Research Article

The Protective Effects of Imperatorin on Acetaminophen Overdose-Induced Acute Liver Injury

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Acetaminophen (APAP) toxicity leads to severe acute liver injury (ALI) by inducing excessive oxidative stress, inflammatory response, and hepatocyte apoptosis. Imperatorin (IMP) is a furanocoumarin from Angelica dahurica, which has antioxidant and anti-inflammatory effects. However, its potential to ameliorate ALI is unknown. In this study, APAP-treated genetic knockout of Farnesoid X receptor (FXR) and Sirtuin 1 (SIRT1) mice were used for research. The results revealed that IMP could improve the severity of liver injury and inhibit the increase of proinflammatory cytokines, oxidative damage, and apoptosis induced by overdose APAP in an FXR-dependent manner. We also found that IMP enhanced the activation and translocation of FXR by increasing the expression of SIRT1 and the phosphorylation of AMPK. Besides, single administration of IMP at 4 h after APAP injection can also improve necrotic areas and serum transaminase, indicating that IMP have both preventive and therapeutic effects. Taken together, it is the first time to demonstrate that IMP exerts protective effects against APAP overdose-induced hepatotoxicity by stimulating the SIRT1-FXR pathway. These findings suggest that IMP is a potential therapeutic candidate for ALI, offering promise for the treatment of hepatotoxicity associated with APAP overdose.

1. Introduction

Acetaminophen (APAP), one of the most widely used analgesic and antipyretic drug in the United States, European countries, and Asia, exerts a safe and reliable effect at proper therapeutic doses. However, when in excess, it can cause severe acute liver injury (ALI) and even acute liver failure (ALF). Currently, cases of APAP hepatotoxicity have been reported due to unintentional overdoses of more than 4 g/day of package recommendations while self-medicating for pain or fever, especially in China, where APAP is one of the three leading causes of acute liver failure [1, 2]. It is generally accepted that primary hepatocellular toxicity caused by APAP is mainly due to the production of the highly cytotoxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI), under the action of CYP-dependent cytochrome P450 [3]. When

APAP is present in large amounts, its metabolite NAPQI depletes GSH in the liver, and then, the remaining NAPQI will bind to mitochondrial proteins, which causes mitochondrial dysfunction, increases the production of mitochondrial reactive oxygen species (ROS), triggers inflammatory response, and ultimately contributes to liver injury [4–6]. N-acetylcysteine (NAC) is currently the only drug used for APAP-induced liver injury and is only effective in the early stages of ALI [7]. There still remains a lack of effective therapeutic drugs for clinical treatment. Therefore, it is imperative to discover novel and effective therapies against APAP overdose-induced ALI. Reducing the oxidative stress and inflammatory response induced by APAP may be a feasible strategy.

Farnesoid X receptor (FXR, NR1H4) is a member of the nuclear receptor superfamily of bile acid-activated transcription factors, which is highly expressed in the liver [8]. In addition, CYP27A1, CYP7A1, and CYP8B1 are key enzymes in the bile acid synthesis pathway. When FXR binds to bile acid, it can directly induce small heterodimer partner (SHP) in the liver to combine with liver receptor homolog-1 (LRH-1) and inhibit CYP7A1 transcription. Induced SHP also can reduce the expression of CYP27A1 and CYP8B1 through Hepatocyte Nuclear Factor 4 alpha (HNF4A) [9]. Besides, FXR can regulate the bile acid transporters distributed in the liver, such as FXR-target genes BSEP, MRP2, MDR3, and NTCP [10]. Previous studies revealed that FXR plays a regulatory role in mitochondrial dysfunction, oxidative stress, and inflammation, thereby protecting against liver damage [11–14]. Therefore, FXR has an anti-inflammatory effect and may be the target protein for mediating drug-induced liver injury as it contributes to inhibit the development of drug-induced liver injury. As a 6α-ethyl derivative of chenodeoxycholic acid (CDCA) of FXR agonist, increasing evidences demonstrated that obeticholic acid (OCA, also known as INT-747) inhibits inflammation via activating FXR activity [15].

Imperatorin (IMP) is a naturally bioactive furanocoumarin from the dry roots of Angelica dahurica (Figure 1(a)), which can be used as an edible flavoring agent in dietary products. It has been proven that IMP possesses various pharmacological effects such as anticancer, anti-inflammatory, and antidiabetic activities [16, 17]. Our previous study found that IMP can attenuate DSS-induced colitis through activating PXR/NF-κB and inhibition of NF-κB signaling in mice [18]. However, its effect on ALI remains unknown. In the present study, we investigated the protective effects of IMP on ALI induced by APAP in vitro and in vivo.

2. Materials and Methods

2.1. Reagents.
IMP (98.8% purity) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Guangzhou, China). Acetaminophen (APAP) and obeticholic acid (OCA) were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Animal Experiment.
Six-week-old male C57BL/6 mice (weight, 20-25 g) were purchased from the Animal Labora-

tory Centre of Guangdong Province (Certificate SCXK2013-0002; Guangzhou, China); FXR$^{-/-}$ knockout mice (007214-B6.129X1(FVB)-Nr1h4tm1Gonz/J) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Liver-specific SIRT1-mutant (SIRT1 knockout) mice were generated by crossing SIRT1$^{fl/fl}$ mice with Alb-cre mice to delete exon 4 of SIRT1 [19]. Mice were housed and maintained under a constant temperature of $22 \pm 2°C$ and a relative humidity of 50-70%. All animal experimental studies were approved by the Animal Ethics Committee of Guangzhou University of Chinese Medicine. Briefly, mice were given a daily gavage of IMP or OCA suspended in 0.5% CMC-Na (0.5 g dissolved in pure water to 100 ml in an edge-bordered mixing, uniform suspension) for 5 days. On the 6th day, all groups, except the control, received APAP (dissolved in 60°C normal saline, 300 mg/kg) by intraperitoneal injection after overnight fasting. All mice were sacrificed 10 h following APAP injection. Fresh liver tissues were collected for analysis. Serum ALT and AST concentrations were detected using Hitachi 7080 automatic biochemical analyzer 132 (Hitachi 7080, Japan). GSH/GSSH was measured using a Total Glutathione/-Oxidized Glutathione Assay Kit (Jiancheng Bioengineering Institute, Nanjing, China). TNF-α, IL-6, and IL-1β were measured using an ELISA (ABclonal, China).

The APAP hepatotoxicity treatment model has been chosen in accordance with previously published studies of glycycoumarin [20]. Comparative experiment for therapeutic effects of IMP or OCA at the indicated dosage was carried out 4 h after APAP injection. Finally, until 10 h post APAP challenge, the mice were sacrificed for subsequent experiments.

2.3. Mouse Survival Experiment.
In the acute inflammation experiments, mice were injected intraperitoneally with LPS (25 mg/kg), and then, different doses of IMP were treated. In drug-induced liver injury experiments, mice were injected intraperitoneally with APAP (300 mg/kg) before the different doses of IMP were administrated. The survival rate experiment was carried out, and the death period was recorded within 72 h.

2.4. Isolation and Culture of Primary Mouse Hepatocytes (PMHs).
Primary hepatocytes were isolated and cultured as previously reported [21]. Briefly, male mice were anesthetized and perfused with type II collagenase (0.5 mg/ml, Sigma Aldrich) via the inferior vena cava for hepatocyte isolation. PMHs were cultured in RPMI 1640 containing 10% FBS, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. After pretreatment with IMP (5, 10 μM) for 24 h, PMHs were incubated with APAP (10 mM) for 24 h.

