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Genipin protects against acute liver injury by abrogating ferroptosis via modification of GPX4 and ALOX15-launched lipid peroxidation in mice

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Abstract

It is essential to further characterize liver injury aimed at developing novel therapeutic approaches. This study investigated the mechanistic basis of genipin against carbon tetrachloride (CCl_4)-triggered acute liver injury concerning ferroptosis, a novel discovered modality of regulated cell death. All experiments were performed using hepatotoxic models upon CCl_4 exposure in mice and human hepatocytes in vitro. Immunohistochemistry, immunoblotting, molecular docking, RNA-sequencing and ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) were conducted. CCl_4 intoxication was manifested with lipid peroxidation-dictated ferroptotic cell death, together with changes in a cascade of ferroptosis-associated events and several regulatory pathways. Both the administration of genipin and ferrostatin-1 (Fer-1) significantly prevented this hepatotoxicity in response to CCl_4 intoxication via upregulating GPX4 and xCT (i.e., critical regulators of ferroptosis). RNA-sequencing unraveled that arachidonic acid metabolism was considerably influenced upon genipin treatment. Accordingly, genipin treatment attenuated arachidonate 15-lipoxygenase (ALOX15)-launched lipid peroxidation in terms of UHPLC-MS/MS analysis and inflammation. In vitro, genipin supplementation rescued erastin-induced hepatocellular inviability and lipid ROS accumulation. The siRNA knockdown of GPX4 partially abrogated the protective effects of genipin on erastin-induced cytotoxicity, whereas the cytotoxicity was less severe in the presence of diminished ALOX15 expression in L-O2 cells. In conclusion, our findings uncovered that genipin treatment protects against CCl_4-triggered acute liver injury by abrogating hepatocyte ferroptosis, wherein the pharmacological modification of dysregulated GPX4 and ALOX15-launched lipid peroxidation was responsible for underlying medicinal effects as molecular basis.

Keywords Genipin · Ferroptosis · Liver injury · GPX4 · Arachidonate 15-lipoxygenase

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Introduction

It has been documented a considerable mortality rate (around 10%) of hospitalized patients due to drug-induced liver injury [1]. Furthermore, acute liver injury partially accounts for the advent, development and progression of various liver diseases which can result in terminal organ failure. Frequent and recurrent liver injury instigates fibrosis, cirrhosis and sometimes the aggressive phenotype as hepatocellular carcinoma. The main reasons of liver injury comprise hepatotoxicant, drug overdose, alcoholism, inappropriate self-medication as well as viral hepatitis infection [2]. Taking into consideration the heavy burden on health systems and public concern pertinent to hepatotoxicant/drug intoxication, increasing attention and many efforts have been dedicated to elucidate the molecular basis of acute liver injury, if available, to develop potential therapeutic approaches.

As for carbon tetrachloride (CCl_4), this hepatotoxic substance has been widely used to establish well-known experimental model regarding acute liver injury, indicative of necrotic alterations involving different zones in the hepatic lobes [3]. Of note, toxicants stemming from CCl_4 metabolite exhibit high affinity to lipids, and in turn remove the $H^{\bullet}$ from the membranous unsaturated fatty acids which are responsible for the subsequent chain process of lipid peroxidation to elicit hepatocellular damages. This toxicant injury model is suited to assess the underlying mechanism in relation to hepatic damages and to identify potential hepatoprotective/anti-hepatotoxic effects of bio-synthetic agents, as well as ingredients derived from natural products [4]. Moreover, CCl_4-triggered liver injury is characterized by similar morphological and biochemical shifts coincided with human liver disorders [5]. Since CCl_4 intoxication to liver accounts for multiple detrimental biological processes, such as oxidative stress, endoplasmic reticulum stress, apoptosis, autophagy and ferroptosis, emerging evidence implicates the potentials to counteract these CCl_4-triggered deleterious effects by identifying therapeutic interventions on the basis of natural/herbal sources [6].

Ferroptosis, as an iron catalysis-mediated novel form of regulated cell death, is appreciated as excessive production of lipid peroxides. Recent researches demonstrate that a wide spectrum of polyunsaturated fatty acids (PUFAs), including arachidonic acid (AA), may participate in the ferroptotic pathways [7]. The peroxidation of lipids encompassing PUFA chains leads to further accumulation of lethal lipid reactive oxygen species (ROS) [8]. Accordingly, lipid peroxidation accounts for the final executor of ferroptosis, which is facilitated by arachidonate lipoxygenase (ALOX)-mediated oxidative response in an enzymatically reactive fashion [9]. Conversely, intracellular glutathione peroxidase 4 (GPX4) is capable of preventing fatal chain reaction through converting resultant 15-hydroperoxyeicosatetraenoyl acid (15-HpETE) to reduced 15-hydroxyeicosatetraenoyl acid (15-HETE) [10]. Until now, a growing body of literature has explored the role of ferroptosis in the context of various liver diseases [11–13]. However, there is scant data concerning the impact of ALOX-launched lipid peroxidation and the combination of divergent ferroptotic pathways in CCl_4-triggered acute liver injury [1, 14, 15].

Despite a variety of bio-synthetic medications have been used to treat liver injury/hepatic dysfunction, their usage are restrained due to limited therapeutic effects, intolerance to prescription and unexpected side effects such as renal and cardiac toxicity in clinical settings. Therefore, aforesaid drawbacks dramatically arouse scientific endeavor to identify hepatoprotective alternatives among the traditional Chinese medicine (TCM). Actually, TCM has been long and broadly used in Oriental countries as efficious complementary therapeutics [16]. Genipin (Fig. 1A) is the aglycone derived from geniposide, the most abundant iridoid glucoside constituent of *Gardenia jasminoides Ellis*, getting conversion through intestinal bacteria-produced enzyme β-D-glycosidase [17]. Thereafter, genipin is absorbed via the intestine and transported to liver across the portal vein. Our previous report implicated that genipin protects against CCl_4-triggered hepatotoxicity, which is linked to the activation of autophagy [18].

Taking into consideration the concept that ferroptosis can determine cell fate in addition to the contribution of hepatocellular iron storage, the current study elaborated on the role of GPX4 pathway and ALOX15-launched lipid peroxidation for acute liver injury in the context of ferroptosis. Herein, our findings unveiled that a cascade of ferroptosis-associated events, including mitochondrial morphology alterations, accumulation of lipid ROS, dysregulation of key regulator GPX4 and accumulation of lipid peroxidation mediated by ALOX15, is observed in mice under CCl_4 exposure. Further, we sought to clarify the medicinal effects of genipin against CCl_4 intoxication. To validate genipin as a potential therapeutic agent, we utilized RNA-sequencing, molecular docking and ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) methods; further analyses showed that the administration of genipin and ferrostatin-1 (Fer-1) effectively mitigates toxicant-induced ferroptosis via upregulating GPX4 and suppressing ALOX15-launched lipid peroxidation. Finally, the shifts in the targeted ferroptotic pathways upon erastin (a pharmacological reagent to induce ferroptosis) exposure were reversed with genipin treatment in vitro, which is then confirmed using transfection knockdown of GPX4 or ALOX15 [19].

