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Dihydromyricetin alleviates acetaminophen-induced liver injury via the regulation of transformation, lipid homeostasis, cell death and regeneration

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ABSTRACT

Aims: We previously reported that *Hovenia dulcis* Thunb. extract, a traditional Chinese medicine rich in dihydromyricetin (DHM), exhibited a significant hepatoprotective effect against acetaminophen (APAP)-induced liver injury. However, whether DHM plays a protective role in APAP hepatotoxicity and what mechanisms are involved remain unclear. In this study, we evaluated the hepatoprotective effects of DHM against APAP-induced liver injury.

Main methods: Male C57BL/6 mice were used for the experiment. LC-MS, q-PCR, immunochemistry and western blot analysis were employed to mechanism analysis.

Key findings: DHM exhibited a protective effect against APAP-induced liver injury. Further mechanistic investigations revealed that the protective effect of DHM against APAP hepatotoxicity had multi-target and multi-pathway characteristics involving APAP metabolism, lipid regulation, and hepatocyte death and regeneration. DHM pretreatment resulted in cytochrome P450 2E1 inhibition and UDP-glucuronosyltransferase 1A1 activation, affecting APAP biotransformation. Moreover, DHM pretreatment significantly ameliorated lipid dysregulation via peroxisome proliferator-activated receptor and sterol regulatory element-binding protein-1c (SREBP-1c) signalling pathways. Furthermore, DHM regulated the expression of cell death- and liver regeneration-associated proteins.

Significance: These results suggested that DHM alleviated APAP-induced liver injury in mice by inhibiting hepatocyte death, promoting p53-related regeneration, and regulating lipid homeostatic imbalance and APAP transformation. Based on these findings, DHM provides a potential and novel approach for preventing and treating APAP-induced liver damage, and SREBP-1c signalling might be a new therapeutic target for APAP hepatotoxicity.

1. Introduction

Acetaminophen (APAP), the most widely used analgesic and antipyretic agent worldwide, can lead to severe liver damage if taken in overdose [1]. Liver injury caused by excessive APAP ingestion is a main cause of acute liver injury in many countries [2]. Considering the seriousness of APAP hepatotoxicity, mechanistic studies have also been valued and thoroughly undertaken [3]. At a therapeutic dose, most APAP is metabolized by UDP-glucuronosyltransferase (UGT) to rapidly produce glucuronidated metabolites and is then excreted into the bile

Abbreviations: ABCA1, ATP-binding cassette transporter A1; ABCG, ATP-binding cassette, sub-family G; ACC1, acetyl-CoA carboxylase 1; AKP, alkaline phosphatase; ALT, alanine aminotransferase; APAP, acetaminophen; APAP-CYS, APAP-cysteine; APAP-GLU, APAP-glucuronide; APAP-GSH, APAP-glutathione; AST, aspartate aminotransferase; BSEP, bile salt export pump; Caspase (Casp), cysteinyl aspartate-specific proteinase; CCND1, cyclin D1; CDK4, cyclin-dependent kinase 4; CD36, cluster of differentiation 36; CPT, carnitine palmitoyltransferase; CYP, cytochrome P450 protein; CytC, cytochrome *c*; DGAT, diglyceride acyltransferase; DHCR24, 24-dehydrocholesterol reductase; FABP, fatty acid binding protein; FASN, fatty acid synthase; FATP, fatty acid transport protein; GSH, glutathione; GST, glutathione S-transferase; HDL-C, high-density lipoprotein cholesterol; H&E, haematoxylin-eosin; HMGCR, HMG-CoA reductase; HMGCS1, 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1; MDA, malondialdehyde; MRM, multiple reaction monitoring; MRP, multi-drug resistance protein; NAPQI, *N*-acetyl-p-benzoquinone imine; OATP, organic anion transporting polypeptide; OSTβ, organic solute transporter β; PCNA, proliferating cell nuclear antigen; PPAR, peroxisome proliferator-activated receptor; SCD1, stearoyl-CoA desaturase 1; SOD, superoxide dismutase; SPF, specific pathogen-free; SREBP, sterol regulatory element-binding protein; TBA, total bile acid; T-CHO, total cholesterol; TG, triglyceride; UGT1A1, UDP-glucuronosyltransferase 1A1

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and urine. Furthermore, approximately 10% of APAP is oxidized by cytochrome P450 (notably CYP2E1, CYP1A2 and CYP3A11) enzymes to the toxic intermediate *N*-acetyl-p-benzoquinone imine (NAPQI), which is detoxified by binding to glutathione (GSH) via the activity of glutathione S-transferase (GST) [4]. Under APAP overdose, NAPQI accumulation leads to GSH depletion, triggers mitochondrial oxidative stress by binding to sulfhydryl groups, and ultimately results in lethal cell death and liver injury [5,6]. *N*-acetylcysteine (NAC) treatment, which allows GSH supplementation and NAPQI detoxification, is now the FDA-approved standard of care for APAP poisoning. However, NAC has certain drawbacks, such as the time of administration and the induction of gastrointestinal reactions, that limit its clinical application [7]. Thus, exploring suitable alternative antidotes is necessary and urgent.

Dihydromyricetin (DHM), a flavonoid extracted from many traditional Chinese medicines such as Rattan tea and *Hovenia dulcis* Thunb. (the dried ripe fruit of the *Hovenia* Thunb. family), confers health benefits with minimum adverse effects. DHM has been reported to possess antioxidative, anti-inflammatory, hepatoprotective, and antitumour properties [8–10]. Regarding hepatoprotection, DHM has been shown to protect against liver-damaging agents, including ethanol and carbon tetrachloride [11,12].

Recently, we found that *Hovenia dulcis* Thunb. extract, which has a DHM content of approximately 30%, conferred a significant hepatoprotective effect against APAP-induced liver injury [13]. However, whether DHM plays a protective role in APAP hepatotoxicity and what mechanisms are involved remain unclear.

On the basis of these studies, the aim of this study was to explore the protective effect and possible protective mechanisms of DHM in APAP hepatotoxicity. We found that DHM markedly attenuated APAP-induced liver damage in mice, potentially through regulating enzymes involved in APAP transformation, altering lipid metabolism mediated by sterol regulatory element-binding protein-1c (SREBP-1c) and peroxisome proliferator-activated receptors (PPARs), inhibiting hepatocyte death and improving hepatocyte regeneration.