2.5. Histopathological Analysis.
Liver tissues were fixed in 4% paraformaldehyde solution and then embedded in paraffin. Subsequently, tissues were cut into 4 μm sections and stained with hematoxylin-eosin (H&E) according to reagent specification. Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) was used to quantitate the percentage of the necrotic area. The percentage of liver necrosis was
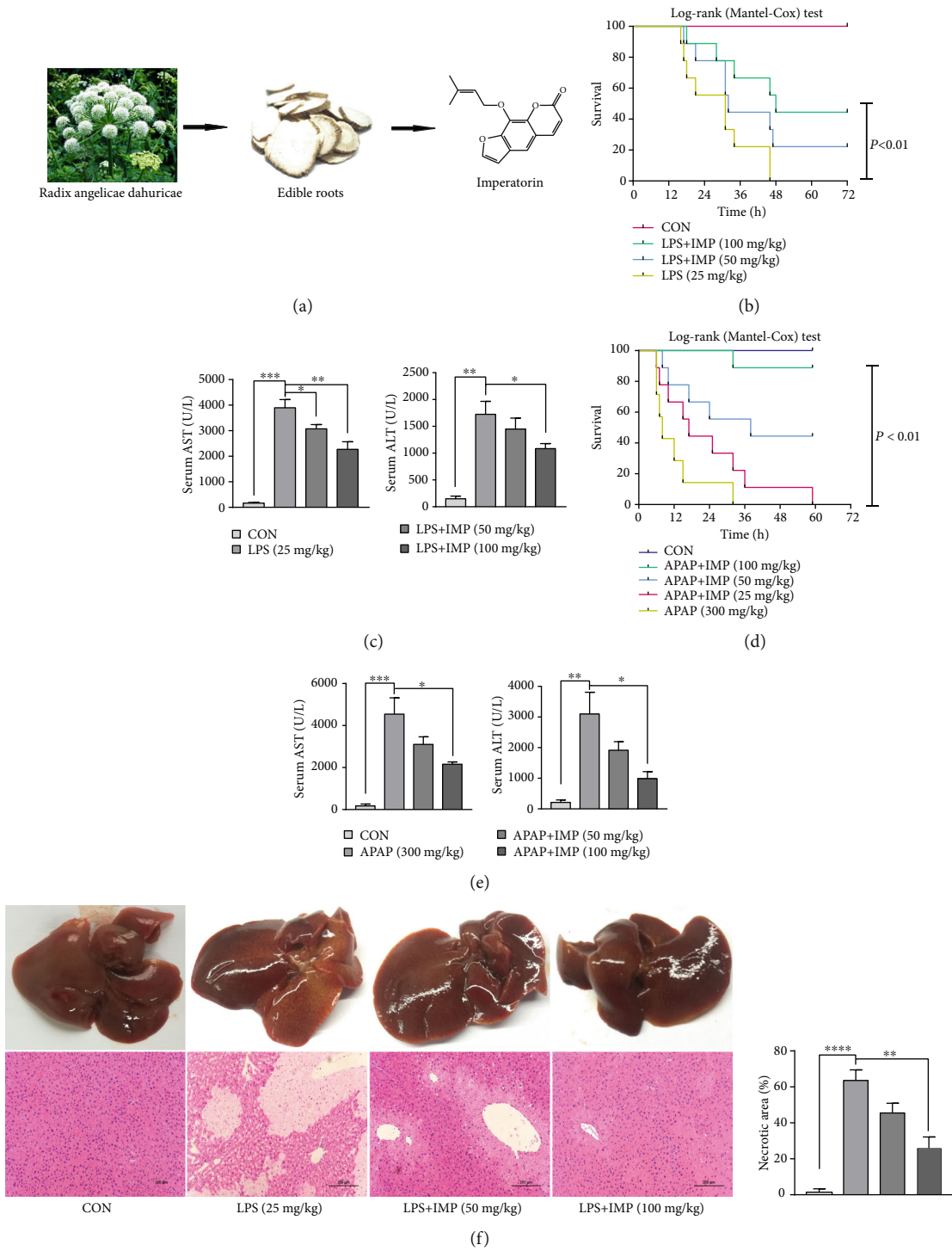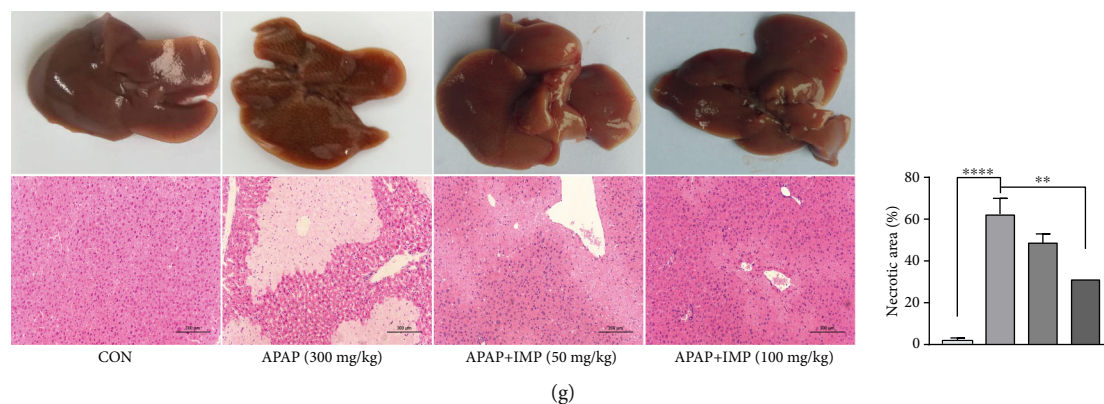
(a)

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Figure 1: Continued.

(g)

FIGURE 1: Pretreatment with IMP protects against APAP overdose-induced acute liver injury and necrotic death. IMP (50 or 100 mg/kg) was intragastrically administrated to mice for 5 days, which then received LPS (25 mg/kg) or APAP (300 mg/kg) by intraperitoneal injection after fasting overnight. Mice were sacrificed after 10 h; serum and liver samples were collected for further analysis. (a) The source of the IMP as a bioactive natural product. (b) The survival rate of mice (with/without IMP pretreatment) after intraperitoneal injection of LPS for 72 h. (c, e) Serum was harvested for measurement of serum ALT and AST levels. (d) The survival rate of mice (with/without IMP pretreatment) after intraperitoneal injection of APAP for 72 h. (f) Changes in the appearance of solid liver tissue and representative images of H&E staining of liver sections (with/without IMP pretreatment) with LPS injection (magnification: 200x). (g) Changes in the appearance of solid liver tissue and representative images of H&E staining in mouse liver sections (with/without IMP pretreatment) with APAP injection (magnification: 200x). $^*p < 0.05$; $^{**}p < 0.01$ versus vehicle-treated control group. IMP: imperatorin; ALT: alanine aminotransferase; AST: aspartate transaminase.

determined by measuring the total dimension of the field and comparing it with the dimension of the necrotic area.

2.6. Western Blot Analysis. Proteins extracted from fresh liver tissues and PMHs were lysed in radioimmune precipitation assay (RIPA) buffer containing protease and phosphatase inhibitor (Roche, Basel, Switzerland). Protein lysates (80-100 μg) were loaded onto 10% SDS-PAGE gels and electrophoretically transferred to PVDF membranes (Solarbio, Beijing, China). Specific antibodies were used for Western blotting, including SIRT1 (CSB-PA004095, Cusabio), FXR (sc-25309, Santa Cruz), anti-phospho-AMPK (p-AMPK) (ab133448), AMPK (ab32047), β-actin (ab179467), horseradish peroxidase-conjugated goat anti-mouse, and rabbit IgG antibodies purchased from Abcam (Cambridge, MA). The fluorescence of signal intensity of Western blot protein bands was quantitatively analyzed using ImageJ.

2.7. RNA Isolation and Quantitative Real-Time PCR. Total RNA was isolated from fresh liver tissues and PMHs using a TRIzol Kit (Invitrogen, USA). Reverse transcription was performed on each sample according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed on a PCR Detection System (Bio-Rad CFX96, USA) following the manufacturer's instructions. The experimental results were analyzed using the $2^{-\Delta Ct}$ method. β-Actin was employed as the internal standard. The primers are listed in Table 1.

2.8. Immunofluorescence Staining. Cells were harvested and fixed with 4% paraformaldehyde for 20 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature after washing with PBS. After blocking with 5% BSA for 30 min, cells were

incubated with rabbit anti-FXR antibody (1 : 100) at 4°C overnight, together with incubated Alexa Fluor 488-conjugated anti-rabbit IgG antibody (A-21206, Invitrogen) for 1 h at room temperature. DAPI (100 ng/ml) in PBS was added to stain the nuclei. Fluorescence images were obtained using a confocal laser scanning microscope (Leica, Wetzlar, Germany) or a fluorescence microscope (Olympus, Tokyo, Japan).

2.9. Assessment of Mitochondrial Membrane Potential. JC-1 assay kits were used to detect the mitochondrial membrane potential of PMHs. Briefly, PMHs were cultured on sterile glass cover slips in 6-well plates and incubated with IMP prior to APAP for 24 h. The cells were fixed in 4% paraformaldehyde for 20 min at room temperature, and mitochondrial membrane potential was measured according to the manufacturer's instructions (C2006, Beyotime, Shanghai, China). Imaging was performed with a fluorescence microscope (Leica Microsystems Ltd., Wetzlar, Germany).

2.10. Detection of ROS. In brief, PMHs were incubated with IMP prior to incubation with APAP for 24 h. DCFH-DA with a final concentration of 10 μm was then added for a 0.5 h incubation at 37°C, protected from light. Finally, the cells were washed 3 times with PBS, and then, the emission of fluorescence was detected by fluorescence microscopy or measured by flow cytometry (FCM).

2.11. TUNEL Assay. Liver tissues were fixed in 4% paraformaldehyde solution, cut into 4 μm sections, and embedded in paraffin. TUNEL levels were detected with a TUNEL assay kit (C1089, Beyotime) according to the manufacturer's instructions. Imaging was performed using a fluorescence microscope (Leica Microsystems Ltd., Wetzlar, Germany).

TABLE 1: Primer information for gene amplification.

Primer	Sequences
FXR	F1: 5′-CAGAAATGGCAACCAGTCATGTA-3′
	R1: 5′-AAATCTCCGCCGAACGAA-3′
SHP	F1: 5′-CGACCTGTCACTCTCGGTATC
	R1: 5′-AGGGCTTGGATGATTCTAGTCA
SOD2	F1: 5′-CAGACCTGCCTTACGACTATGG-3′
	R1: 5′-CTCGGTGGCGTTGAGATTGTT-3′
IL-1β	F1: 5′-GAAATGCCACCTTTTGACAGTG-3′
	R1: 5′-TGGATGCTCTCATCAGGACAG-3′
IL-6	F1: 5′-CTGCAAGAGACTTCCATCCAG-3′
	R1: 5′-AGTGGTATAGACAGGTCTGTTGG-3′
Nrf2	F1: 5′-CTTTAGTCAGCGACAGAAGGAC
	R1: 5′-AGGCATCTTGTTTGGGAATGTG
SIRT1	F1: 5′-TGATTGGCACCGATCCTCG
	R1: 5′-CCACAGCGTCATATCATCCAG
Bcl2	F1: 5′-GCTACCGTCGTGACTTCGC
	R1: 5′-CCCCACCGAACTCAAAGAAGG
Bax	F1: 5′-AGACAGGGGCCTTTTTGCTAC
	R1: 5′-AATTCGCCGGAGACACTCG
COX2	F1: 5′-GACCGCAATGAACTTCGGGA
	R1: 5′-TCCATTAGGTCTCTAAAGCCGAG
β-Actin	F1: 5′-ATGACCCAAGCCGAGAAGG
	R1: 5′-CGGCCAAGTCTTAGAGTTGTTG

2.12. Molecular Docking. The structure of IMP was obtained from the ChemSpider database of the Royal Society of Chemistry (ChemSpider ID 9797) and drawn using Chem-Draw Std 14. The three-dimensional crystal structure of hFXR was obtained from the RCSB Protein Data Bank (PDB code: 3HC5). The hFXR ligand, chenodeoxycholic acid, served as the template molecule to evaluate the ligand affinity for hFXR, and the residues around the template ligand were selected as the binding pocket for docking IMP into hFXR-LBD. The cocrystalized structure and docking complex were prepared using SYBYL2.1.1 to correct structural errors, such as broken bonds and missing loops. Docking results were analyzed, and figures were created in Discovery Studio Visualizer.