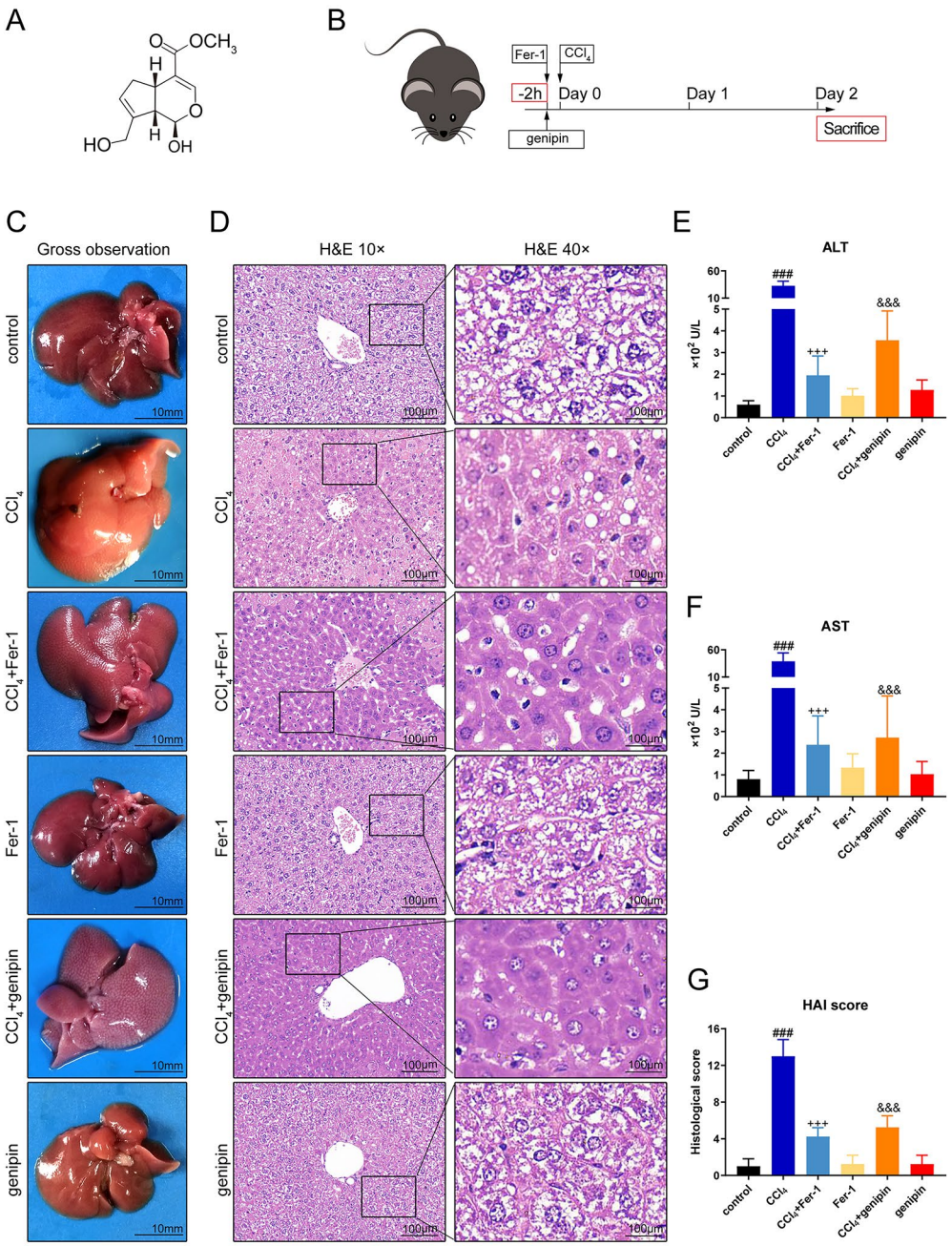

Fig. 1 Genipin protects against acute liver injury subjected to CCl_4 intoxication in vivo. **(A)** The chemical structure of genipin. **(B)** Schematic illustration of the experimental protocols in detail. **(C, D)** Alterations of macroscopic (scale bar: 10 mm) and microscopic (scale bar: 100 μm) appearance in terms of H&E staining. **(E, F)** Measurement of serum ALT/AST levels. **(G)** Evaluation of histopathological changes in terms of HAI score. ###$p < 0.001$ vs. control group, +++$p < 0.001$ vs. CCl_4 group, &&&$p < 0.001$ vs. CCl_4 group

Materials and methods

We showed additionally available materials and experimental protocols in detail referring to the supplementary files (Supplementary File).

Animal toxicant injury models, treatment and experimental design

All animal studies were approved by the Institutional Animal Care and Use Committee at Tianjin Medical University General Hospital (IRB2021-DWFL-142). Mice were purchased from the National Institutes for Food and Drug

Control (Beijing, China). The mice were allowed for one week of acclimation period to minimize environmental differences. The mice were housed in a pathogen-free room maintained under specific conditions: a temperature of 23 ± 2 °C and relative humidity of 50 ± 10% with a 12 h light-dark cycle, together with water and food *ad libitum*. Male C57BL/6 mice aged 6–8 weeks and weighed 20–22 g, unless otherwise indicated, were employed. As for the acute liver injury model, the mice were subjected to a dose of 2 ml/kg combining CCl_4 (50%) and olive oil (50%) via an intraperitoneal (*i.p.*) injection. The control group was indicative of an *i.p.* injection for the same value of olive oil as the CCl_4 group. The liver and blood samples were collected 48 h afterward. As for the pharmacological modification, an intravenous injection of genipin or saline (vehicle) was performed via the tail vein 2 h prior to CCl_4 exposure. In this experiment, we chose a 48 h time point and an optimally effective dose of 2.5 mg/kg genipin for the whole protocols coincided with previous reports (Fig. 1B) [18, 20]. Fer-1 was dissolved in saline (1 mg/kg) along with an *i.p.* injection to mice for once 1 h prior to CCl_4 challenge aimed at elaborating on the medicinal effects of genipin on CCl_4-triggerd liver injury in the context of ferroptosis. The mice were randomly divided into six groups (per each group 3 ~ 6 mice): (1) vehicle-treated normal control (control); (2) vehicle-treated CCl_4 challenge (CCl_4); (3) 1 mg/kg Fer-1-treated CCl_4 challenge (CCl_4 + Fer-1); (4) 1 mg/kg Fer-1-treated (Fer-1); (5) 2.5 mg/kg genipin-treated CCl_4 challenge (CCl_4 + genipin); (6) 2.5 mg/kg genipin-treated (genipin).

Cell culture and siRNA knockdown experiment

Normal human hepatocyte L-O2, purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), were incubated in Dulbecco's Modified Eagle Medium encompassing fetal bovine serum (10%), penicillin (100 IU/ml) and streptomycin (100 mg/ml) in 37 °C with 5% CO_2. In this experiment, L-O2 hepatocytes were transfected with non-targeting control siRNA (NC siRNA, 80 pmol/ml), siRNA directed against ALOX15 (80 pmol/ml) or GPX4 (80 pmol/ml) for 48 h by employing Lipofectamine 3000 in terms of the manufacturer's instruction.

Statistical analysis

All data were depicted in the manner regarding mean ± standard deviation (SD). The overall significance of data was compared in terms of one-way analysis of variance, and statistical differences among several groups were considered significance at $p < 0.05$ with the Bonferroni correction in the case of multiple comparisons. We used GraphPad Prism 8.0.1 (Graph Pad Software, Inc. San Diego, CA, U.S.) to analyze data in the current study.

Results

The execution of ferroptosis in response to CCl_4-triggered acute liver injury

First, we sought to clarify the role of ferroptosis in the context of CCl_4-triggerd acute liver injury. In alignment with our previous report, the 48 h time point was of choice coincided with the most pronounced histological damages in the mice liver [18]. Regarding the macroscopic appearance and H&E staining findings, the destruction and hepatic damages due to CCl_4 exposure were consistently alleviated upon Fer-1 treatment, designated as a specific ferroptosis inhibitor by capturing/eliminating lipophilic radicals (Fig. 1C, D). Simultaneously, the aggravation concerning histopathological scores in the injurious mice liver was significantly mitigated by Fer-1 supplementation (Fig. 1G). Moreover, we found similar medicinal effects regarding genipin treatment, mirrored as diminishment of hepatocellular necrosis, hepatic architecture loss as well as inflammatory cell infiltration. Accordingly, our findings unraveled that Fer-1 supplementation considerably reverses increases in ALT/AST concentration in response to CCl_4 challenge as compared with the control group (Fig. 1E, F).