2. Materials and methods

2.1. Chemicals and reagents

APAP (PubChem CID: 1983, purity > 99.0%) was purchased from Aladdin Industrial Corporation (Shanghai, China). DHM (PubChem CID: 161557, purity > 98%) was purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China) and verified by HPLC (Supplementary Fig. 1). The kits for all biochemical assays, such as the alanine aminotransferase (ALT) and aspartate transaminase (AST) assays, were purchased from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). A Hoechst 33258 staining kit was purchased from Beyotime Institute of Biotechnology (Shanghai, China). The primary antibody against UGT1A1 was acquired from Abcam (Cambridge, UK). Primary antibodies against Bax, Cleaved caspase-3, Cytochrome c, Bcl-2, Cyclin D1 (CCND1), p53, Proliferating cell nuclear antigen (PCNA), Cyclin-dependent kinase 4 (CDK4), and GAPDH were purchased from Proteintech Group, Inc. (Chicago, USA). CYP2E1, CYP1A2, and Cleaved caspase-9 were obtained from Boster Biological Technology Co., Ltd. (California, USA). PPARα, PPARγ, SREBP-1c, CPT1A and ACC1 were purchased from Biosynthesis Biotechnology Co., Ltd. (Beijing, China). The horseradish peroxidase-conjugated AffiniPure goat anti-mouse immunoglobulin G and anti-rabbit immunoglobulin G secondary antibodies were purchased from Proteintech Group, Inc. (Chicago, USA).

2.2. Animals

All animal study procedures were approved by the Animal Ethics Committee of Shandong University and performed in accordance with the Guide for the Care and Use of Laboratory Animals. Male C57BL/6 mice [6–8 weeks old, 20 ± 2 g, specific pathogen-free (SPF)] were obtained by the Shandong University Laboratory Animal Center (Shandong, China) and housed in standard plastic cages with sawdust as bedding under standardized conditions (12 h light/dark cycle, 22–25 °C, 50–60% humidity) in the Laboratory of Pharmacology, School of Pharmaceutical Sciences, Shandong University. The mice had free access to standard rodent chow and water and were acclimatized for 1 week prior to the experiments. Male C57BL/6 mice are widely used for experimental APAP-induced liver damage research [14,15].

The animals were weighed and randomly divided into the following six groups: (1) control, (2) APAP ($300\ mg{\cdot}kg^{-1}$), (3) DHM200 ($200\ mg{\cdot}kg^{-1}$), (4) APAP + D25 ($25\ mg{\cdot}kg^{-1}$), (5) APAP + D100 ($100\ mg{\cdot}kg^{-1}$), and (6) APAP + D200 ($200\ mg{\cdot}kg^{-1}$). Mice in the treatment groups were gavaged with DHM suspended in water at doses of $200\ mg{\cdot}kg^{-1}$, $25\ mg{\cdot}kg^{-1}$, $100\ mg{\cdot}kg^{-1}$, $200\ mg{\cdot}kg^{-1}$ body weight, respectively, every day for 5 consecutive days. The control group and APAP group were administered water at the same time. All mice were fasted for 15 h before the intraperitoneal injections and then resumed feeding. In all but the control and DHM200 groups, APAP ($300\ mg{\cdot}kg^{-1}$) dissolved in 0.9% saline was intraperitoneally injected 1 h after the final DHM or water treatment. Nine hours after APAP administration, the animals were weighed again, and blood was collected for the relevant biochemical evaluations. At the end of the experiment, all mice were euthanized, and the livers were harvested. A small segment of the liver was collected in 4% paraformaldehyde for histology, and the remaining tissue was frozen in liquid nitrogen and stored at −80 °C for subsequent use.

Blood stored for 1 h was centrifuged at 3000 rpm for 12 min to obtain serum. The supernatant was gathered and stored at −20 °C for subsequent assays.

A portion of fresh liver tissue was homogenized with cold saline and centrifuged at 3000 rpm for 15 min. The supernatant was used for biochemical assessment. Liver homogenate protein was quantitated by a bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology, Shanghai, China).

2.3. Biochemical and histological analysis

Serum ALT, AST, alkaline phosphatase (AKP), triglyceride (TG), total cholesterol (T-CHO), high-density lipoprotein cholesterol (HDL-C), total bile acid (TBA), liver homogenate GSH, malondialdehyde (MDA), and superoxide dismutase (SOD) levels were detected by a Thermo Scientific Multiskan FC Microplate Photometer (Thermo Fisher Scientific, USA).

For histopathological studies, fresh livers from mice in each group were immediately fixed with paraformaldehyde, embedded in paraffin, and cut into 5 μm thick sections. The slides were examined for histological architecture by haematoxylin-eosin (H&E) staining. Hoechst 33258 staining was performed following the standard protocol [16]. Briefly, the liver tissues were harvested and fixed in 4% paraformaldehyde. These samples were cut into 5 μm sections and stained with Hoechst 33258 ($10\ \mu g{\cdot}mL^{-1}$). After washing with PBS, stained nuclei were visualized with UV excitation and imaged under a fluorescence microscope (Olympus, Tokyo, Japan).

2.4. Analysis of APAP and APAP metabolites

APAP and its in vivo metabolites, including APAP-cysteine (APAP-CYS), APAP-glucuronide (APAP-GLU) and APAP-glutathione (APAP-GSH), were analysed by LC-MS as reported previously, with slight modifications [17]. Briefly, 50 μL of serum was deproteinized with 150 μL of methanol. After centrifugation at 12000 rpm for 12 min, 150 μL of the supernatant was evaporated under a gentle stream of nitrogen. The residue was reconstituted in 150 μL of methanol:water (50:50, v/v), and 5 μL of the sample was injected into an HPLC/AB