2.13. Dual-Luciferase Reporter Assay. hFXR expression plasmid was constructed by cloning genes encoding FXR into pCDNA3.1(+)-3Flag-C vector. hBSEP promoter reporter was constructed by cloning a genomic DNA fragment upstream of the transcription start site (-1448 to +83) containing an FXR response element into pGL3-basic vector. HEK293T cells seeded in a 96-well culture plate were transiently transfected with pCDNA3.1-vector, pCDNA3.1-hFXR (100 ng per well), pGL3-basic-hBSEP (200 ng per well), and pGL3-CMV Renilla luciferase plasmid (10 ng per well) as an internal control with a Lipofectamine 2000™ transfection

reagent (Invitrogen, Carlsbad, CA, USA). Transfected cells were changed to 0.1 ml of fresh supplemented culture medium containing DMSO (0.1% v/v; vehicle control), with DMSO, OCA (10 μM), IMP (10 μM), or IMP (20 μM) as indicated, respectively, for another 24 h. Then, firefly and Renilla luciferase activities were determined on an EnSpire Multimode Plate Reader (PerkinElmer, CA, USA) with the dual-luciferase report assay system (Promega, Madison, WI, USA). Results were normalized to the Renilla luciferase controls.

2.14. Statistical Analysis. All results in the figures and text are expressed as the mean ± SEM from at least three independent experiments. The data were evaluated using GraphPad Prism Version 7.0 (GraphPad Software, La Jolla, CA, USA). The statistical significance of two group comparisons was performed by Student's t-test and multiple comparisons analyzed using one-way ANOVA followed by LSD or Dunnett's test. A value of $p < 0.05$ was considered statistically significant for all tests.
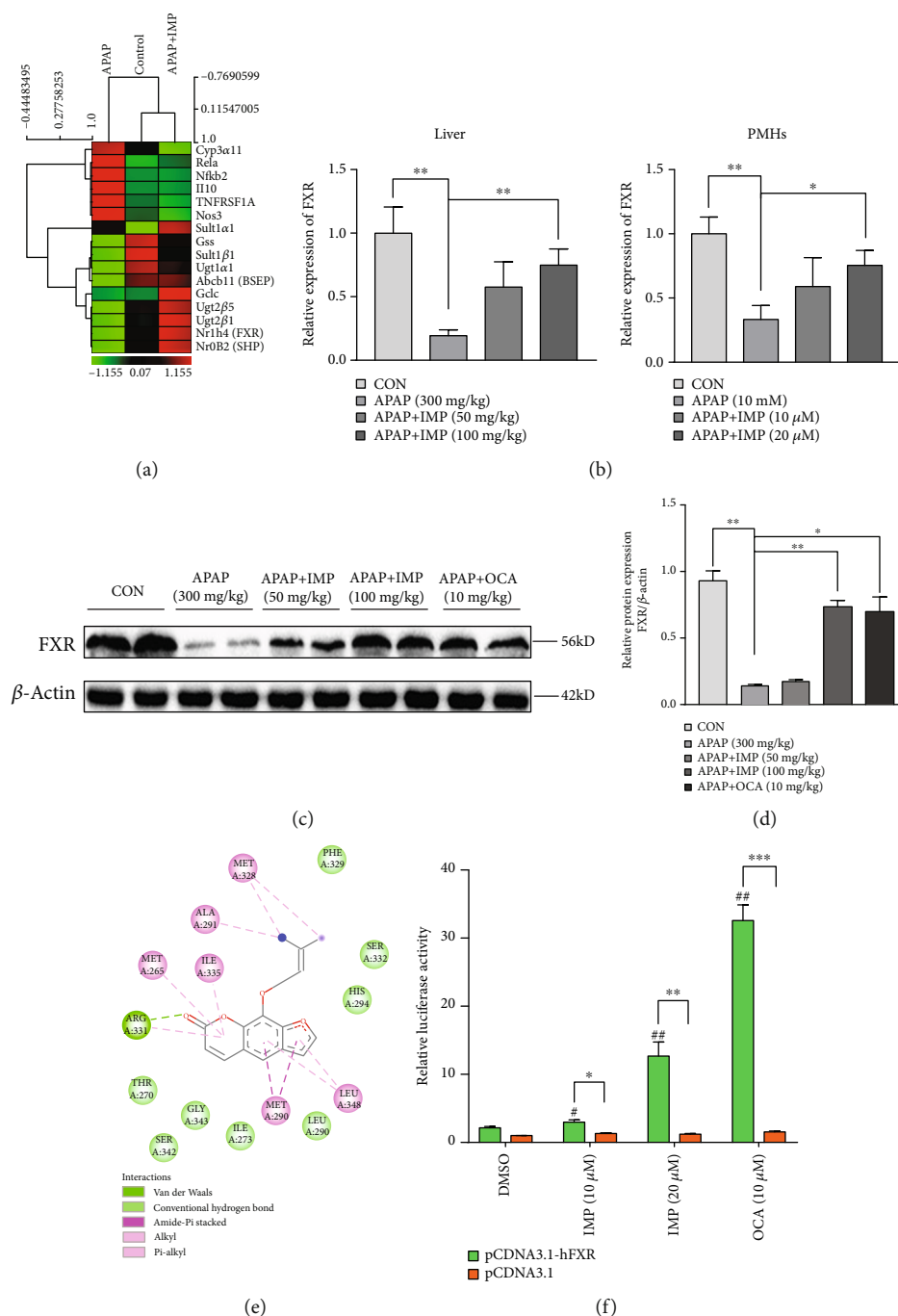
3. Results

3.1. IMP Attenuates LPS or APAP Overdose-Induced ALI in Mice. The liver protective effects of IMP were initially verified on the liver injury mouse model with inflammatory response and acute oxidative stress, which were established by intraperitoneal injection of LPS or APAP, respectively.

IMP treatment significantly decreased LPS or APAP overdose-induced mortality (Figures 1(b) and 1(d)). As expected, exposure of APAP or LPS resulted in severe liver damage, as indicated by the increase in serum ALT and AST. However, IMP treatment effectively decreased these parameters, especially when a high dose was administered (Figures 1(c) and 1(e)). With IMP treatment, APAP overdose-induced centrilobular hepatic necrosis and the necrotic area were also reduced as shown by H&E staining (Figures 1(f) and 1(g)). These findings indicate the potential protective effect of IMP on the prevention of LPS or APAP overdose-induced ALI.

3.2. FXR Serves as a Candidate Target for IMP against ALI. To explore the underlying mechanism of IMP against ALI, transcriptome analysis was performed with liver tissue. The expression of FXR and its target genes, SHP (NROB2) and BSEP (ABCB11), was significantly inhibited in APAP-treated mice. However, treatment with IMP rescued their expression (Figure 2(a)). Genes related to antioxidation and liver metabolic enzymes showed the same trend (Figure 2(a)). Moreover, treatment with IMP significantly reversed the APAP-induced decline of FXR expression in vivo and in vitro (Figure 2(b)). This finding was similar to that for OCA, an FXR-specific activator (Figures 2(c) and 2(d)).

Docking analysis between IMP and FXR was carried out to investigate the binding mode of IMP within the binding pocket of hFXR and further understand the structure activity relationship. The three-dimensional crystallographic structure of FXR (Supplement Figure S1), deposited in the

(a)

(b)

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(d)

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(f)

FIGURE 2: Results of transcriptome in vivo analysis (tiVA) and in vitro experimental verification of activation of FXR signaling with IMP. (a) Heatmap representation of the transcriptome analysis of 19 differentially expressed genes (IMP pretreatment mediated a reversal of inhibition of antioxidant gene expression and FXR-targeted genes induced by APAP overdose). (b) Pretreatment of IMP improves the impaired mRNA expression of FXR and FXR-dependent downstream target genes in liver tissue and PMHs. The mRNA levels of FXR were determined by qRT-PCR. (c) Pretreatment of IMP reverses the downregulation of the protein levels of FXR in liver tissue induced by APAP. (d) Quantitative analysis of scanning densitometry of protein bands for FXR in liver tissue. (e) The 2D structure with the predicted binding details of IMP to hFXR ligand-binding domain (LBD) (rendered in colored sticks). (f) A standard dual-luciferase assay was carried out on HEK293T cell lysates, transiently transfected with pCDNA3.1-vector, pCDNA3.1-hFXR, and pGL3-basic-hBSEP (200 ng per well), and pGL3-CMV *Renilla* luciferase control reporter vectors were used as the transfection control. Relative luciferase activity was determined by the ratio of firefly luciferase/Renilla luciferase activity ($n = 3$). $^*p < 0.05$; $^*p < 0.01$; $^{***}p < 0.001$, compared to the same treatment group transfected with empty vector or with vehicle-treated control group, and $^{##}p < 0.01$ versus the DMSO control group transfected with the receptor expression plasmid. FXR: Farnesoid X receptor; mRNA: messenger RNA; PMHs: primary mouse hepatocytes; qRT-PCR: quantitative real-time PCR; BSEP: bile salt export pump.