As for TEM analysis, we showed that the mitochondria are smaller in size alongside reduced cristae; the outer membrane is torn in mice liver due to CCl_4 exposure (Fig. 2A). In addition, our findings uncovered that, via TUNEL assay, both administration of Fer-1 and genipin remarkably repress hepatocyte death (TUNEL (+) cells) in the injurious mice livers (Fig. 2B, C). In line with therapeutic potentials against liver damages, genipin and Fer-1 treatment dramatically restored CCl_4-triggered mitochondrial destruction relative to untreated samples. Altogether, these data strongly supported the hypothesis that execution of ferroptosis is responsible for CCl_4-triggered acute liver injury.

Genipin represses CCl_4-triggered ferroptotic process in vivo

As for typically biochemical features in the context of ferroptosis, for instance, Fe^{2+} accumulation, extensive ROS generation and resulting lipid peroxides, we demonstrated that genipin effectively represses these detrimental activities. The expression levels of 4-HNE and MDA, suggestive of toxicant-induced lipid peroxidation serving as critical executors for ferroptosis, were significantly diminished in response to the genipin treatment (Fig. 3A, D). Using

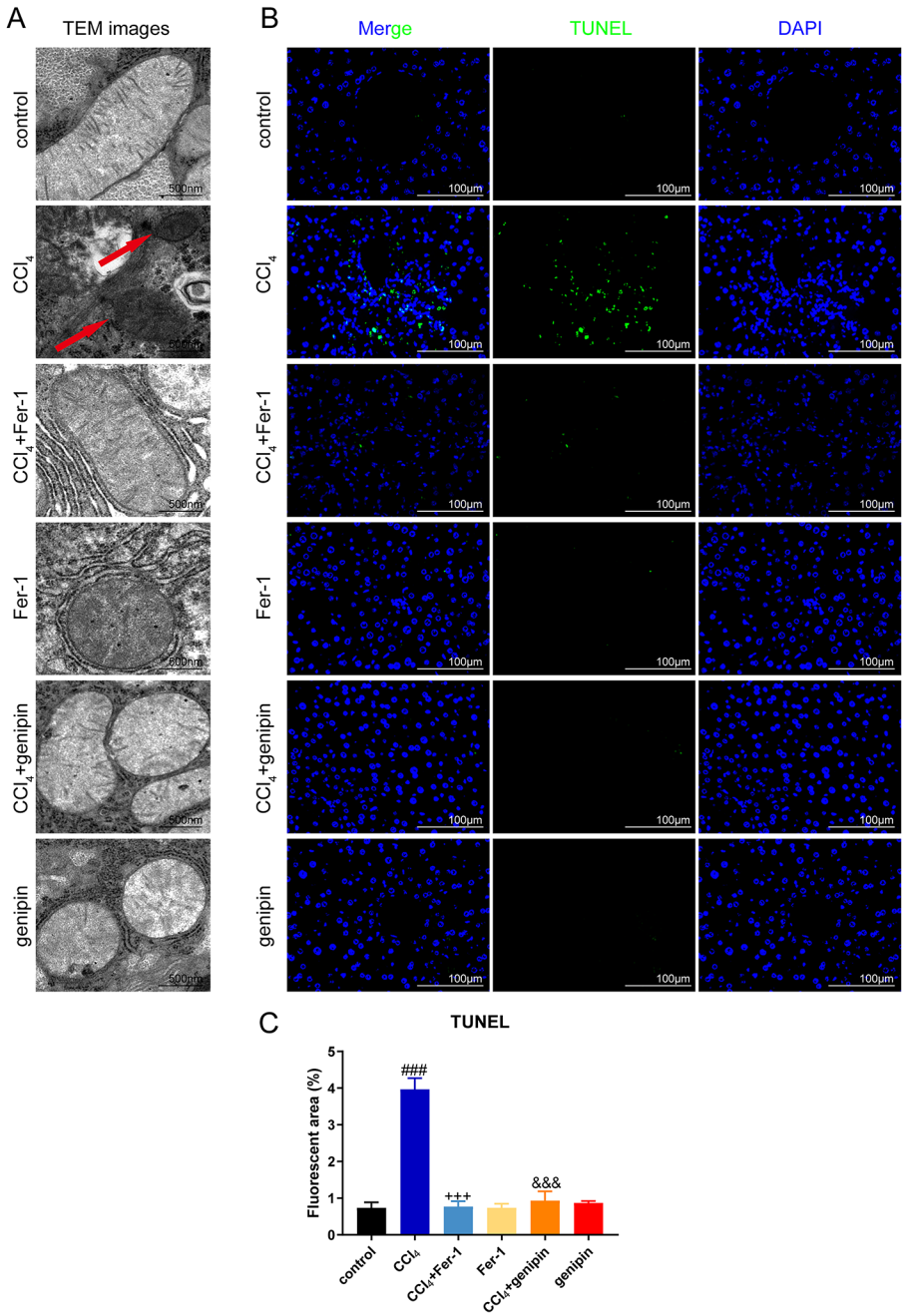

Fig. 2 Genipin represses hepatocyte death in vivo. **(A)** Analysis of TEM for mitochondrial morphology of ferroptotic cells in the liver tissue (red arrow; scale bar: 500 nm). **(B)** Measurement of dead hepatocytes in terms of TUNEL staining in the mice livers (scale bar: 100 μm). Representative images were present. **(C)** Quantification of TUNEL (+) cells was present. Data were expressed as mean ± SD. ###$p < 0.001$ vs. control group, +++$p < 0.001$ vs. CCl_4 group, &&&$p < 0.001$ vs. CCl_4 group

specific 581/591 C11-BODIPY fluorescent probe, a marked production of lipid ROS was observed in the liver tissue challenged by CCl_4. By contrast, no lipid ROS accumulation was found in the control group. Additionally, both supplementation of genipin and Fer-1 significantly reverse increases of lipid ROS/hepatic ROS levels compared to CCl_4 challenged group (Fig. 3B, C).

Dysregulated iron metabolism contributes to the induction of ferroptosis among a wide spectrum of liver diseases [21–23]. Our findings showed that Fe^{2+} levels increase in the liver of CCl_4-triggered mice, whereas Fer-1 and genipin ameliorate these deleterious iron overload (Fig. 3E). Moreover, a validated biomarker of ferroptotic pathway *Ptgs2* gene expression was diminished upon genipin treatment relative to the injurious liver models (Fig. 3F) [24, 25].