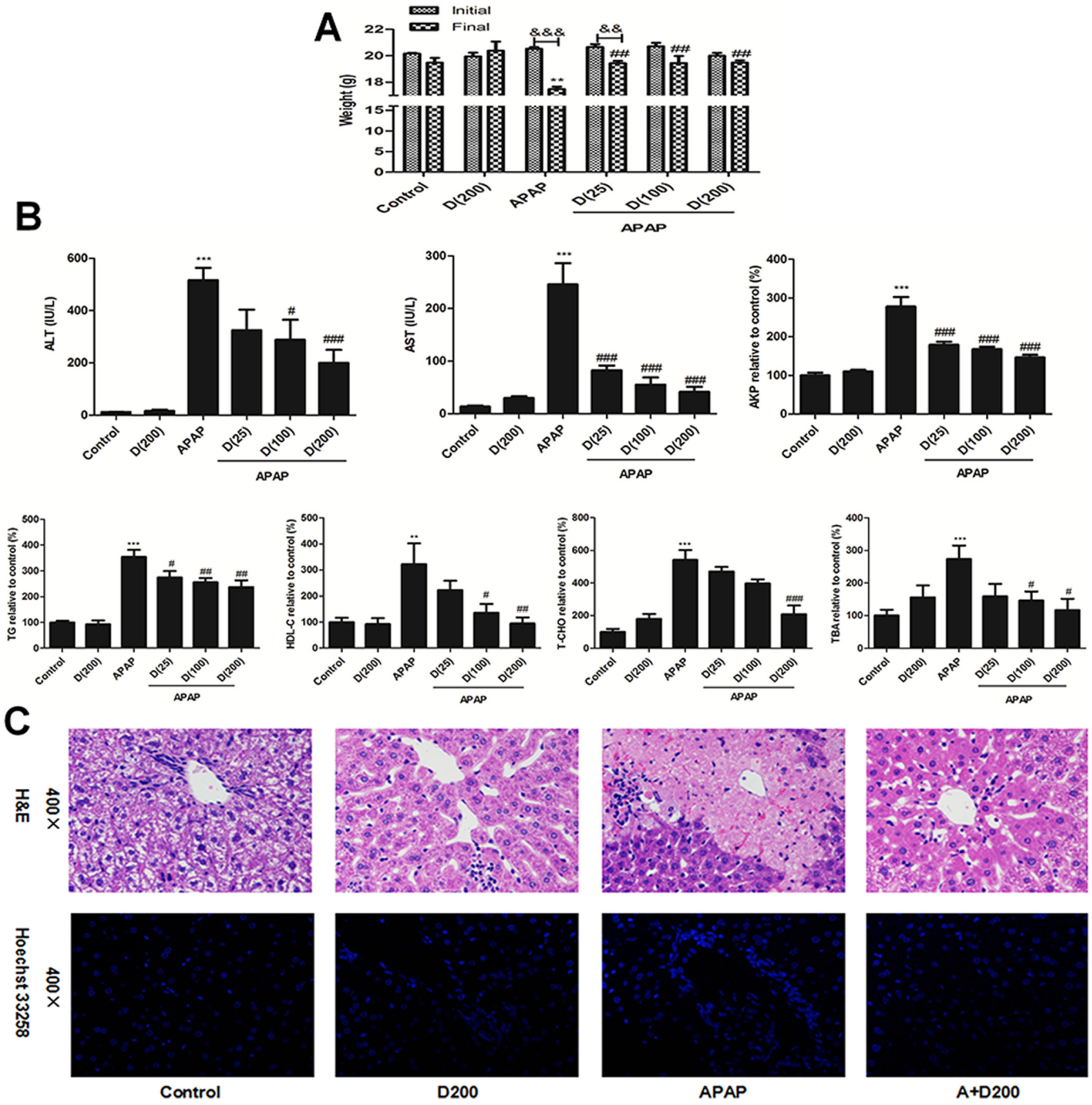

Fig. 1. DHM exerts hepatoprotective effects against APAP-induced liver injury. (A) Initial and final mouse body weights during the five-day experiment. (B) Serum ALT, AST and AKP levels as well as serum T-CHO, TG, HDL-C and TBA levels were measured as indicated. The data are expressed as the mean ± S.E.M.; n = 7 for the APAP group, n = 6 for the other groups. $^{**}P < 0.01$ and $^{***}P < 0.001$ versus the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus the APAP group; $^{\&\&}P < 0.01$ and $^{\&\&\&}P < 0.001$ initial weight versus final weight. (C) Representative images of H&E- and Hoechst 33258-stained liver sections at 400× magnification (n = 3).

SCIEX API 4000 triple-quadrupole mass spectrometer instrument (AB SCIEX, Foster City, CA, USA) for the analysis of APAP and its metabolites. Chromatographic separation was achieved using a Phenomenex Gemini C18 column (4.6 mm × 25 cm, 5 μm) at 35 °C. The mobile phase consisted of acetonitrile containing 0.1% formic acid (phase A) or 0.1% aqueous formic acid (phase B) at a flow rate of 400 μL/min under the following gradient program: a linear gradient from 5% to 30% A (0–7 min), 30% to 50% A (7–10 min), 50% to 95% A (10–13 min), 95% to 50% A (13–17 min), and 50% to 5% A (17–19 min), followed by equilibration with 5% A for 5 min. The samples were analysed in positive mode, and quantification was performed using the multiple reaction monitoring (MRM) mode monitoring the *m/z* transitions 152.1 → 110.2 for APAP, 328.1 → 152.1 for APAP-GLU, 271.1 → 184.1 for APAP-CYS, and 457.2 → 182.1 for APAP-GSH. The optimal parameters for MS operation were as follows: collision energy (CE): 22 V, ion spray (IS) voltage: 5000 V, and temperature (TEM): 500 °C. The pressures for gas 1 and gas 2 were set to 50 and 40 psi, respectively; the nitrogen curtain gas pressure was set at 10 psi. Data acquisition was performed with Analyst software (AB SCIEX, Foster City, CA, USA). The metabolite peak area of the acetaminophen model mice was 100%, and