Brookhaven Protein Data Bank (3HC5), had a good binding affinity with IMP mainly through stable hydrogen-bond interactions with residues of Arg331.

As depicted in Figure 2(e), Van der Waals interactions with residues Thr270, Phe329, Leu287, Gly343, Ser332, and Tyr260; His294 and Ser342; the amide-π stacking with MET 290; and the hydrophobic interactions with MET 328, ALA 291, ILE 335, MET 265, and LEU 348 may also contribute to the high binding interactions of hFXR with surrounding residues.

For further elucidation, hFXR transactivation experiment was performed to demonstrate that IMP could significantly elevate hBSEP luciferase activity in a dose-dependent manner, with similar effects to OCA (a specific FXR agonist as positive control) (Figure 2(f)).

3.3. Treatment with IMP Protects Mice against APAP Overdose-Induced Hepatoxicity via a FXR-Dependent Manner.
Based on our previous studies, FXR$^{-/-}$ mice were used to assess the crucial role of FXR in IMP protection against ALI in vivo. Aligning with previous studies, FXR expression was proved to be absent in FXR$^{-/-}$ mouse livers (Supplement Figure S2).

The absence of FXR largely abrogated the hepatoprotective effects of IMP against APAP overdose-induced ALI, as shown by the similar serum levels of ALT and AST, and extensive histological liver necrosis among the APAP group, IMP group, and OCA group (Figures 3(a) and 3(b)).

3.4. Treatment with IMP Effectively Alleviates APAP Overdose-Induced Inflammation by Upregulation of FXR.
Treatment with IMP could significantly inhibit the elevated expression of proinflammatory cytokines, including TNF-α, IL-1β, IL-6, and COX2 levels in serum and liver tissue, and cause a sharp decline in their mRNA levels both in liver tissues and PMHs (Figures 4(a)–4(c)). The IMP-induced inhibitory effects of APAP overdose-induced inflammation in the liver were diminished in FXR$^{-/-}$ mice. TNF-α, IL-1β, IL-6, and COX2 showed no significant changes in FXR$^{-/-}$ mice after APAP overdose, even with a high dose of IMP (Figures 4(d)–4(f)).

3.5. Treatment with IMP Effectively Attenuates APAP Overdose-Induced Oxidative Damage by Enhancing FXR and Antioxidant Gene Expression.
The content of serum GSH/GSSG and the expression levels of Nrf2 and SOD2 have remarkable restoration after IMP treatment with hepatotoxic mice (Figures 5(a) and 5(c)). However, the expression of these antioxidants was not altered significantly in FXR$^{-/-}$ mice after IMP treatment nor did the Nrf2 protein (Figures 5(c) and 5(e)). Aligning with the results in the liver tissues, IMP treatment rescued the expression of Nrf2 and SOD2 in PMHs after APAP exposure, and this effect was abolished in PMHs from FXR$^{-/-}$ mice (Figures 5(b) and 5(f)). Meanwhile, FCM data and fluorescent figure showed that treatment with IMP administration could significantly reduce ROS levels in WT PMHs after APAP exposure (Figures 5(d) and 5(h)), suggesting that IMP could alleviate APAP overdose-induced ALI through the ame-

lioration of antioxidant defense systems. However, the IMP-induced inhibitory effects of ROS after APAP exposure were diminished due to the depletion of FXR gene (Figures 5(g) and 5(h)).

3.6. Antiapoptosis and the Prevention of Mitochondrial Dysfunction Are Closely Linked with the Regulation of FXR in IMP-Treated ALI Mice.
As shown in Figures 6(a) and 6(b), APAP upregulated Bax and decreased Bcl-2 expression at the protein and mRNA levels in liver tissues and PMHs; IMP increased the Bcl-2/Bax ratio in a concentration-dependent manner. However, IMP failed to remodel the expression of Bcl-2 and Bax in FXR$^{-/-}$ mice and its PMHs, even at a high dose (Figures 6(c) and 6(d)). Besides, IMP treatment significantly protected mice from APAP overdose-induced apoptosis, as shown by the reduction in TUNEL levels in the liver (Figure 6(e)). Conversely, there was no change in TUNEL levels in FXR$^{-/-}$ mice after APAP overdose with or without IMP treatment, even at a high concentration (Figure 6(e)).

JC-1 translocated to the mitochondria through the mitochondrial membrane and aggregated into a polymer with red fluorescence in the control group. Contrariwise, exposure to APAP significantly damages mitochondria, as revealed by the increase in green fluorescent monomer (Figure 6(f)). Notably, IMP treatment effectively protected against APAP-induced mitochondrial dysfunction, thereby displaying a decreased green fluorescent monomer number and increased red fluorescence polymer number (Figure 6(f)). Unsurprisingly, no marked changes were observed in the PMHs derived from FXR$^{-/-}$ mice after APAP exposure with or without IMP treatment, suggesting that the protective effect of IMP or OCA on mitochondrial membrane potential was dependent on FXR activation (Figure 6(g)).

3.7. IMP Treatment Ameliorates APAP Overdose-Induced ALI through Modulation of SIRT1-FXR Signaling.
IMP administration significantly rescued the phosphorylation of AMPK and expression of SIRT1 in APAP-exposed mice and PMHs (Figures 7(a) and 7(b)). Additionally, the mRNA levels of SIRT1 were dramatically improved both in the liver tissues and in PMHs (Figure 7(c)). Conversely, IMP failed to activate the FXR signaling pathway both at the protein and mRNA levels after APAP exposure in the PMHs isolated from SIRT1$^{-/-}$ mice (Figures 8(a) and 8(b)).

As previously reported, SIRT1 controlled the regenerative response of the liver by suppression of FXR acetylation [22, 23], in accordance with our finding that the effect of nuclear translocation of FXR activation is inhibited with SIRT1$^{-/-}$ mice PMHs (Figure 8(c)). Therefore, it is possible that IMP exerted a hepatoprotective effect through direct activation of FXR and indirect SIRT1-mediated FXR deacetylation to induce transactivation (Figure 9, diagrammatic sketch).

3.8. Treatment with IMP at 4 h after Overdose APAP Can Also Reduce Liver Damage Significantly.
To assess whether IMP could be used as a potential therapeutic reagent to treat APAP-induced liver damage, WT mice were administrated
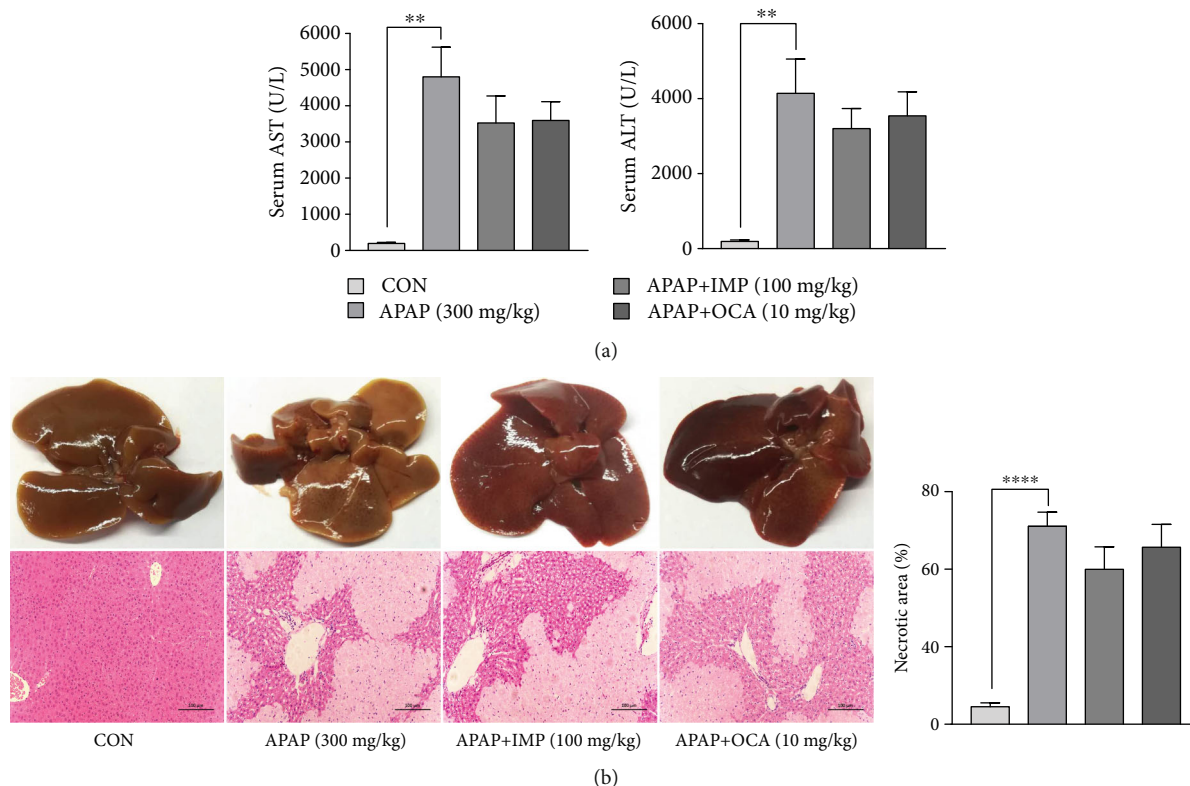
(a)

(b)

FIGURE 3: FXR knockout abolishes the protective effects of IMP against APAP overdose-induced ALI and necrotic death. (a) Serum was harvested for measurement of ALT and AST levels. (b) Changes in the appearance of solid liver tissue and representative images of H&E staining in FXR$^{-/-}$ mouse (with/without IMP pretreatment) liver sections at 10 h after APAP injection (magnification: 200x). Results are compared to the vehicle-treated control group. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ versus vehicle-treated control group. ALT: alanine aminotransferase; AST: aspartate transaminase.

with APAP and orally received a single dose of IMP 4 h later. The major reduction in liver damage after treatment with IMP was further demonstrated by the decrease of serum transaminase levels, as well as the improvement of genes involved in apoptosis and antioxidant (Supplement Figure S3A-B). Analysis of APAP-induced liver damage by hepatocyte necrosis and liver histology showed minimal necrotic areas in livers from mice treated with IMP relative to the control group (Supplement Figure S3C). Together, these results suggest that treatment with IMP at 4 h after overdose APAP can also reduce liver damage significantly.