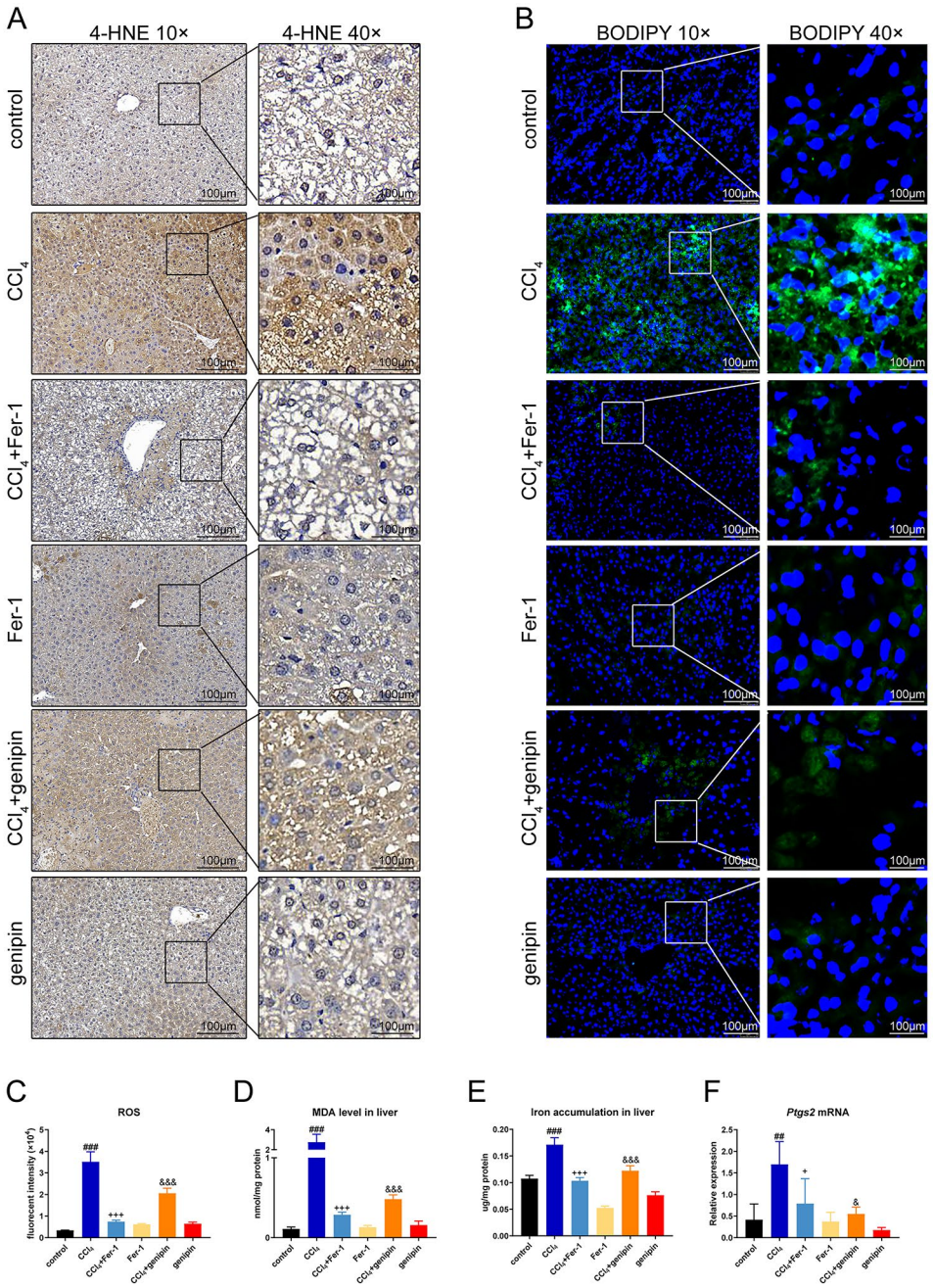

Fig. 3 Genipin represses CCl_4-triggered ferroptotic process in vivo. **(A)** Detection of 4-HNE protein adducts expression in fixed liver tissue sections (scale bar: 100 μm). **(B)** Detection of accumulated lipid ROS in terms of 581/591 C11-BODIPY fluorescent probe (scale bar: 100 μm). **(C)** Measurement of hepatocellular ROS in the liver tissue. **(D)** Assessment of the content of MDA in the liver tissue. **(E)** Evaluation of hepatic Fe^{2+} levels. **(F)** Determination of *Ptgs2* gene expression in terms of real-time PCR analysis. Data were expressed as mean ± SD. ##p < 0.01, ###p < 0.001 vs. control group, +p < 0.05, +++p < 0.001 vs. CCl_4 group, &p < 0.05, &&&p < 0.001 vs. CCl_4 group

Genipin upregulates hepatic expression of GPX4 and xCT in vivo

It has been built that GPX4 and xCT (a multipass transmembrane protein encoded by the *solute carrier family 7 member 11* gene) serve as pivotal regulators of ferroptosis, thus we sought to determine the pertubations in protein expression levels. Our findings revealed that CCl_4 exposure downregulates the protein levels of GPX4 around 26.5% relative to the control group, but genipin rescued these decreases by upregulating around three fold over the injured group (Fig. 4A, B). Furthermore, the expressed xCT levels significantly decreased to 24.3% in CCl_4-intoxicated livers compared to the controls. In the CCl_4 + genipin group, the