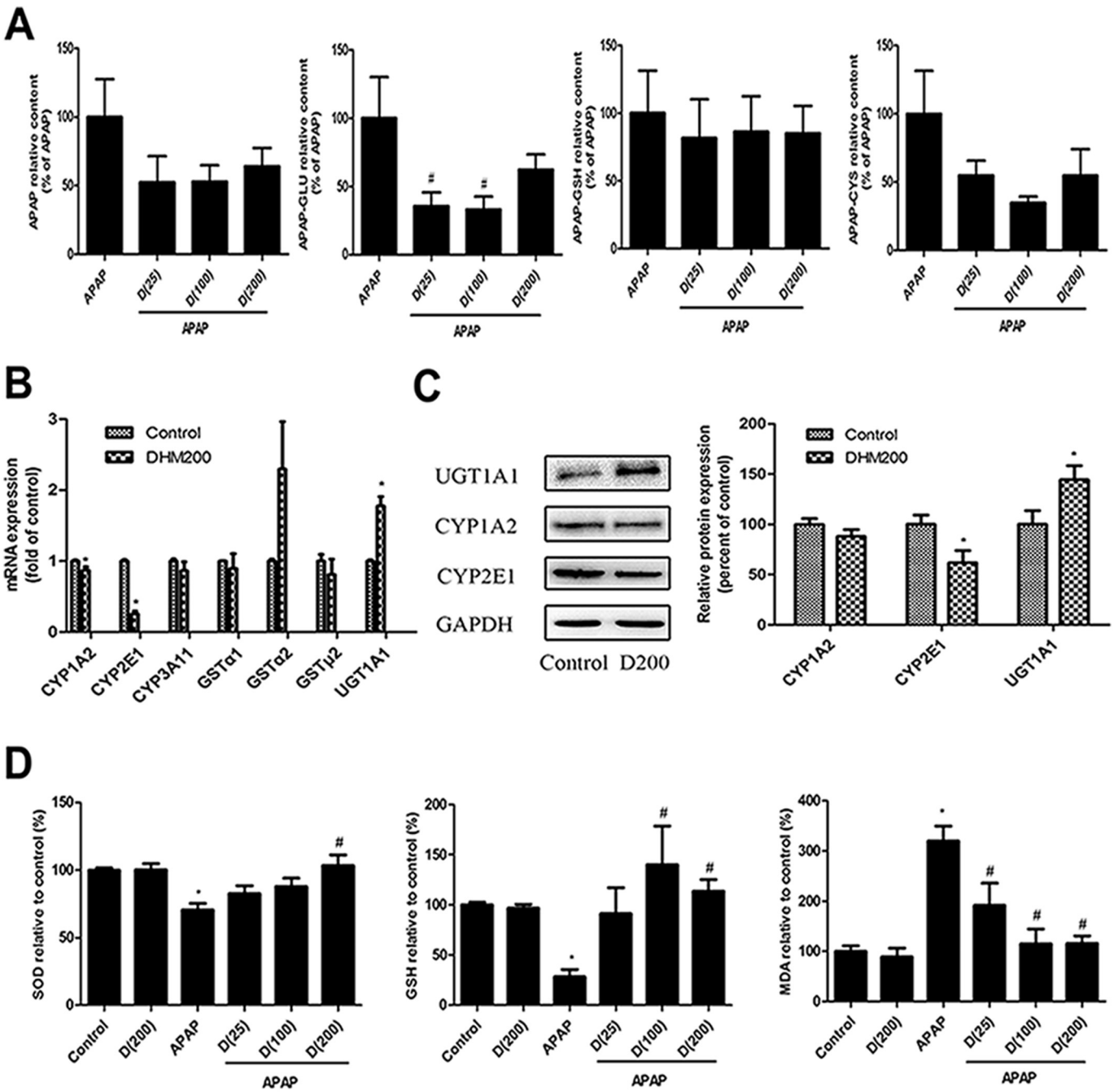

Fig. 2. DHM activates UGT1A1 and inhibits CYP2E1 enzymes involved in APAP bioactivation. (A) Relative serum levels of APAP and its metabolites. (B) The levels of APAP metabolism-related genes in mouse liver were determined by RT-PCR (n = 5). (C) UGT1A1, CYP2E1 and CYP1A2 protein expression levels were compared between the control and DHM200 groups (n = 6). (D) Liver SOD, total GSH and MDA activities (n = 7 for the APAP group, n = 6 for the other groups). The data are expressed as the mean ± S.E.M. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the APAP group.

the relative contents of the remaining groups were compared.

2.5. Quantitative PCR (qPCR) analysis

qPCR analysis was performed as described in a previous report [18]. Total mRNA was extracted from frozen liver tissues by TRIzol reagent (Invitrogen, Carlsbad, CA) and analysed by a BioDrop (Biochrom, Cambridge, UK). Total RNA (2.5 μg) was reverse transcribed to cDNA with M-MLV reverse transcriptase according to the manufacturer's protocol. All qPCR primer sequences are listed in Table S1.

Total cDNA (2.5 μL), the forward primer (1.25 μL), the reverse primer (1.25 μL) and LightCycle 480 SYBR Green I Master Mix (5 μL) (consisting of FastStart Taq DNA polymerase, reaction buffer, dNTP mix, SYBR Green I dye, and $MgCl_2$) constituted the qPCR reaction mixture. All reactions were performed on a qTOWER 3.0G real-time PCR system (Analytik Jena AG, Jena, Germany). GAPDH was used for internal normalization.

2.6. Immunohistochemistry

Paraffin sections were used for immunohistochemical detection of PCNA as previously described [19,20]. Briefly, paraffin-embedded liver sections (5 μm thick) were deparaffinized and rehydrated. A primary antibody against PCNA was used for immunohistochemistry. Antibody

binding was visualized using a peroxidase-conjugated second antibody and 3,3′-diaminobenzidine (DAB) solution.

2.7. Western blot analysis

Total liver protein was extracted in radioimmunoprecipitation assay (RIPA) lysis buffer containing 1 mM phenylmethanesulfonyl fluoride (PMSF) and quantitated by a BCA protein assay kit (Beyotime Biotechnology, Shanghai, China). Protein samples were separated by 10% or 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto 0.22 μm polyvinylidene difluoride membranes (Bio-Rad, Germany). Then, the membranes were blocked with 5% non-fat milk in TBS containing 0.1% Tween-20 (TBST) for 1.5 h. Primary antibodies against UGT1A1, CYP2E1, CYP1A2, Cytochrome c, Cleaved caspase-3, Cleaved caspase-9, Bcl-2, Bax, p53, PCNA, CDK4, CCND1, PPARα, PPARγ, SREBP-1c, CPT1A, ACC1 and GAPDH were used. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Finally, the membranes were washed 3 times with TBST before immunoblot analysis was performed.

2.8. Statistical analysis of data

Statistical analysis was performed only when $n \geq 5$ independent samples were acquired. All data are expressed as the mean ± S.E.M. An unpaired Student's *t*-test was used for comparing two groups, and one-way ANOVA followed by Dunnett's post hoc multiple comparisons test was used to compare data for more than two groups. Statistical significance was set at $P < 0.05$. GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA) was employed to generate the resulting data charts.

