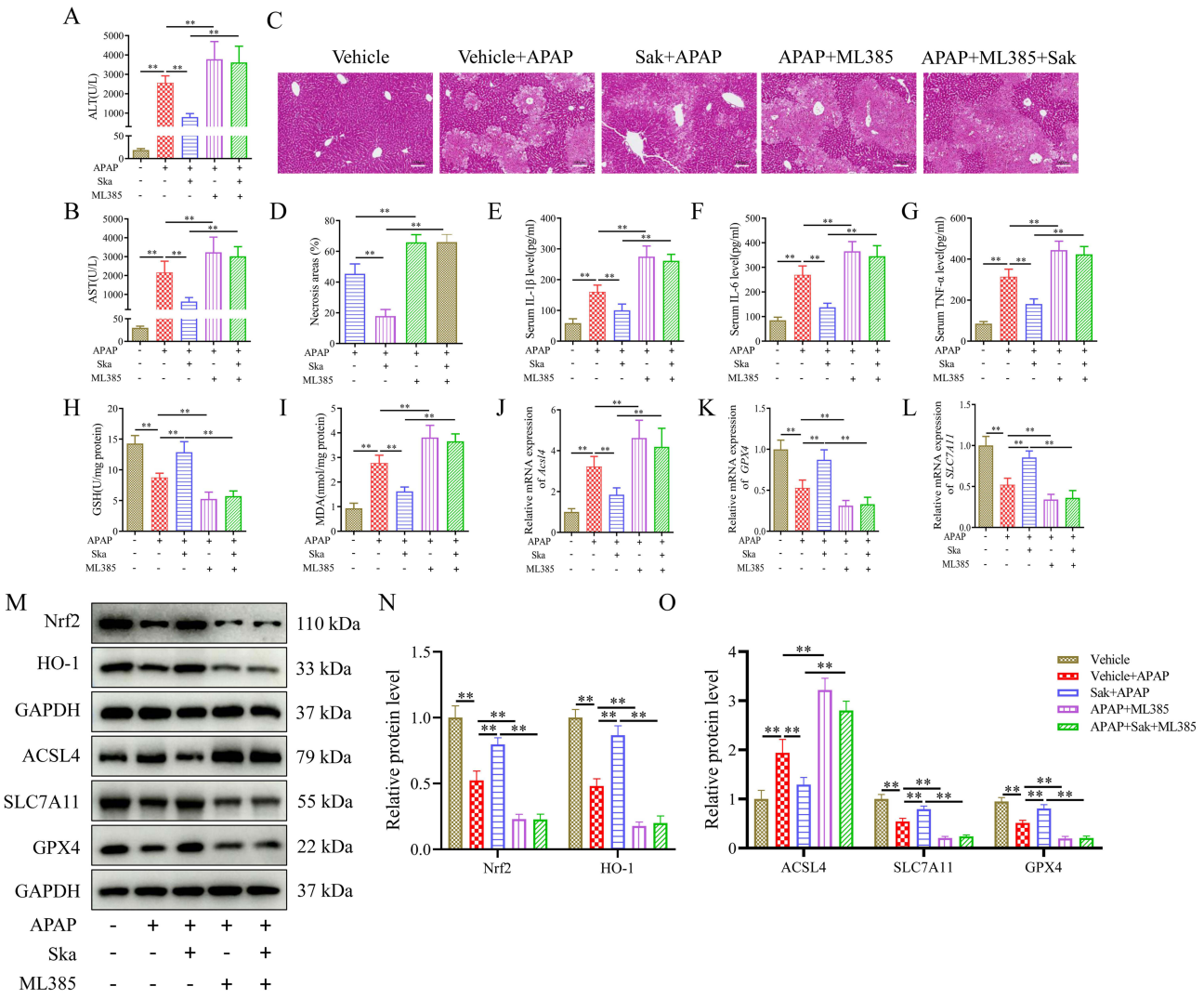

Figure 6 The protection of Sakuranetin against APAP hepatotoxicity is dependent on the Nrf2 signaling pathway (**A** and **B**) Serum ALT and AST levels (n = 6/group). (**C**) HE staining and (**D**) necrotic area statistics (n = 6/group). (**E–G**) Serum contents of IL-1β, IL-6, and TNF-α were detected by ELISA (n = 6/group). (**H**) GSH activity in liver tissue (n = 6/group). (**I**) MDA levels in liver tissue (n = 6/group). (**J–L**) mRNA expression of ACSL4, SLC7A11 and GPX4 in liver tissues (n = 6/group). (**M**) Protein detection and (**N** and **O**) statistical analysis of Nrf2, HO-1, ACSL4, SLC7A11 and GPX4 in mouse liver tissues (n = 6/group). **P < 0.01.