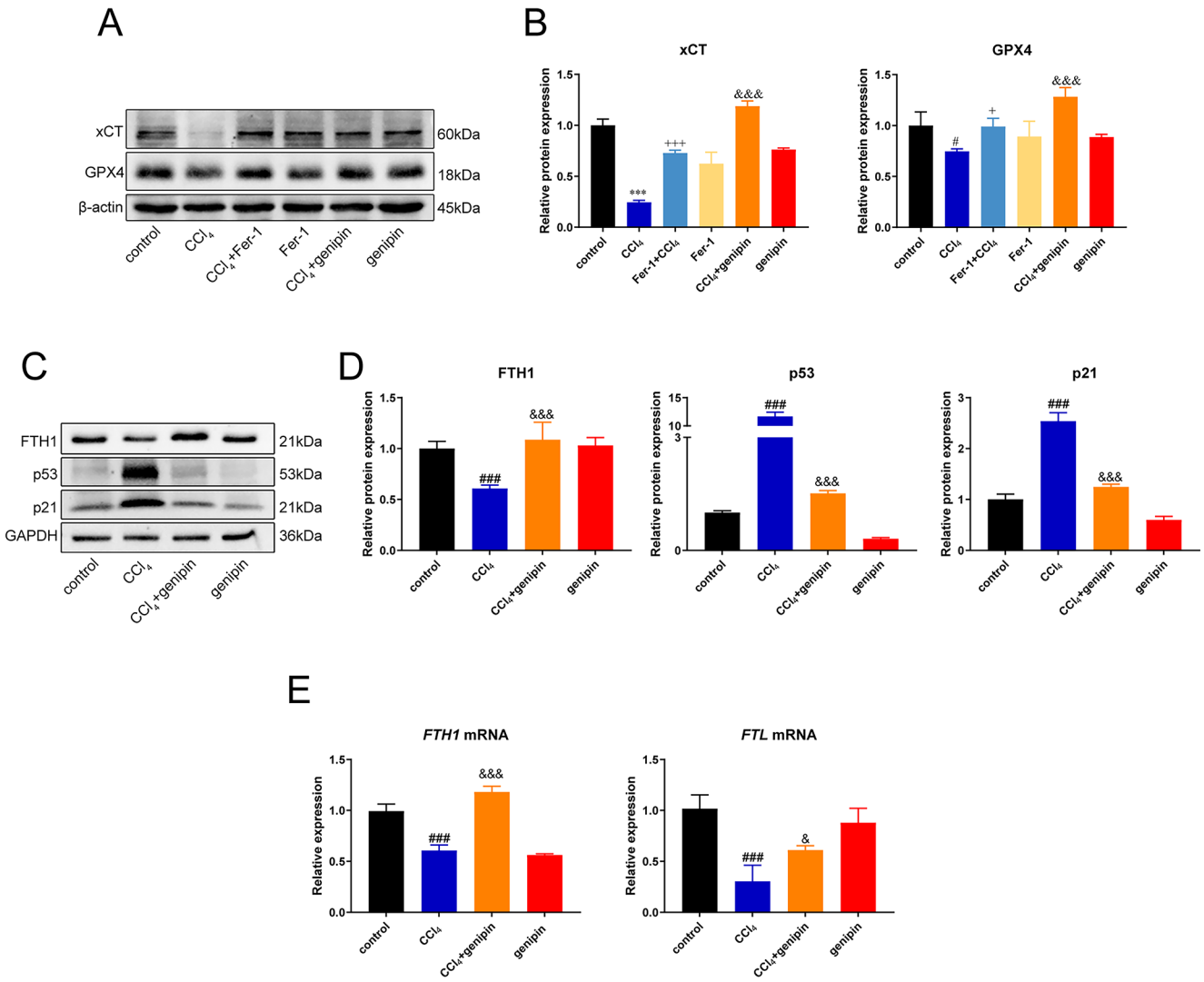

Fig. 4 Genipin regulates hepatic expression of GPX4/xCT and several key mediators in relation to ferroptotic process in vivo. **(A)** Determination of protein levels for xCT and GPX4 in the liver tissue subjects to CCl_4 challenge. **(B)** Quantification of the band intensities for xCT and GPX4 relative to β-actin in terms of image J software. **(C)** Determination of hepatic protein levels for FTH1, p53 and p21 upon genipin treatment in the context of toxicant-induced ferroptosis. **(D)** Quantification of the band intensities for FTH1, p53 and p21 relative to GAPDH. **(E)** Determination of *FTH1* and *FTL* gene expression in terms of real-time PCR analysis. Data were expressed as mean ± SD. **p < 0.01 vs. control group, ***p < 0.001 vs. control group, +++p < 0.001 vs. CCl_4 group, &&p < 0.01 vs. CCl_4 group, &&&p < 0.001 vs. CCl_4 group

expressed GPX4 levels increased closely to two fold relative to CCl_4 exposure group. Of note, Fer-1 also restored the decreases of GPX4/xCT due to CCl_4 intoxication. Taken together, these data argued a critical role of hepatocyte ferroptosis in the context of CCl_4-triggered acute liver injury and the therapeutic potentials to suppress ferroptosis with genipin in vivo.

Genipin affects several key mediators in relation to ferroptotic process

Accumulating evidence suggests that multiple mediators responsible for the ferroptotic process concerning distinct pillars. For instance, the role of p53-p21 pathway in the context of ferroptosis appears to be complicated. It is suggested that p53-KR abrogates cystine import and in consequence leads to ferroptotic cell death, whereas p53-p21 activation may also potentiate glutathione (GSH) recycle along with decrease in cellular GSH export/consumption [26, 27]. The ferritin heavy chain 1 (FTH1) is associated with sensitivity to ferroptosis, indicative of silencing FTH1 towards aggravated erastin-induced ferroptosis [28]. As shown in Fig. 4C, D, the positive ferroptosis mediators p53-p21 were significantly upregulated in CCl_4-intoxicated livers, whereas the expression of FTH1 proteins decreased in the CCl_4 exposure group relative to the control group. The gene expression of

hepatic *FTH1* and ferritin light chain (*FTL*) also decreased in the CCl_4 exposure group (Fig. 4E). Particularly, genipin treatment effectively restored these alterations. Thereafter, we sought to measure inflammation and lipid peroxidation in the context of ferroptosis in vivo. As for F4/80 staining positive macrophages in response to CCl_4 exposure, the increases were mitigated by supplementation with genipin and Fer-1 (Figure S1A, B). Hepatic *p21* and *ALOX15* gene expressions were upregulated in CCl_4-triggered mice, whereas administration of genipin and Fer-1 inhibited these increases (Figure S1C, D). Given CCl_4 can result in liver damages through extensive inflammatory response and a potential link between necroinflammation and ferroptosis, we detected the serum levels of typical cytokines. As shown in Table S1, CCl_4 significantly stimulated the production of IL-1β, IL-6 and TNF-α in the circulation relative to the control group, whereas both genipin and Fer-1 supplementation suppressed these proinflammatory cytokines.

Genipin inhibits erastin-induced hepatocyte ferroptosis in vitro

In an attempt to elucidate the role and molecular basis pertaining to ferroptosis in the context of liver injury in vitro, we applied several pharmacological inhibitors including Fer-1, Z-VAD-FMK and Nec-1 to identify subroutines of regulated cell death upon erastin treatment. Our findings implicated that both genipin and Fer-1 ameliorate erastin-induced hepatocyte death to some extent (Fig. 5A). In contrast, neither Z-VAD-FMK nor Nec-1 rescued cell inviability. The accumulation of LDH and MDA was also mitigated in response to genipin and Fer-1 treatment compared with the erastin group (Fig. 5B, C). As for cellular ROS, the DCFH-DA probe demonstrated that genipin remarkably reduces the severity of lipid peroxidation elicited by erastin in L-O2 cells (Fig. 5D). As for altered morphology via TEM evaluation, genipin restored mitochondrial shrinkage induced by erastin (Fig. 5E). As for iron homeostasis, the excessive cellular and mitochondrial Fe^{2+} accumulation due to erastin challenge were observed in terms of higher FerroOrange and Mito-FerroGreen signals relative to the controls, but these changes were significantly attenuated upon genipin and Fer-1 administration (Fig. 5F, G).

Modifying GPX4 partially accounts for hepatoprotective effects of genipin

Given the essential role of GPX4 to counteract the generation of lipid ROS in the presence of Fe^{2+} (catalytically active iron), a molecular docking analysis of GPX4 with genipin was implemented (Fig. 6A). Using this approach, genipin was considered to embrace relatively high binding affinity with amino acid residue Val-125 of GPX4 to orchestrate an intermolecular hydrogen bond (XP docking score: -3.422).

Next, the medicinal effects of genipin were validated by siRNA targeting GPX4. As shown in Fig. 6B, we selected si-GPX4#2 with high transfection efficiency for all further experiments. Accordingly, GPX4 knockdown remarkably aggravated erastin-induced cytotoxicity, and genipin administration failed to rescue cell cytotoxicity/damages in this circumstance (Fig. 6C, D). As for DCFH-DA and TEM analyses, these findings also supported the pivotal role of GPX4 contributing to the therapeutic potential of genipin (Fig. 6E-H). Collectively, all in vitro data verified that genipin has hepatoprotective effects by modifying GPX4 pathway to counteract toxicant-induced ferroptosis.

Inhibiting ALOX15-launched lipid peroxidation partially accounts for hepatoprotective effects of genipin

To further elaborate on the mechanistic basis by which genipin exerted hepatoprotective effects, we analyzed an RNA-sequencing dataset for CCl_4-triggered and genipin treatment mice. The KEGG pathway analysis showed that several biological processes, including fatty acid degradation, biosynthesis of unsaturated fatty acids, fatty acid elongation as well as AA metabolism were enriched within the top 10 pathways (Fig. 7A, B). Actually, those pathways are in close relation to ferroptosis execution, since n-6 PUFAs like AA in phospholipid membranes can react with ROS [29]. Given the significance of lipoxygenase, known as a non-heme, iron-containing enzyme capable of catalyzing the PUFAs deoxygenation, we further detected ALOX15-launched lipid peroxidation. By using UHPLC-MS/MS, we found that several oxylipins associated with ALOX15 catabolism including AA, 15-HpETE and 15-HETE are upregulated in CCl_4-intoxicated livers, and increases in these lipid peroxides were reversed with genipin treatment (Fig. 7C). Furthermore, we conducted in vitro experiments to validate the role of ALOX15 to facilitate ferroptotic process. In terms of ALOX15 protein silencing by siRNA approach (Fig. 7D), we showed that ALOX15 knockdown (using si-ALOX15#2 with high transfection efficiency) significantly suppresses erastin-induced cytotoxicity/damages and partially abolishes the hepatoprotective effects of genipin in L-O2 cells (Fig. 7E).