3. Results

3.1. DHM protects against APAP-induced liver damage

Body weight changes and histopathological and biochemical assays were used to test whether DHM has a protective effect against APAP-induced liver damage. The changes in the body weights of the mice are shown in Fig. 1A. The final weight remained constant in all groups except the APAP group, which displayed a significant weight decrease. Comparing the initial and final mouse weights, we observed that weight loss was most evident in mice treated with APAP alone ($P < 0.0001$). Regarding the biochemical assays, the serum ALT, AST, and AKP levels significantly increased in mice treated with APAP alone compared with these levels in mice from the other groups, implying that APAP caused severe liver damage in mice. Serum TBA and lipid markers also varied in the same pattern displayed by ALT and AST (Fig. 1B). Pretreatment with DHM significantly restored these indexes to normal compared with the index values in the APAP group. In addition, 300 mg·kg^{-1} APAP caused noticeable hepatocyte necrosis (Fig. 1C). DHM pretreatment for 5 days ameliorated the effects of APAP-related hepatocyte necrosis. As indicated by these results, DHM pretreatment protected against APAP-induced liver damage, which manifested as an increase in the body weights and the normalization of the serum biochemical indexes.

3.2. DHM influences the metabolism of APAP in vivo

To determine whether DHM pretreatment affected APAP bioactivation, the contents of APAP, APAP-GLU, APAP-GSH and APAP-CYS in serum were analysed by LC-MS. Pretreatment with DHM reduced the content of APAP and its metabolites in serum (Fig. 2A). Since UGT1A1 is a major enzyme in APAP glucuronidation and CYP and GST enzymes also play important roles in the toxicity-related APAP biotransformation process, the hepatic expression levels of UGT1A1, CYPs and GSTs were investigated [5,21]. After DHM (200 mg·kg^{-1}) pretreatment for 5 days, the expression of UGT1A1 was increased, while that of CYP2E1 was suppressed (Fig. 2B and C). In addition, the relative liver contents of GSH and SOD were dramatically lower in the APAP group than in the other groups (Fig. 2D), but the level of MDA increased. Taken together, these findings suggest that DHM markedly inhibited CYP2E1 and activated UGT1A1 to influence APAP biotransformation but had no significant effect on GST family enzymes.

3.3. DHM regulates SREBP-1c and PPAR-mediated lipid dysregulation caused by APAP

Based on changes in serum lipids and bile acids (Fig. 1B), the expression of genes related to lipid processing, including lipid absorption, synthesis, metabolism and transport, was also quantified (Fig. 3A–H). Compared with the APAP group, the APAP + D200 group exhibited dramatic reductions in the expression of fatty acid transport protein (FATP2), cluster of differentiation 36 (CD36), acetyl-CoA carboxylase 1 (ACC1), multi-drug resistance proteins 3 and 4 (MRP3, MRP4), organic solute transporter β (OSTβ), and diglyceride acyltransferase 1 (DGAT1). However, the expression of carnitine palmitoyltransferases 1A and 2 (CPT1A, CPT2), CYP7A1, CYP27A1, ATP-binding cassette transporter A1 (ABCA1), MRP2, HMG-CoA reductase (HMGCR), 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 (HMGCS1), 24-dehydrocholesterol reductase (DHCR24) and stearoyl-CoA desaturase 1 (SCD1) was increased in the APAP + D200 group. In addition, compared with the control group, the DHM200 group exhibited significantly upregulated expression of CYP7A1 and organic anion transporting polypeptides 1 and 2 (OATP1, OATP2) but downregulated expression of SCD1; ATP-binding cassette, sub-family G 2 and 5 (ABCG2, ABCG5); and bile salt export pump (BSEP). In summary, these data suggested that DHM regulated the lipid imbalance caused by APAP via genes which involves lipid synthesis, absorption, metabolism and transport. Compared with the APAP group, the APAP + D200 group exhibited increased expression of PPARα and PPARγ but decreased expression of SREBP-1c. Further, the SREBP-1c and PPARα/γ signalings, which were reported to be closely related to lipidolysis and lipid synthesis, were validated by the western blot method [22]. The analysis is consistent with PCR (Fig. 3I). These data indicated that DHM regulated APAP-induced lipid metabolism dysregulation by modulating SREBP-1c and PPARα/γ signalings. Notably, the hepatoprotective action mechanism of DHM affected multiple targets. In addition, we performed a post-treatment strategy to explore the underlying protective mechanisms. Specifically, we treated mice with DHM (200 mg·kg^{-1}) 2 h after APAP administration and measured serum parameters at different times (6, 12, 24, 48 h) to verify that the effects of dihydromyricetin on liver injury and lipid homeostasis. The result suggested that DHM post-processing has a certain protective effect (Supplementary Fig. 2A). It will greatly help to elucidate the potential off-target effect of DHM to P450 enzymes in pretreatment study. These data also suggested that DHM still reduced the APAP-induced lipid upregulation by post-treatment, suggesting that DHM might directly regulate APAP-induced lipid homeostasis (Supplementary Fig. 2B).

3.4. DHM prevents APAP-related hepatocyte death and activates p53-related regeneration signals

To investigate the effect of DHM on APAP-related hepatocyte death and regeneration, western blot analysis was used to investigate cell death- and regeneration-related proteins [20]. As shown in Fig. 4A, the expression of the released Cytochrome c, Bax, Cleaved caspase-3 and Cleaved caspase-9 was notably higher in the APAP group than in the control group but was significantly lower in the APAP + D200 group than in the APAP group; this tendency was opposite to that observed for the expression of Bcl-2, an anti-apoptotic factor. The relationship between DHM and hepatocyte regeneration was also investigated. The expression of p53 was upregulated in the 300 mg·kg^{-1} APAP group, but