4. Discussion

We report for the first time that IMP alleviates ALI via FXR-mediated anti-inflammatory, antioxidant, and antiapoptotic effects. Previous studies show that APAP toxicity is mediated by multiple elements such as APAP metabolism, endoplasmic reticulum stress, oxidative stress, and aseptic inflammation. Among them, APAP-provoked mitochondrial dysfunction and excessive oxidative stress play a vital role in the development of ALI [24]. At normal therapeutic doses, APAP is primarily metabolized by sulfotransferases (SULTs) and UDP-glucuronosyltransferases (UGTs), converted to nontoxic metabolites, and then excreted into the urine and bile. During APAP overdose, UGT and SULT are exhausted,

and APAP is mainly converted to NAPQI by cytochrome P450 2E1 (CYP2E1). The excess NAPQI depletes GSH, resulting in the covalent attachment of excess NAPQI to sulfhydryl groups in other proteins, particularly in mitochondrial proteins. This leads to mitochondrial dysfunction, which produces oxidative stress and excess superoxide free radicals, ultimately resulting in liver damage [24–27]. Fortunately, we observed that IMP protected mitochondrial membrane potential, decreased ROS expression, and increased the expression of Nrf2 and its downstream antioxidant protein SOD2. Besides, treatment with IMP increased liver metabolic enzyme expression (e.g., Gclc, Gss). This indicates that IMP not only alleviates the oxidative stress caused by NAPQI but also accelerates APAP metabolism at the source to reduce the production of NAPQI via upgrading the expression of APAP-related metabolic enzymes.

In this research, the expression of FXR and its target genes, NROB2 and ABCB11, was significantly inhibited in APAP-treated mice and rescued by IMP treatment. FXR is a ligand-mediated transcription factor that is highly expressed in the liver and plays a critical role in bile acid metabolism, glucose metabolism, and stress response to hepatotoxicity, such as exogenous chemicals and cholestasis induced by endogenous bile acid [28]. FXR is thus often suppressed in liver injury [29]. Studies have shown that FXR activation can protect against liver injury caused by APAP
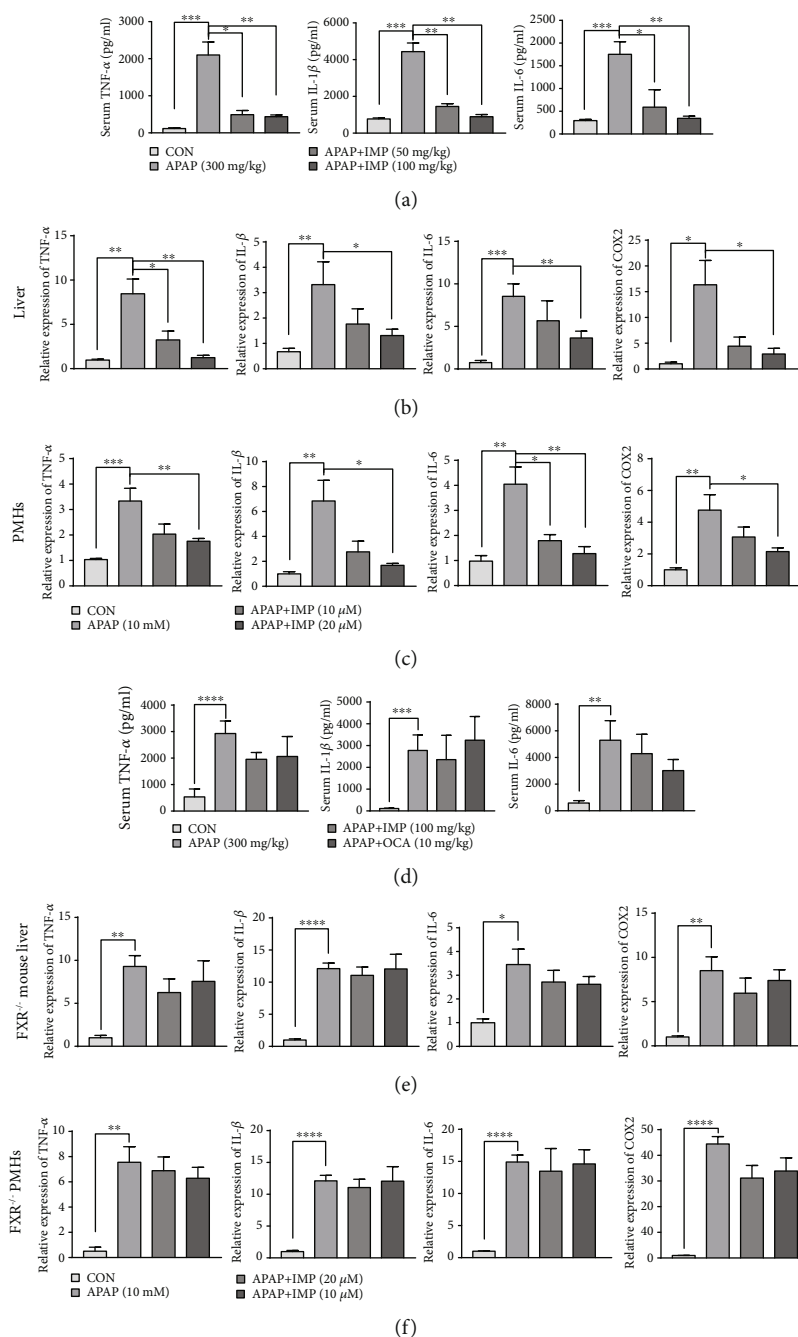
Figure 4: Pretreatment with IMP attenuates APAP overdose-induced proinflammatory cytokine elevation and expression of related genes. In vivo, IMP (50 or 100 mg/kg) or FXR agonist OCA (10 mg/kg) was also intragastrically administrated to mice for 5 days, which then received APAP (300 mg/kg) by intraperitoneal injection after fasting overnight. In vitro, after pretreatment with IMP (10 or 20 μM) or OCA (10 μM) for 24 hours, PMHs were incubated with APAP (10 mM) together with IMP/OCA for 24 hours prior to hepatocyte harvest. (a) IMP pretreatment alleviates APAP overdose-induced elevation of serum proinflammatory cytokines, including TNF-α, IL-1β, and IL-6. (b) The mRNA expression of TNF-α, IL-1β, IL-6, and COX2 in liver tissue. (c) The mRNA expression of TNF-α, IL-1β, IL-6, and COX2 in PMHs after APAP incubation with IMP. (d) The mRNA expression of TNF-α, IL-1β, IL-6 and COX2 in FXR$^{-/-}$ liver tissue. The serum protein levels of TNF-α, IL-1β, and IL-6 were measured by ELISA. (e) The mRNA expression of TNF-α, IL-1β, IL-6, and COX2 in FXR$^{-/-}$ liver tissue. (f) The mRNA expression of TNF-α, IL-1β, IL-6, and COX2 in FXR$^{-/-}$ PMHs. The mRNA levels of TNF-α, IL-1β, IL-6, and COX2 from tissue and cells were determined by qRT-PCR. Beta-actin served as the internal control. Data are expressed as the means ± SEM. Results are expressed as fold changes compared to the vehicle-treated control group. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. IMP: imperatorin; OCA: obeticholic acid; APAP: acetaminophen; TNF-α: tumor necrosis factor alpha; IL-1β: interleukin-1 beta; IL-6: interleukin-6; COX2: cyclooxygenase-2; qRT-PCR: quantitative real-time PCR; ELISA: enzyme-linked immunosorbent assay.
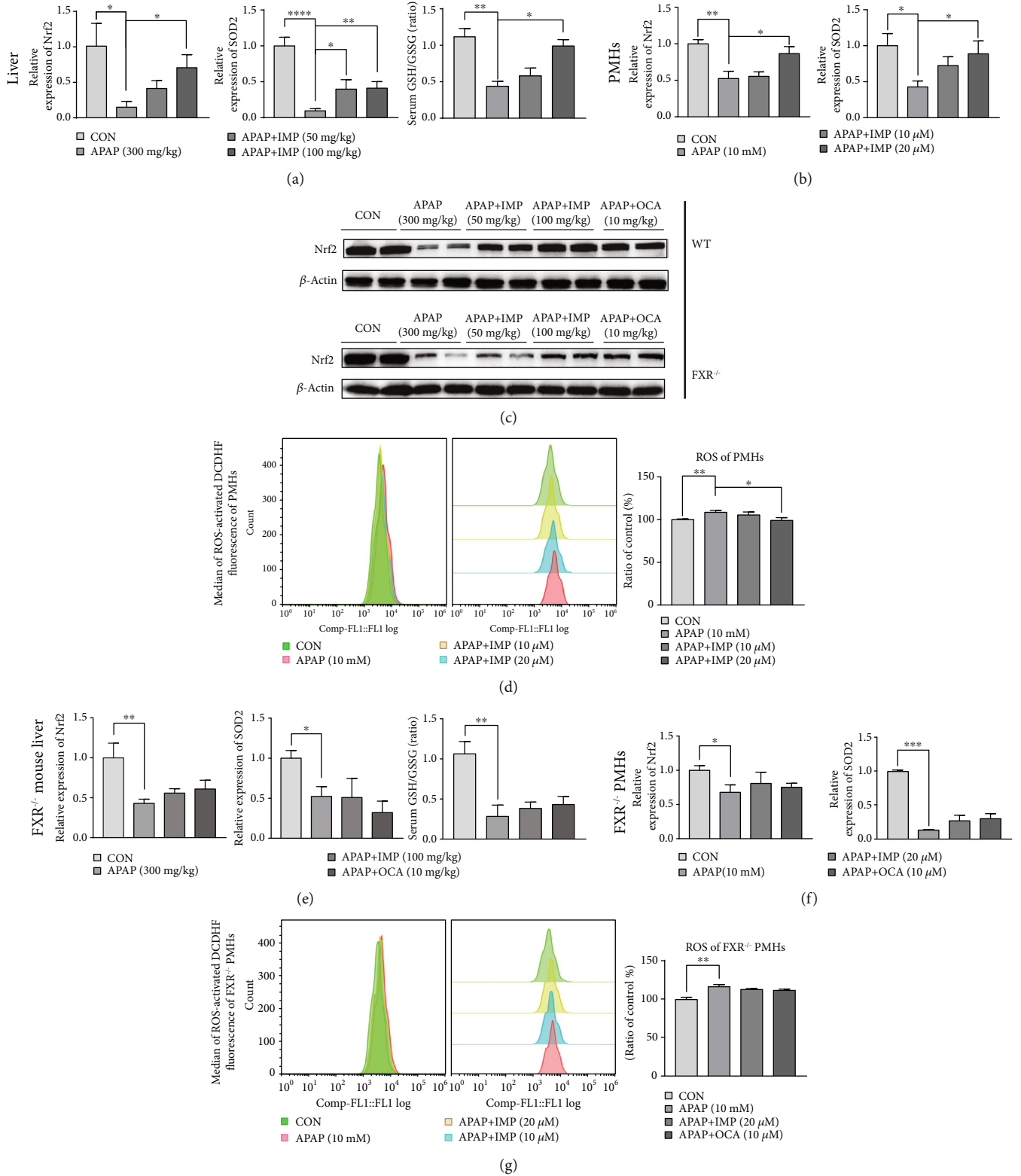
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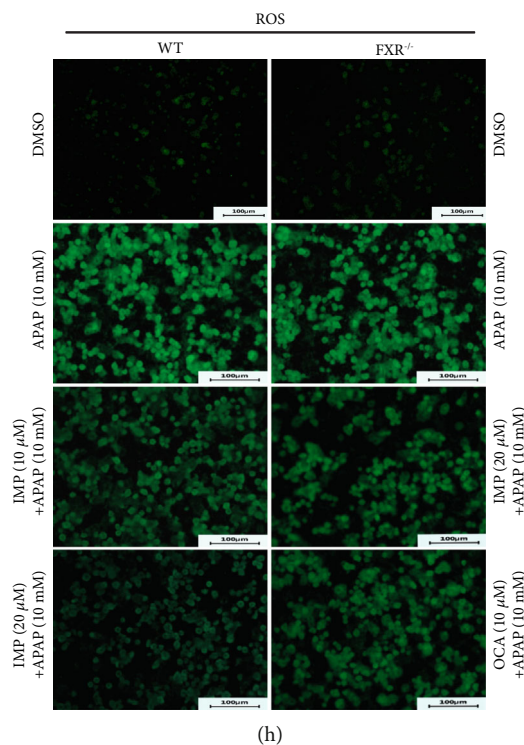
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FIGURE 5: Continued.