Discussion

Our findings in the current study implicated that genipin, a natural ingredient extracted from *Gardenia jasminoides Ellis*, exhibited hepatoprotective effects by abrogating

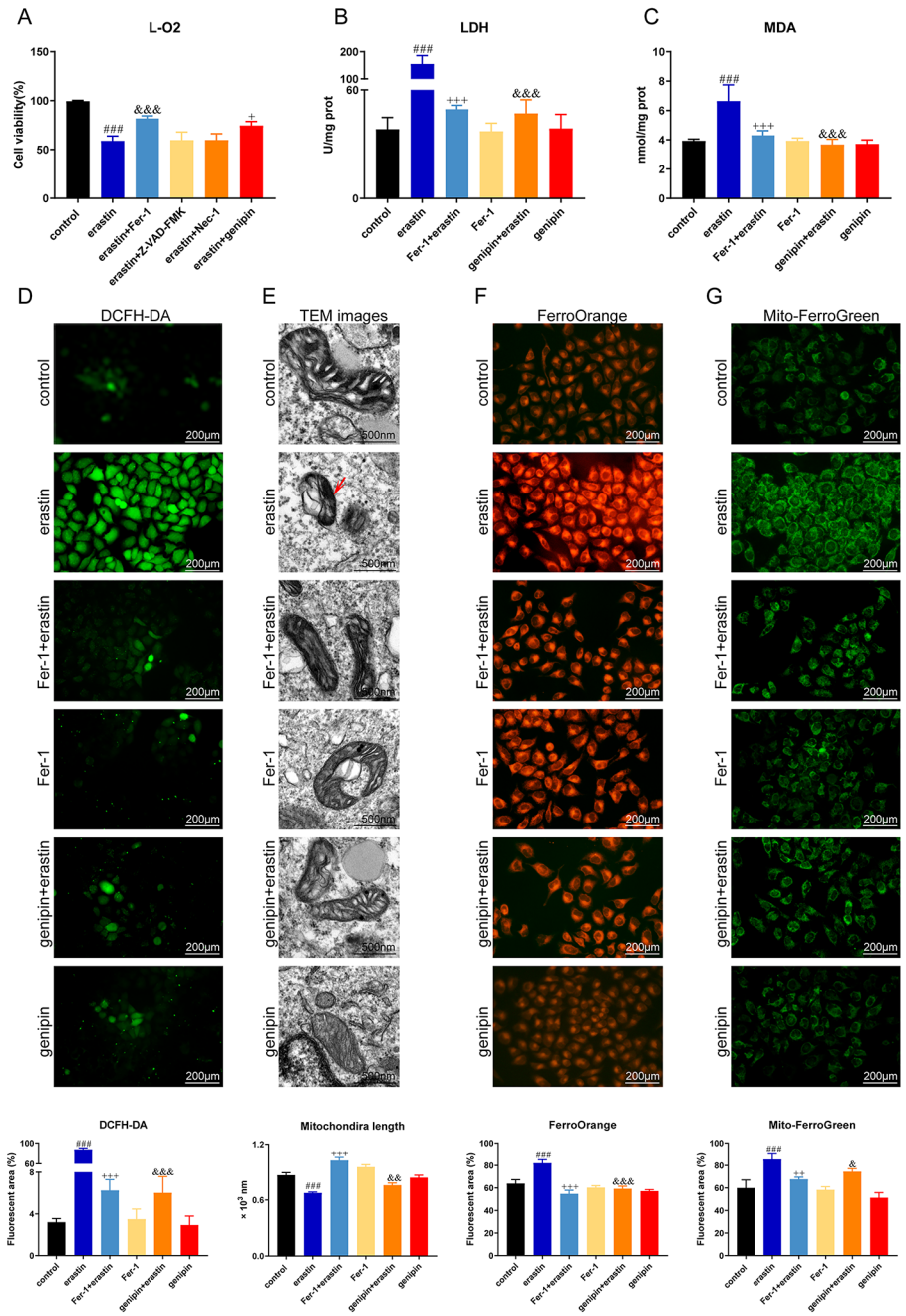

Fig. 5 Genipin inhibits erastin-induced hepatocyte ferroptosis in vitro. **(A)** Assessment of the viability of L-O2 cells challenged with erastin and divergent pharmacological inhibitors including Z-VAD-FMK, Nec-1, Fer-1 and genipin in terms of CCK-8. **(B, C)** Evaluation of the accumulation of LDH and MDA in L-O2 cells. **(D)** Quantification of cellular ROS content in terms of DCFH-DA probe (scale bar: 200 μm). **(E)** Analysis of TEM pertinent to mitochondrial morphology of ferroptotic L-O2 cells (scale bar: 500 nm). **(F, G)** Detection of cellular and mitochondrial Fe^{2+} culmination in terms of FerroOrange probe and Mito-FerroGreen probe of cultured L-O2 cells (scale bar: 200 μm). ###p < 0.001 vs. control group, ++p < 0.01 vs. erastin group, +++p < 0.001 vs. erastin group, &&p < 0.01, &&&p < 0.001 vs. erastin group

ferroptosis in the context of CCl_4-triggerd acute liver injury. Moreover, mechanistic research revealed that a cascade of ferroptosis-associated events is influenced upon genipin administration in the injurious mice livers. Also, in-depth investigation regarding molecular mechanisms indicated that pharmacological modification of dysregulated GPX4 and ALOX15-launched lipid peroxidation accounts for medicinal effects of genipin to be a promising therapeutic approach for specific liver pathology. Previously, we denoted that genipin counteracts hepatotoxicity by