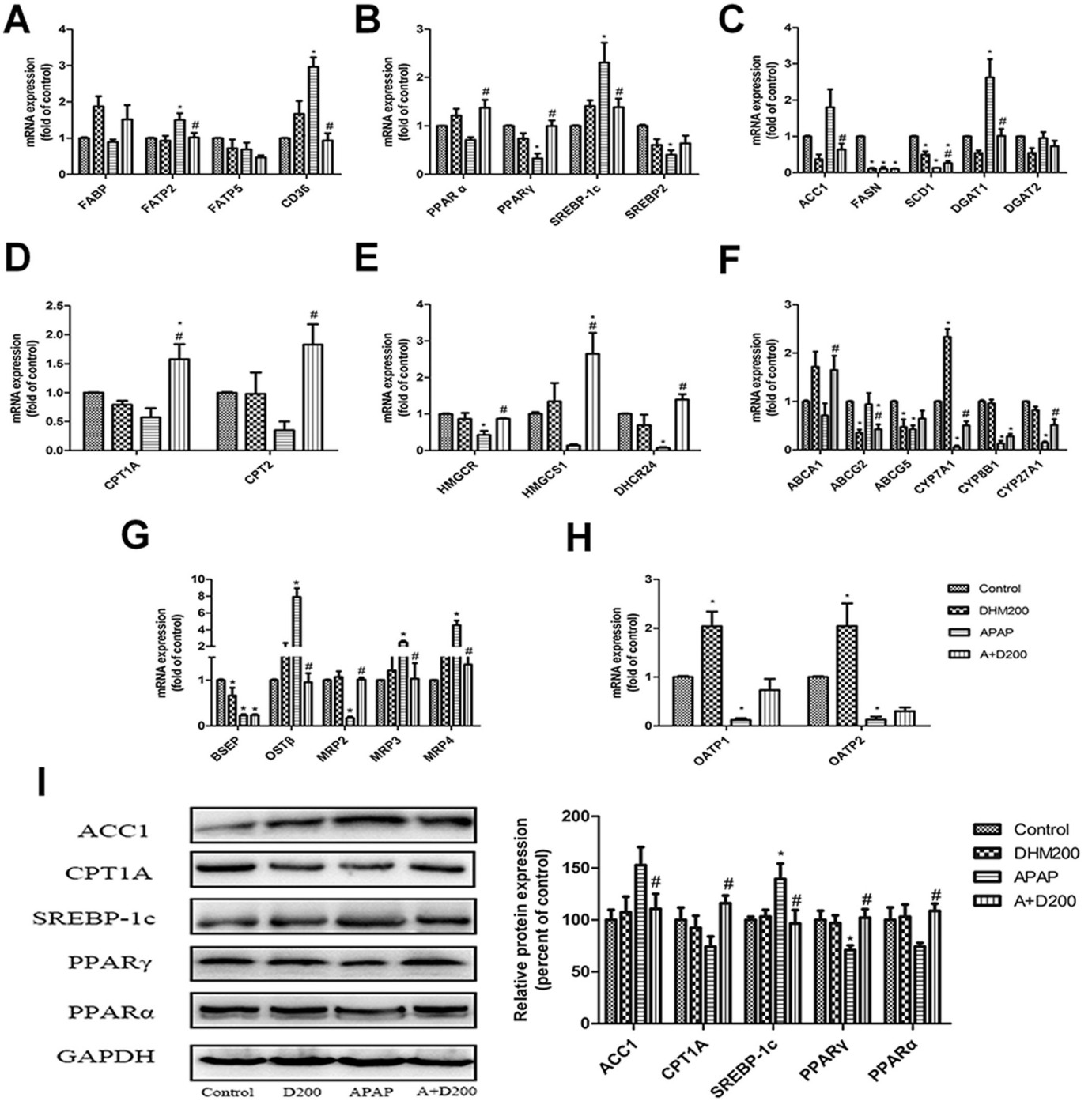

Fig. 3. DHM regulates APAP-induced lipid dysregulation by improving the expression profiles of genes and proteins involved in lipid homeostasis. (A) Fatty acid uptake mediators. (B) Transcription factors. (C) TG synthesis mediators. (D) Fatty acid oxidation enzymes. (E) Cholesterol synthesis mediators. (F) Cholesterol metabolism mediators. (G) Bile acid efflux transporters. (H) Bile acid uptake transporters. (I) Western blot of important lipid-related proteins. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the APAP group. The data are expressed as the mean ± S.E.M., n = 5.

this increase was attenuated by DHM pretreatment before APAP administration (Fig. 4C). DHM (200 mg·kg^{-1}) pretreatment increased the expression of the cell regulatory proteins CCND1 and CDK4 (Fig. 4C). In addition, the immunohistochemical results showed that PCNA expression increased in the DHM and APAP + D200 groups (Fig. 4B). These results suggested that DHM pretreatment could efficiently reduce APAP-induced liver injury by preventing cell cycle arrest and cell death.

4. Discussion

APAP overdose has long been a major cause of drug-induced liver injury, and its related adverse events are still a global issue because of the limited therapeutic approaches [7]. The mouse model of APAP-induced liver injury, which is considered a commonly used animal model, has often been used to research the molecular mechanisms of APAP-induced liver injury [23]. Recently, considerable effort has been directed at developing therapeutically effective agents from herbal and natural products [24]. Schisandrol B, celastrol, and betaine have been