(h)

FIGURE 5: Effects of IMP pretreatment protect mice against APAP overdose-induced oxidative stress damage. (a) The relative expression of Nrf2 and SOD2 in WT mouse liver tissue and the serum level of GSH/GSSG. (b) The relative expression of Nrf2 and SOD2 in WT PMHs. The mRNA levels of Nrf2 and SOD2 from tissue and cells were determined by qRT-PCR. (c) The protein expression of Nrf2 in FXR$^{-/-}$ mice. (d) The expression of ROS in WT PMHs. The expression of ROS was measured by flow cytometry. (e) The relative expression of Nrf2 and SOD2 in FXR$^{-/-}$ mouse liver tissue and the serum level of GSH/GSSG. (f) The relative expression of Nrf2 and SOD2 in FXR$^{-/-}$ PMHs. The mRNA levels of Nrf2 and SOD2 from tissue and cells were determined by qRT-PCR. (g) The expression of ROS in FXR$^{-/-}$ PMHs. The expression of ROS was measured by flow cytometry. The ratio of each group to the control group was obtained. (h) ROS fluorescent in FXR$^{+/+}$ and FXR$^{-/-}$ PMHs. Results are expressed as fold changes compared to the vehicle-treated control group. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. GSH: reduced glutathione; GSSG: oxidized glutathione; Nrf2: nuclear factor erythroid 2-related factor 2; SOD2: superoxide dismutase-2; qRT-PCR: quantitative real-time PCR; ROS: reactive oxygen species.

overdose-induced oxidative stress by increasing the level of liver GSH [30]. Some drugs have been proven to ameliorate liver damage by upregulating FXR [12, 31]. Hence, activation of FXR may be a promising therapeutic concept in the treatment of drug-induced liver injury. It is worth noting that the antioxidant effect of IMP on APAP overdose-induced oxidative damage in FXR$^{-/-}$ mice was inconspicuous, suggesting that the protective effect of IMP is related to the enhanced expression of FXR. Here, we initially defined IMP as an FXR agonist based on the following aspects. First, the molecular docking results demonstrated that IMP could closely interact with hFXR-LBD. Second, IMP activated hFXR and regulated hFXR signaling. Third, the dual-luciferase assay verified that IMP can significantly increase the expression of FXR-dependent ABCB11, the downstream target genes of FXR. Finally, FXR knockout could eliminate the expression of IMP-mediated NROB2 and ABCB11. Collectively, the data reveal that IMP could closely interact with hFXR-LBD to regulate hFXR signaling. Our results suggest that the protective effects of IMP against APAP overdose-induced liver injury may be dependent on the activation of the FXR signaling pathway.

A previously accepted concept revealed that stable mitochondrial membrane potential helps to maintain the mitochondrial microenvironment. Damage of the mitochondrial membrane structure can elevate the generation of ROS and release cytochrome C and apoptosis-inducing factor to promote apoptosis, which in turn continuously activate and enhance mitochondrial ROS, forming a positive feedback [32]. In addition, Bax could be activated and translocated to the mitochondria, contributing to the opening of the mitochondrial membrane pores and ruining mitochondrial membrane potential [33]. The subsequent change in mitochondrial membrane potential facilitates the release of cytochrome C and apoptosis-inducing factor, thereby activating caspase and finally inducing apoptosis [34]. We found that IMP stabilized mitochondrial membrane potential and reduced apoptosis caused by APAP in vivo and in vitro in an FXR-dependent manner. As expected, the antiapoptosis effect of IMP in FXR$^{-/-}$ mouse hepatocytes was not as obvious as that in WT mouse hepatocytes. Therefore, we speculate that the antiapoptotic effect of IMP on hepatocytes may be related to the stability of the mitochondrial membrane potential.

(a)

(b)