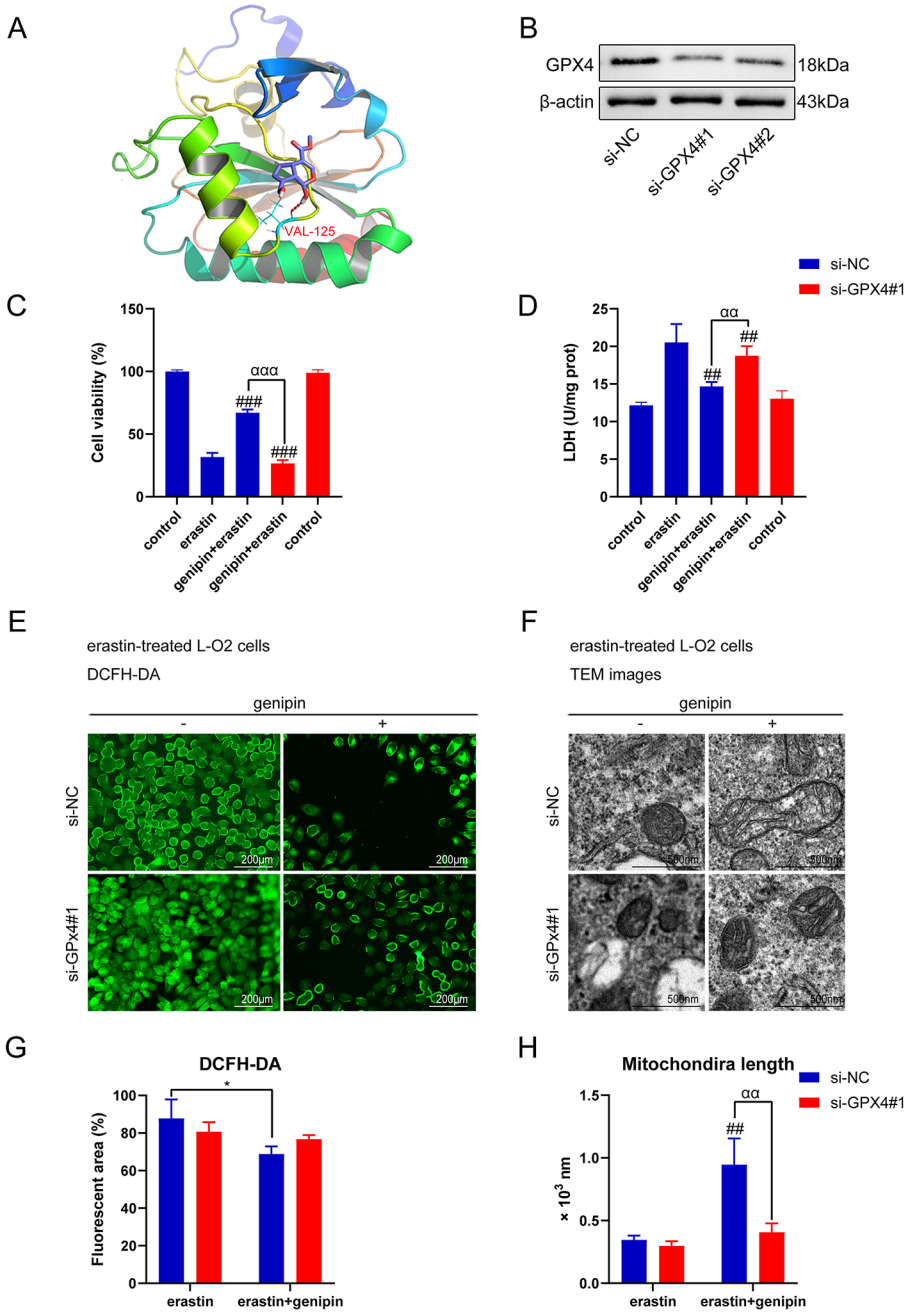

Fig. 6 Genipin exerts hepatoprotective effects by modifying GPX4. **(A)** Molecular docking analysis of GPX4 with genipin. **(B)** Determination of protein levels of GPX4 in L-O2 cells transfected with NC siRNA or GPX4 siRNA. **(C)** Assessment of the viability of L-O2 cells transfected with NC siRNA or GPX4 siRNA. **(D)** Evaluation of the accumulation of LDH in L-O2 cells transfected with NC siRNA or GPX4 siRNA. **(E, G)** Detection of cellular ROS content in L-O2 cells transfected with NC siRNA or GPX4 siRNA in terms of DCFH-DA probe (scale bar: 200 μm). **(F, H)** Detection of TEM pertinent to mitochondrial morphology of ferroptotic L-O2 cells (scale bar: 500 nm). ###p < 0.001 vs. control group, αp < 0.05 vs. GPX4 siRNA group, αααp < 0.001 vs. GPX4 siRNA group

enhancing autophagy among toxicant injury models [18]. In alignment with lately published report, genipin may exhibit its protective effects by modifying divergent modalities in relation to regulated cell death. It is tempting to target ferroptotic cell death in conjunction with impaired autophagic flux to potentiate hepatic damages recovery.

In the current study, we elaborate on the role of GPX4 dysregulation in the context of toxicant-induced ferroptosis. Previously, we argued that GPX4 and xCT serve as essential components to counteract the generation of specific lipid hydroperoxides in the presence of overwhelming iron load [13]. Inactivation of GPX4 or depletion of cellular

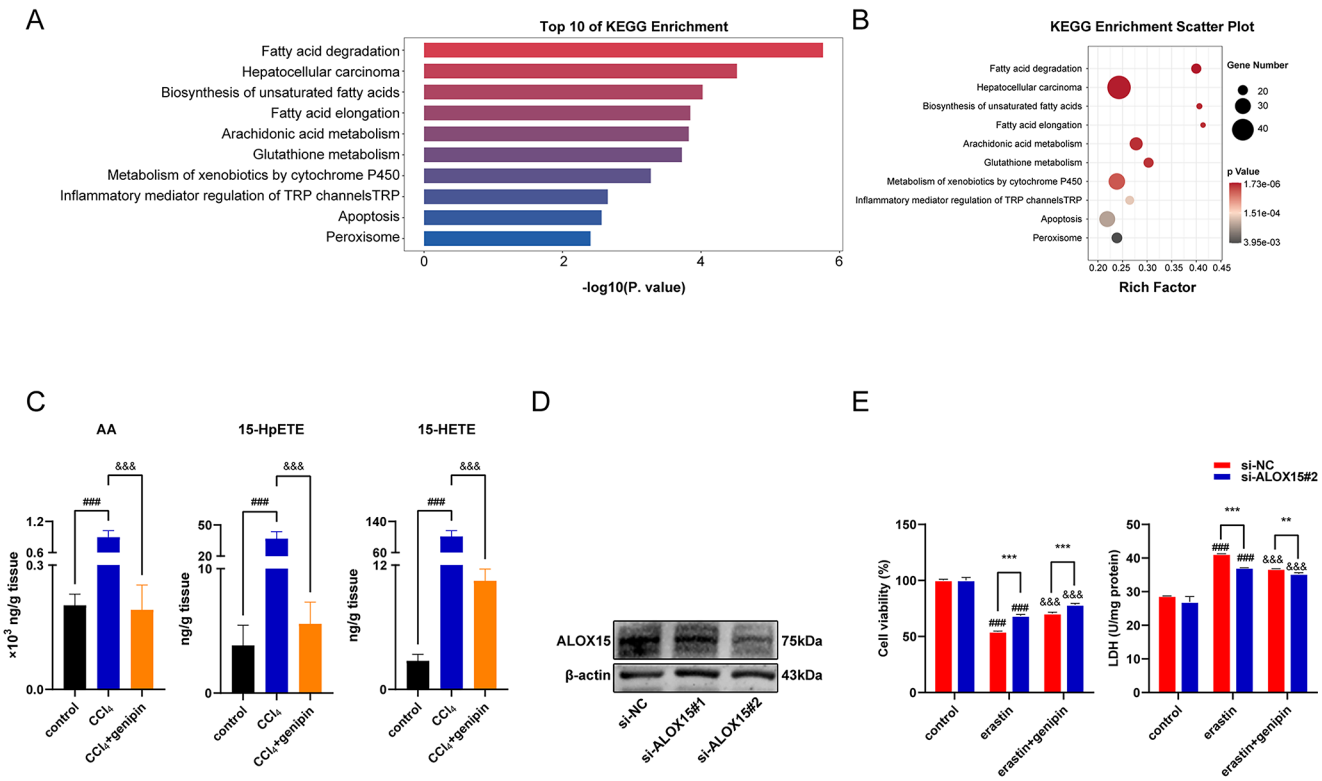

Fig. 7 Genipin exerts hepatoprotective effects by inhibiting ALOX15-launched lipid peroxidation. **(A, B)** The KEGG pathway analysis using liver tissues of mice subjected to CCl_4 intoxication with and without genipin in terms of RNA-sequencing. **(C)** Evaluation of several oxylipins associated with ALOX15 catabolism including AA, 15-HpETE and 15-HETE in terms of UHPLC-MS/MS. **(D)** Determination of protein levels of ALOX15 in L-O2 cells transfected with NC siRNA or ALOX15 siRNA. **(E)** Assessment of the viability of L-O2 cells and the accumulation of LDH in L-O2 cells transfected with NC siRNA or ALOX15 siRNA. ###$p < 0.001$ vs. control group, &&&$p < 0.001$ vs. erastin group, *** $p < 0.001$ vs. ALOX15 siRNA group