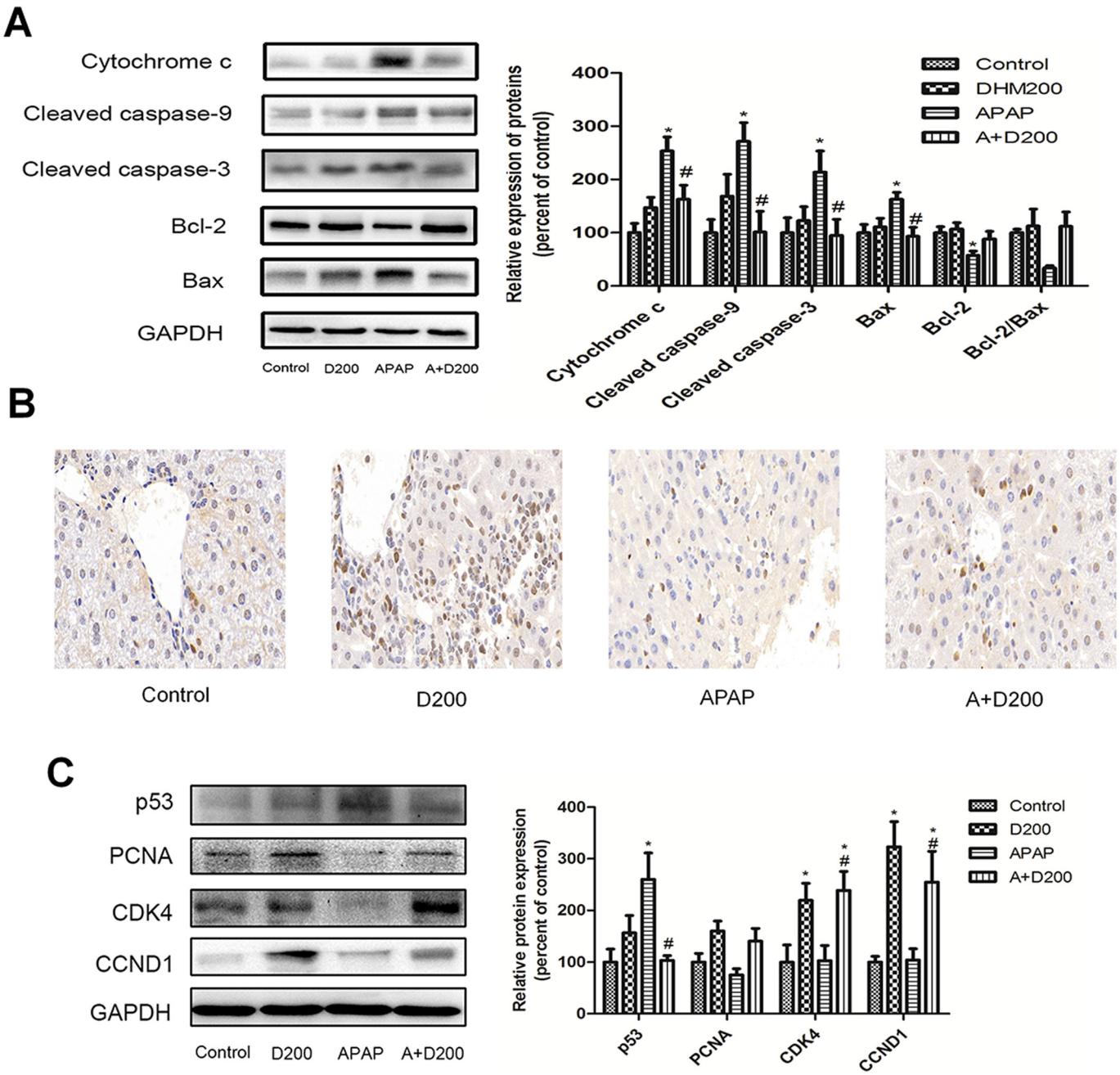

Fig. 4. DHM attenuates APAP-induced liver damage through preventing hepatocyte death and activating regeneration signals. (A) Western blot of hepatocyte death-related proteins in mice (n = 6). (B) PCNA immunohistochemistry at 400× magnification (n = 3). (C) Western blot of hepatocyte regeneration-related proteins (n = 6). The data are expressed as the mean ± S.E.M. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the APAP group.

indicated to be potential alternative agents for the treatment of APAP hepatotoxicity [14,25,26]. In previous studies, we demonstrated that *Hovenia dulcis* Thunb. extract ameliorates APAP-induced liver damage [13]. DHM is a major component of *Hovenia dulcis* Thunb. extract. However, whether DHM exerts protective effects against APAP hepatotoxicity remains unclear. Therefore, this research was carried out to explore whether DHM ameliorates APAP-induced liver damage and to investigate the possible underlying molecular mechanism. The histopathological analysis and the biochemical assessment of indexes such as ALT, AST and GSH indicated that DHM exerts a protective effect against APAP-induced liver injury.

APAP bioactivation in vivo directly contributes to severe liver toxicity [14,27]. Thus, studying the role of DHM in the biological activation of APAP is essential. The changes in the serum levels of APAP and its related metabolites demonstrated that DHM influenced the biotransformation of APAP in vivo. UGT1A1, a crucial enzyme in APAP glucuronidation, is responsible for the metabolism of APAP to APAP-GLU. Current reports show that keampferol pretreatment increases UGT1A1 expression and enhances APAP glucuronidation to attenuate APAP-induced liver injury [5,28]. In this study, the decrease in APAP-GLU may be related to the accelerated metabolism of APAP after DHM pretreatment, which is consistent with the decrease in serum APAP (Fig. 2A). More importantly, CYP enzyme-mediated metabolism of APAP is the initial step in the development of APAP liver toxicity. CYP1A2, CYP2E1 and CYP3A11 have been proven to be crucial for the generation of NAPQI, the toxic metabolite of APAP in vivo [14]. Many studies have noted that chemical agents affect APAP hepatotoxicity by influencing its bioactivation via the inhibition of CYP450 enzyme

expression [14,29]. GST-mediated GSH conjugation quickly results in the conversion of NAPQI to APAP-GSH and APAP-CYS, which inhibits GSH consumption and APAP toxicity [21]. Collectively, our results suggested that the hepatoprotective effects of DHM against APAP toxicity might be achieved by the inhibition of CYP2E1 activity and the stimulation of UGT1A1 activity, a finding consistent with the reduced depletion of GSH (Fig. 2D). This process eventually relieves the oxidative stress caused by excessive APAP ingestion.

APAP treatment disrupts lipid homeostasis in vivo [30,31], and DHM has been reported to improve lipid metabolism in nonalcoholic fatty liver disease [32]. However, the effect of DHM on the lipid dysregulation caused by APAP is still unknown. In this study, the TG, T-CHO, HDL-C and TBA levels in mice increased appreciably after treatment with 300 $mg \cdot kg^{-1}$ APAP (Fig. 1B). According to the analysis of the expression of numerous lipid-associated genes, the hepatoprotective effect of DHM is closely related to the regulation of lipid homeostasis, involving fatty acid absorption, TG and T-CHO synthesis, metabolism and bile acid transport (Fig. 3A–H). PPARα and PPARγ, pivotal nuclear hormone receptors, regulate the transcription of several genes associated with lipid metabolism. Many drugs have been reported to exert hepatoprotective effects by interacting with PPAR receptors [33,34]. PPARα activates its downstream target genes related to fatty acid oxidation and promotes fatty acid β-oxidation [35]. SREBP-1c, a key transcriptional regulator, can control the lipogenic enzyme ACC1 [36]. The PPARα and PPARγ upregulation and SREBP-1c downregulation observed in our experiments clearly showed that DHM exhibits a multitarget reversal effect against APAP-induced liver injury. DHM decreased ACC1 and increased CPT1A expression to correct the imbalance between lipid synthesis (lipogenesis) and decomposition (lipolysis or β-oxidation), eventually regulated lipid homeostasis (Fig. 5). The present work showed that the protective effect of DHM against APAP liver damage was achieved through the modulation of PPAR and SREBP-1c signalling. Prior to this report, drugs have rarely been reported to ameliorate the hepatotoxicity of APAP via the SREBP-1c signalling pathway. Thus, our findings may provide a new therapeutic direction for APAP hepatotoxicity. In addition, the hepatic levels of MRP3 and MRP4 mRNA dramatically increased after APAP treatment, consistent with the findings in previous studies [37]. The hepatoprotective effect of DHM may thus be related to the regulation of MRP-related genes.