sakuranetin could significantly ameliorate the decrease of GSH antioxidant enzymes and reduce MDA and ROS production induced by APAP, suggesting that sakuranetin may play a protective role in APAP-induced liver injury by inhibiting oxidative stress.

Ferroptosis, a form of programmed cell death characterized by iron accumulation and depletion of plasma membrane polyunsaturated fatty acids, has been implicated in several pathological conditions including neurological disorders, myocardial infarction, ischemia/reperfusion injury, and inflammation.[28–30] In AILI, a key aspect of ferroptosis is the depletion of GSH, leading to decreased GPX4 levels.[31] Many studies have shown that inhibition of ferroptosis can ameliorate AILI.[32–34] The present study provided evidence for the involvement of ferroptosis in AILI. Our research showed that mice had increased levels of ferroptosis after AILI, as indicated by increased levels of ACSL4, and decreased levels of SLC7A11 and GPX4. Conversely, sakuranetin pretreatment showed a reduction in ferroptosis markers after AILI, with increased levels of SLC7A11 and GPX4, and decreased levels of ACSL4. These findings were further supported by in vitro experiments, confirming the protective role of sakuranetin in attenuating APAP-induced ferroptosis in the liver.

The Nrf2 signaling pathway plays an important role in resisting AILI. In AILI, Nrf2 dissociates from Keap1 and translocates to the nucleus to where it binds to antioxidant response elements, activating the gene expression of a series of antioxidant proteins and detoxification enzymes, enhancing the ability of cells to neutralize NAPQI, and reducing liver cell injury and death.[13] Therefore, many studies are investigating the activation of the Nrf2 signaling pathway by drugs or other means to prevent and treat AILI.[35–37] In addition, studies have shown that activation of the Nrf2 signaling pathway ameliorates liver injury by regulating ferroptosis pathway.[38,39] Li et al showed that APAP treatment reduced NRF2 expression in mouse liver tissue and promoted ferroptosis, while kaempferol treatment promoted Nrf2 pathway activation and inhibited ferroptosis to alleviate AILI.[40] Similarity, in the present study, we observed that Nrf2 signaling was inhibited after APAP treatment in mouse liver and HepG2 cells. However, sakuranetin pretreatment significantly increased the activation of Nrf2 signaling pathway and promoted the nuclear translocation of Nrf2 after APAP injury in mouse liver and HepG2 cells. To further investigate whether the hepatoprotective effect of sakuranetin was mediated by the promotion of Nrf2 signaling pathway, we used the Nrf2 pathway inhibitor ML385 in mice in vivo. Consistent with previous reports, the liver injury was more severe after inhibition of the Nrf2 signaling pathway,[41,42] while the protective effect of sakuranetin on liver injury in mice with APAP, oxidative stress and ferroptosis was significantly reduced. These results suggest that sakuranetin may mediate the ferroptosis pathway through the Nrf2 signaling pathway to ameliorate APAP-induced liver injury.

Conclusion

In conclusion, our study demonstrated that sakuranetin pretreatment can significantly reduce oxidative stress and ferroptosis in APAP-induced liver injury, and the protective mechanism may be related to the promotion of Nrf2 signaling pathway and inhibition of ferroptosis. However, whether sakuranetin ameliorates APAP-induced liver injury in mice by regulating other signaling pathways needs to be further investigated.

Data Sharing Statement

Data used to support the findings of this study are available from the corresponding author upon request.

Funding

The present study was supported by the Natural Science Foundation of Hubei Province (2022CFB346).

Disclosure

The authors report no conflicts of interest in this work.

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