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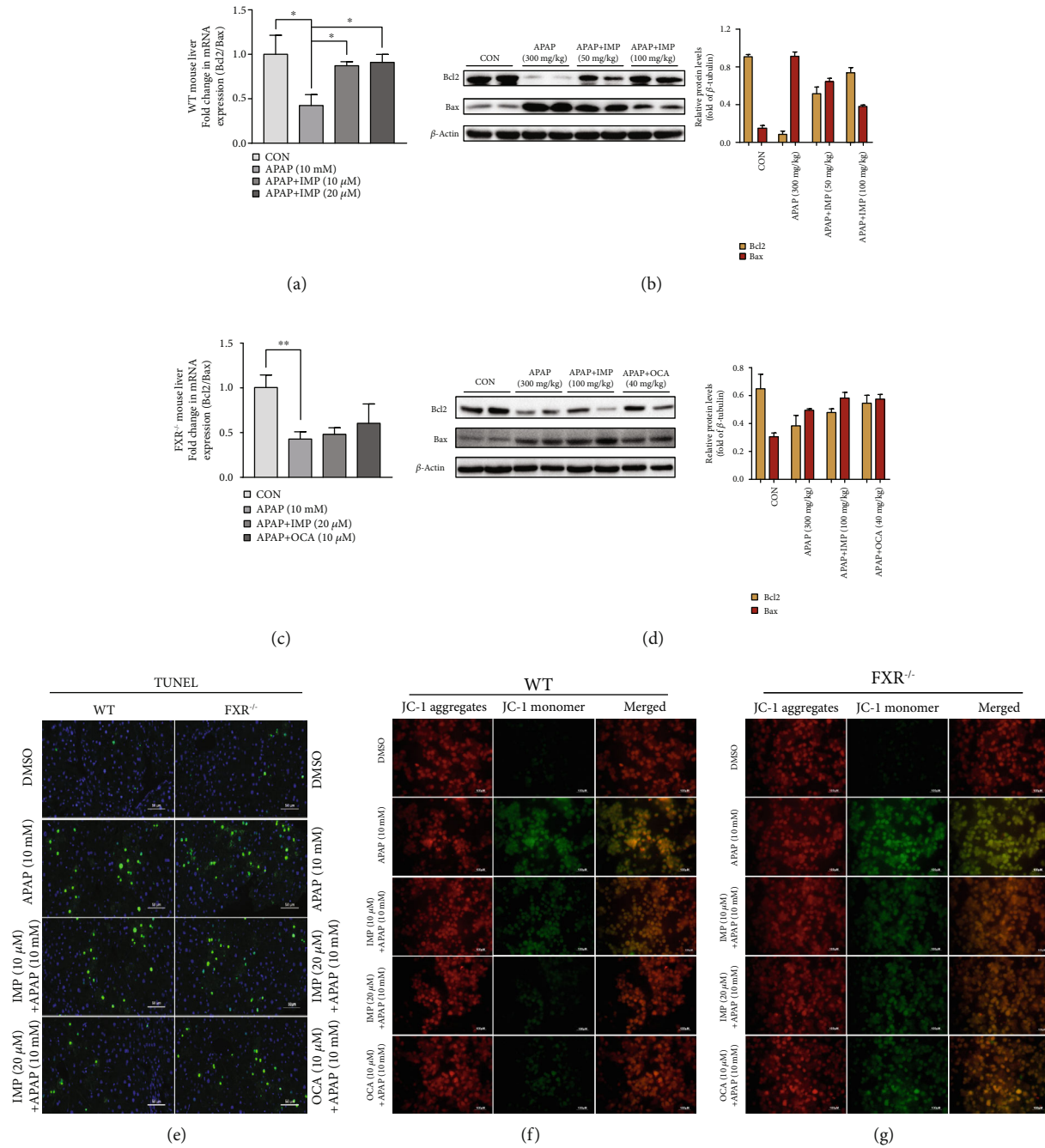
FIGURE 6: Pretreatment with IMP attenuates APAP overdose-induced apoptosis in WT mice but not in FXR$^{-/-}$ mice. (a, c) The mRNA ratio of Bcl2 to Bax in WT PMHs and FXR$^{-/-}$ PMHs. The mRNA levels of Bcl2 and Bax from cells were determined by qRT-PCR. (b, d) Proteins of Bcl2 and Bax from WT liver tissue and FXR$^{-/-}$ liver tissue were analyzed by Western blot. (e) The TUNEL levels in WT liver tissue and FXR$^{-/-}$ liver tissue after the treatment of IMP or OCA. The nucleus is stained with DAPI. (f, g) Mitochondrial membrane potential changes following the treatment of IMP or OCA. In the control group, JC-1 translocated to mitochondria through the mitochondrial membrane and aggregated into a polymer with red fluorescence. Conversely, in the APAP group, the concentration was reduced and the JC-1 polymer appeared as a green fluorescent monomer. As expected, IMP or OCA treatment decreased the green fluorescent monomer number and increased the red fluorescence polymer number. Unsurprisingly, the protective effect of IMP or OCA on mitochondrial membrane potential in FXR$^{-/-}$ mouse hepatocyte was not as obvious as that in WT mouse hepatocyte. Fluorescence intensity was measured by flow cytometry, and the brown fluorescence represents apoptosis. All data are expressed as the means ± SEM. Results are expressed as fold changes compared to the vehicle-treated control group; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. Bcl2: B-cell lymphoma 2; Bax: Bcl2-associated X protein; TUNEL: TdT-mediated dUTP nick-end labeling; DAPI: 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride; JC-1: JC1-mitochondrial membrane potential assay to determine mitochondrial transmembrane potential; WT: wild-type mice.
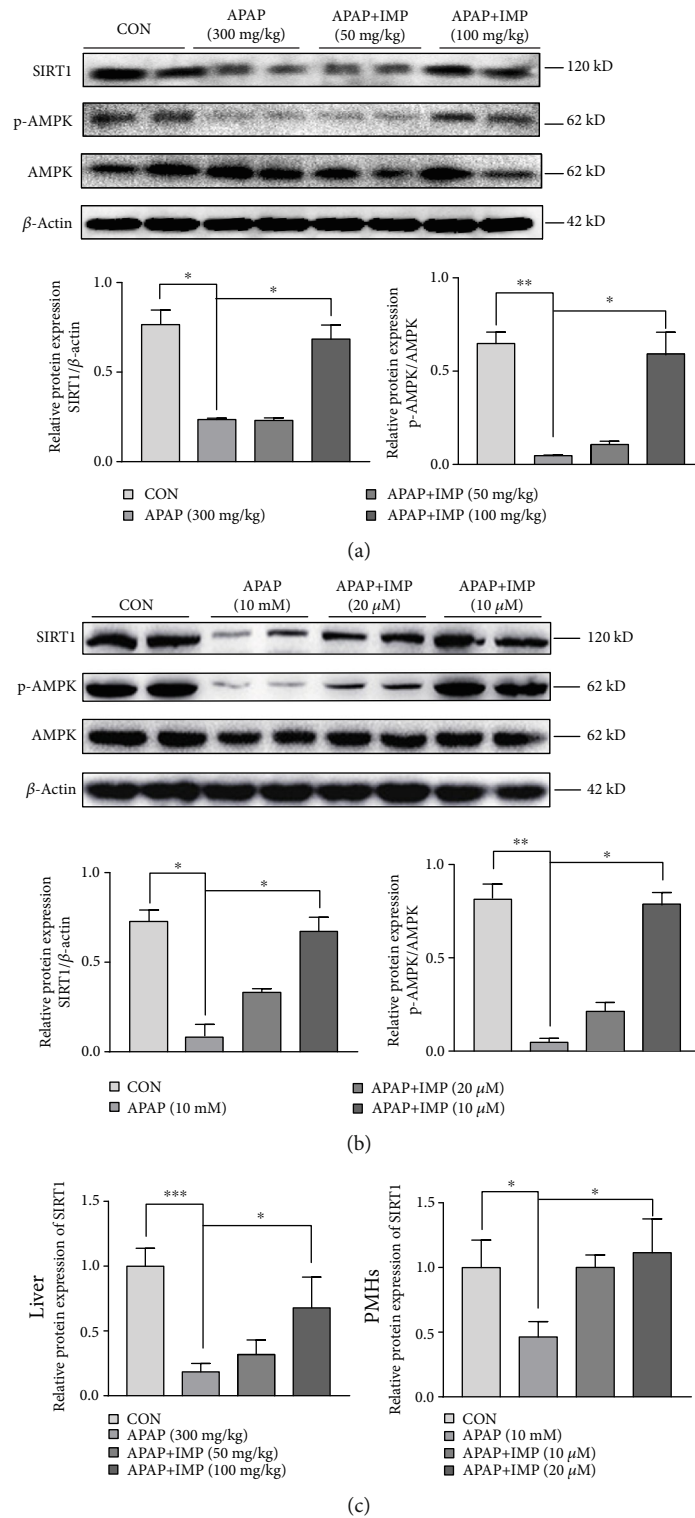
Figure 7: Pretreatment with IMP alleviates APAP overdose-induced oxidative damage with activation of SIRT1 and promotion of the phosphorylation of AMPK. (a, b) The protein expression of SIRT1 and p-AMPK in WT mouse liver tissue and PMHs was analyzed by Western blot. Quantitative analysis of scanning densitometry of protein bands for SIRT1, p-AMPK, and AMPK. (c) The relative expression of SIRT1 in WT liver tissue and WT PMHs was determined by qRT-PCR. IMP: imperatorin; APAP: acetaminophen; SIRT1: Sirtuin 1; AMPK: adenosine $5'$-monophosphate- (AMP-) activated protein kinase; p-AMPK: phosphorylation of adenosine $5'$-monophosphate- (AMP-) activated protein kinase; PMHs: primary mouse hepatocytes. Results are expressed as fold changes compared to the vehicle-treated control group. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.
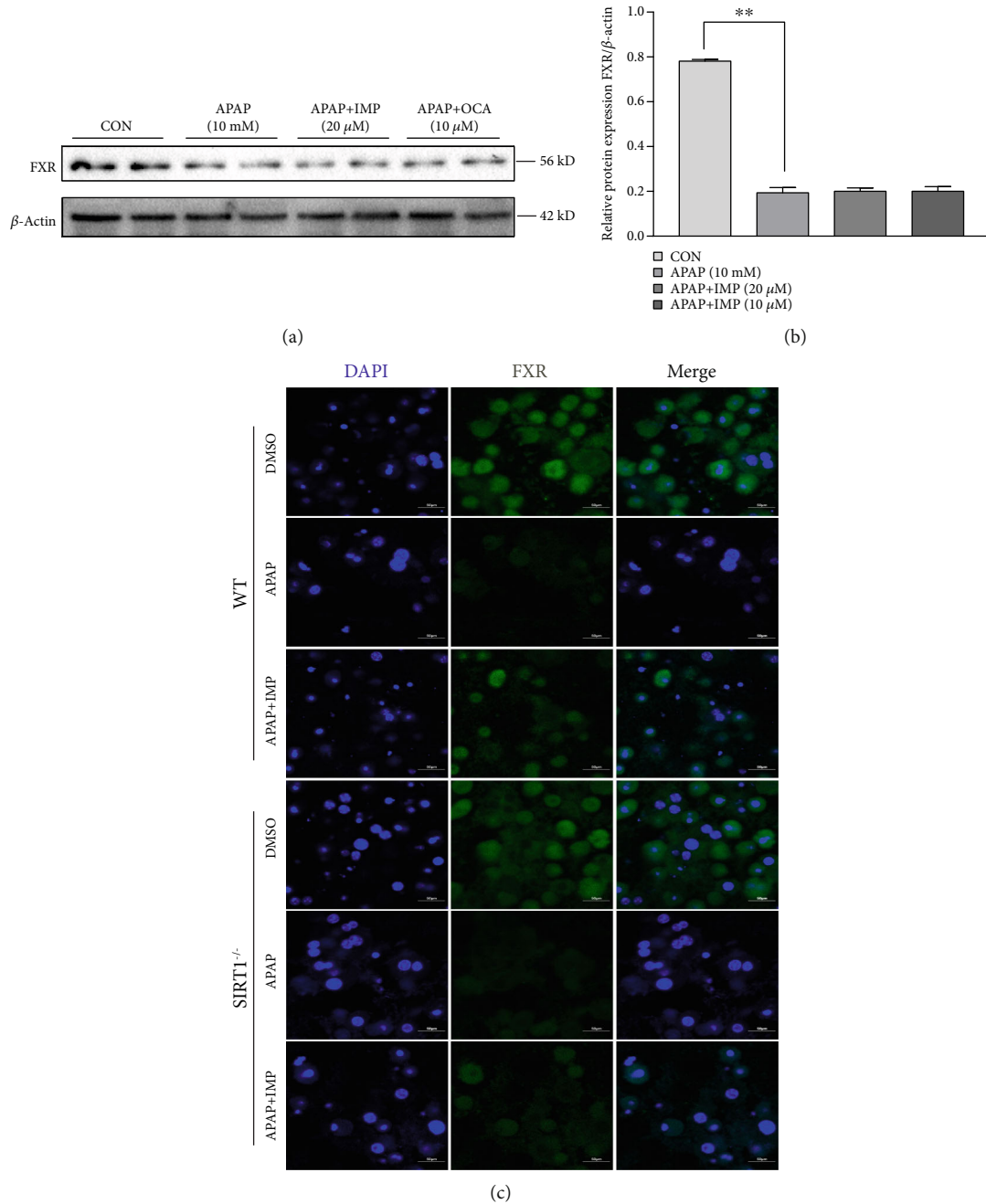
(a)