Necrosis is considered as the main mode of cell death induced by APAP overdose [38,39], which also be shown in the study (Fig. 1C). On the basis of these studies, we analysed relevant proteins involved in hepatocyte death and found that DHM pretreatment before APAP administration reduced the expression of Bax, Cytochrome c but increased the expression of Bcl-2. Furthermore, DHM pretreatment partially maintained the balance between Bax and Bcl-2. Western blot results suggested the cleavage of caspases 3 and 9, which may be associated with apoptosis (Fig. 4A). However, in view of the apoptosis results (Fig. 1C) and the severe liver injury of APAP (Fig. 1B and C), the APAP-induced cell death pattern is still mainly necrosis. Accurate conclusions require more experimental proof. Liver regeneration, an important recovery process, is a potential clinical strategy for APAP-induced liver damage [14]. Indeed, many genes and signalling pathways have been demonstrated to trigger and facilitate liver regrowth [15,40]. The tumour suppressor p53 plays key roles in cell proliferation and death [41]. In particular, p53 is activated to regulate the initiation of liver regrowth via proliferative signalling after APAP overdose [42]. A recent study investigated the expression levels of p53 and its downstream targets CDK4, CCND1 and PCNA. As shown in Fig. 4C, the protein level of p53 in the APAP group was significantly increased, but this increase was reversed by DHM pretreatment. In addition, the expression of CCND1 and CDK4, which are involved in cell cycle activation, was

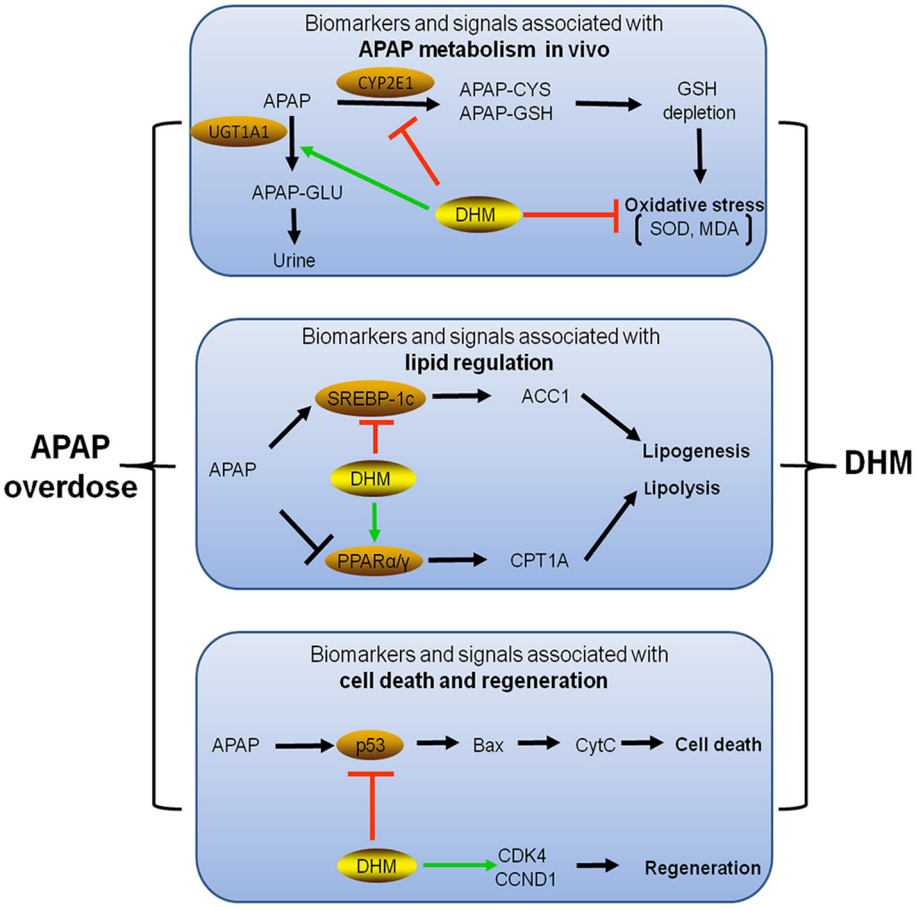

Fig. 5. Proposed mechanism underlying the hepatoprotective effect of DHM against APAP-induced liver injury. DHM alleviated APAP-induced liver injury in mice by inhibiting hepatocyte death, promoting regeneration, regulating the lipid homeostasis imbalance mediated by of PPARs and SREBP-1c and regulating APAP transformation.

significantly elevated in the DHM200 and APAP + D200 groups. PCNA expression also increased. Taken together, our present data revealed that the hepatoprotective effect of DHM was achieved via the inhibition of hepatocyte death and the activation of p53-associated regeneration signals.

5. Conclusion

In conclusion, this research demonstrates for the first time that DHM protects against APAP-induced liver damage. Through regulating APAP biotransformation and p53-related hepatocyte death and regeneration, as well as rectifying lipid dysregulation, DHM exerts a satisfactory protective effect against the hepatotoxicity induced by APAP by affecting many targets and networks (Fig. 5). The multi-pathway and multi-target characteristics of this protective effect provide wider application possibilities for DHM. Given the noticeable hepatoprotective effect of DHM, the regulation of lipid homeostasis via SREBP-1c signalling might be a new potential therapeutic target for APAP hepatotoxicity. Future work will focus on identifying potential targets via network pharmacology and other approaches.

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Conflicts of interest

All the authors confirm that there are no conflicts of interest related to this article.

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Author contributions

Haina Wang designed and revised the manuscript. Sijing Dong performed the experiments and wrote the manuscript. Jianbo Ji and Lingyun Hu participated in the animal experiments.

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