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FIGURE 8: Activation of FXR mediated by IMP or OCA treatment is partially lost in SIRT1$^{-/-}$ PMHs. (a) The protein expression of FXR in SIRT1$^{-/-}$ PMHs was analyzed by Western blot. (b). Quantitative analysis of scanning densitometry of protein bands for FXR in SIRT1$^{-/-}$ PMHs. (c) FXR localization was performed using immunofluorescence staining and observed using a confocal laser scanning microscope (magnification: 400x) stained by an anti-FXR antibody (1 : 100) with Alexa Fluor 488-conjugated detection antibody. The nucleus is stained with DAPI. Results are expressed as compared to the DMSO-treated control group. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. IMP: imperatorin; APAP: acetaminophen; OCA: obeticholic acid; SIRT1: Sirtuin 1; FXR: Farnesoid X receptor; DAPI: 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride; SEM: standard error of mean.

APAP overdose-induced hepatotoxicity could activate proinflammatory cytokines and chemokines and then recruit neutrophils and monocytes to the injured area of the liver, causing the production of more proinflammatory cytokines, aggravating inflammatory response [35]. In the early stages of liver injury, this process helps to remove dead cells and debris, and in the later stages, it contributes to liver repair.

However, inflammation is a double-edged sword. Excessive inflammatory response is bound to cause more serious injury or even hepatocellular death [36]. Therefore, whether APAP overdose-induced inflammation promotes the progression of liver damage or acts as a cellular defense against toxicity remains controversial. Nonetheless, most studies identified anti-inflammation as a therapeutic strategy to protect against
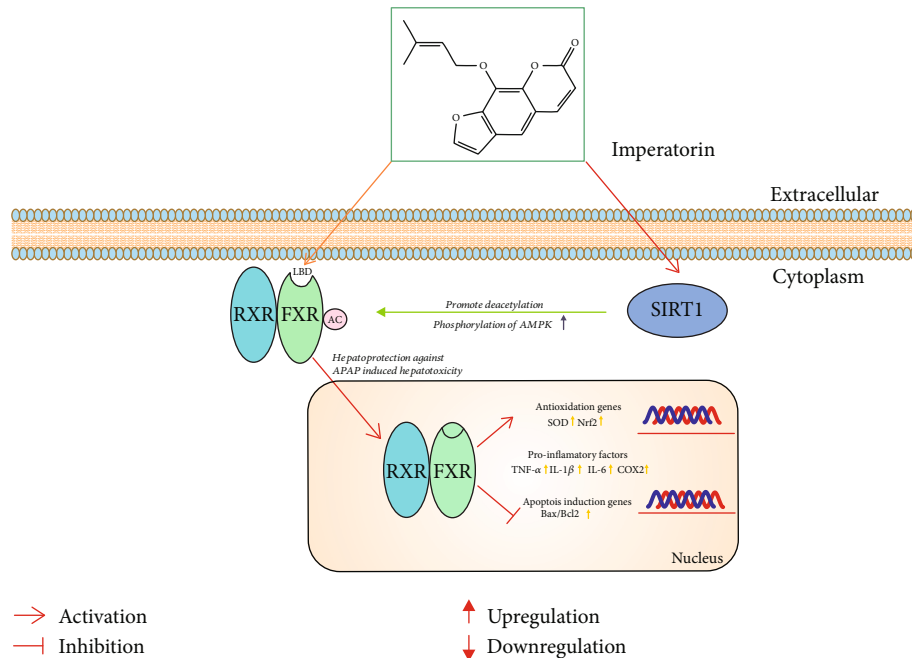
FIGURE 9: Proposed mechanisms of IMP mediating activation of SIRT1 and FXR to protect mice against APAP overdose-induced liver injury. IMP exerts the physiological effect of hepatoprotection against APAP overdose-induced liver injury via directly activating FXR signaling and indirectly facilitating the SIRT1-dependent deacetylation of FXR. Meanwhile, IMP can also promote the SIRT1-dependent phosphorylation of AMPK, which leads to FXR heterodimerization with the retinoid X receptor (RXR) and binds to FXR response elements (FXREs) to upgrade expression of FXR-targeted genes involved in antioxidant, anti-inflammatory, and antiapoptosis effect.

APAP overdose-induced liver damage [37–40]. In this study, we found that IMP could significantly inhibit the increase in proinflammatory cytokines including TNF-α, IL-1β, and IL-6 in the liver of APAP-WT mice in an FXR-dependent manner. To determine the relationship between IMP and FXR, we performed the same experiment on FXR$^{-/-}$ mice and their primary hepatocytes, using the FXR activator as a positive control. Consistent with the hypothesis, the protective effects of IMP and OCA were reduced.

As an endogenous activator of FXR in liver cells, SIRT1 modulates the FXR-stimulated transcriptional signaling via the deacetylation of this nuclear receptor and neighboring histones that strictly control the target gene transcription, and it has been confirmed that SIRT1 is downregulated in liver injury [41, 42]. The energy sensor AMPK has been found to act as the prime initial sensor through phosphorylation that helps translate the adaptability to stress response into SIRT1-dependent deacetylation of the transcriptional regulators for modulation of mitochondrial function and energy metabolism genes, possibly by increasing intracellular NAD+ levels [43]. Currently, studies have shown that SIRT1 protects mice from APAP overdose-induced hepatotoxicity by remodulating inflammation and oxidative stress [44]. Activation of the SIRT1-FXR signaling pathway has been proven to mediate the protective effects of celastrol on a-naphthyl isothiocyanate (ANIT) and thioacetamide (TAA) overdose-induced liver injury [42]. Whether and how SIRT1 is involved in IMP-induced activation of FXR in ALI remain unknown. In this research, SIRT1$^{-/-}$ mice were also employed to reveal the involvement of SIRT1 in alleviation of ALI with IMP treatment. We found that IMP significantly enhanced SIRT1 and FXR expression in APAP overdose-induced WT mice while the activation of expression of FXR with IMP or OCA treatment was partially lost in SIRT1$^{-/-}$ PMHs, indicating that SIRT1-AMPK activation contributes to the IMP-mediated FXR signaling against APAP overdose-induced liver injury.

Based on clinical cases, we conducted a study on the therapeutic efficacy of IMP in protecting against APAP-induced acute liver injury. Our results suggested that treatment with IMP at 4 h after a toxic APAP administration can also reduce liver tissue damage significantly (Supplementary Figure 3). In view of the close relationship between intestinal flora and liver, we will further explore the mechanism of IMP regulating intestinal flora in the future [45–48]. IMP is administered by gavage to mice, and the restoration of intestinal flora balance may also be one of the mechanisms used by IMP to improve APAP overdose-induced liver injury.

Nevertheless, the following limitation of our work must be considered while interpreting our results. Our data were derived from mice and have not been validated in clinical trials. Therefore, future studies are needed to confirm its efficacy in clinically relevant treatment models.

5. Conclusion

In summary, our findings confirm that IMP can be considered a new FXR agonist that protects against APAP overdose-induced ALI in mice. This protective mechanism

is mainly exerted via activation of FXR signaling, which then produces anti-inflammatory, antioxidant, and antiapoptosis effects, eventually relieving liver damage. It suggests that IMP offers promise for the treatment of APAP overdose-induced hepatotoxicity.

Data Availability

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

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Zhao Gao and Jiecheng Zhang are co-first authors.

Acknowledgments

Supplementary Materials

Supplement Figure S1: FXR molecular docking. Supplement Figure S2: identification of FXR knockout C57BL/6 mice (FXR$^{-/-}$). Supplement Figure S3: therapeutic efficacy of IMP in protecting against APAP-induced acute liver injury. *(Supplementary Materials)*

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