GSH, a cofactor indispensible for selenocysteine-containing enzyme, can instigate a cascade of ferroptosis-associated events. Although acetaminophen (APAP)-triggered liver injury has been under extensive investigations for decades, the relationship between ferroptosis and APAP hepatotoxicity is still under debate. Particularly, Jaeschke H et al. argued that there is a lack of credible evidence to support the notion that APAP elicits ferroptotic hepatocellular death, taking into consideration that superoxide dismutase mimetics exhibit highly effective protection against APAP hepatotoxicity [30]. The CCl_4-triggerd acute liver injury represents typical hallmarks in relation to ferroptosis, which is characterized by intensively oxidative stress, lipid peroxidation and expression of Ptgs2/p53 in addition to GSH consumption [31, 32]. As for oxidative stress phase, CCl_4-derived toxicants and deleterious metabolites give rise to disruption and impairment to antioxidative defense systems including GPX members and GSH [33, 34]. For instance, Shah et al. found that over-production of ROS in CCl_4-induced hepatic dysfunction model may result in protein inactivation manifested as a significant decrease pertaining to the levels of GPX [34]. Another report implicated that toxic metabolites of CCl_4 (e.g., $^{\bullet}CCl_3$) can considerably impact the activity of indicative glutathione-metabolizing enzyme, meanwhile, direct reaction with sulfhydryl groups of GSH was responsible for its reduced functionality and concentration [33]. Accordingly, our findings aligned with aforesaid data in terms of significant reduction of GPX4 protein levels, which was restored upon genipin supplementation (Fig. 4A). Moreover, it was highlighted that there is a similar pattern concerning pertubation in xCT protein levels. Mounting evidence indicates that xCT-GSH-GPX4 signalings orchestrate tightly to exert antioxidant effects [35]. Notably, the negative regulation of xCT expression can be mainly attributed to transcription factor p53, mirrored as p53 diminishment contributing to the upregulation of xCT levels [26]. In the present investigation, we observed a remarkable increase of p53 protein level due to CCl_4 intoxication, while genipin effectively restored its expression. Therefore, the synergistic effect of upregulated xCT-GPX4 pathway partially accounts for the hepatoprotective activities of genipin. As for lipid peroxidation phase, unneutralized CCl_4 radicals orchestrating a covalent link with membranous proteins/lipids and mitochondria in hepatocytes to produce plenty of

lipid radicals, all of which lead to the morphological and functional pertubations in liver cells [4, 36]. Given that GPX4 serves as the core inhibitor of ferroptosis, capable of eliminating lipid peroxides, and in consequence determines cell fate, the molecular docking analysis clearly supported a potential molecule interactions between genipin and GPX4 protein. Intriguingly, recent study reported exactly similar findings to the present investigation: apigenin supplementation, a natural plant flavonoid, repressed the levels of ROS and concentrations of MDA alongside recovery manifesting a combined decrease of GPX4 and xCT in di(2-ethylhexyl) phthalate-induced liver injury in the context of ferroptosis execution [37]. In that study, further molecular docking also revealed an interaction between the ligand apigenin and the amino acid residues of GPX4. On the other hand, Li et al. identified a series of substituted compound on the basis of combined computational (i.e., molecular docking program) and experimental screen, one of which activates GPX4 to serve as antioxidative (inhibition of intracellular ROS), anti-inflammatory (repression of AA oxidation) and cytoprotective (suppression of ferroptosis) agent [38]. Consistently, our results denoted that genipin supplementation effectively regulates a cascade of ferroptosis-associated events. The iron homeostasis, destruction of mitochondrial structure, production of lipid ROS and cell death were modulated upon genipin treatment in CCl_4-intoxication models in light of determining catalytically active Fe^{2+}, TEM imaging, BODIPY probe as well as TUNEL staining. These findings were also confirmed in vitro concerning multistage ferroptosis alongside GPX4 silencing experiments. Taken together, the hepatoprotective effects of genipin, to some extent, can be attributed to restore GPX4 expression levels aimed at preventing toxicant-induced ferroptosis.

It is well documented that lipid peroxidation represents hallmarks in liver pathology subjected to CCl_4 challenge [39]. Plenty of bioactive carbonyls, such as MDA and 4-HNE, are produced in response to reaction between lipid peroxides and iron, all of which in turn conjugate with GSH and diminish intracellular GSH contents. Accordingly, augmented production of MDA and 4-HNE (both lipid peroxidation biomarkers) were observed in the livers of CCl_4-triggered mice, which were remarkably attenuated by genipin administration. Furthermore, mechanistic research in terms of RNA-sequencing analysis unveiled that several gene clusters concerning biogenesis of unsaturated fatty acids, arachidonic acid metabolism and fatty acid elongation are substantially influenced by genipin treatment, pinpointing the key role of PUFAs metabolism in the context of ferroptotic cell death.

Lipoxygenase accounts for catalyzing the PUFAs peroxidation to corresponding hydroperoxy derivatives, resulting in the bursting of pro-ferroptotic oxidation process [40, 41]. ALOX15 is capable of using preferred substrates such as linoleic acid, docosahexaenoic acid and AA, which are incorporated into cholesterol esters/phospholipids or in free forms [42]. Of note, an n-6 PUFA, designated as AA, exhibits a major component of the membranous phospholipids, which is converted into 15-HpETE and 15-HETE by ALOX15 and metabolized towards various bioactive substances [43]. In agreement with these concepts, our results showed that genipin supplementation significantly downregulates ALOX15 protein expression in the CCl_4-intoxicated livers. In this regard, UHPLC-MS/MS analysis denoted that AA, 15-HpETE and 15-HETE increase in toxicant injury models, and these shifts are reversed by genipin treatment. The participation of ALOX15 in erastin-induced ferroptosis was also validated by in vitro experiments. Taking into account the mechanism of lipid peroxidation as a controversy, these findings provide a novel therapeutic approach to target ALOX15-launched lipid peroxidation in divergent pathological conditions.

As for inflammatory response phase, CCl_4-created free radicals are responsible for the hypertrophy and hyperplasia of macrophages in the livers, which can lead to damages to the hepatic parenchyma via massive release of detrimental and proinflammatory molecules [5, 44]. The results of the current study verified that intoxication of CCl_4 is closely linked to aggravated inflammation. The macrophages identified by F4/80 staining and production of several cytokines (IL-1β, IL-6 and TNF-α) determined by Milliplex increased in CCl_4 intoxication mice livers, whereas these alterations were reversed in the CCl_4 + genipin group, coincided with findings in similar investigations by us and others [4, 18, 45]. As a matter of fact, the relationship between ALOX15 and inflammation appears to be intricate and heterogeneous. It is suggested that ALOX15 may considerably impact inflammatory response by producing lipid mediators [42]. Particularly, a recent report indicated that ALOX15-launched peroxidation of PUFA-phospholipids represents a pivotal initial event among ischemia injuries [29]. Taken together, in-depth investigations are warranted to elucidate underpinning molecular mechanisms regarding protective effects of genipin concerning interaction between lipid peroxidation and inflammation in the context of ferroptosis.

Genipin may influence a wide range of pathophysiological conditions/events to exert its hepatoprotective effects such as modification of mitochondria quality, anti-inflammatory activity, antioxidative activity, antifibrogenic effect, cholagogic effect and regulation of cell death [46]. On the other hand, accumulating evidence implicates that divergent forms of regulated cell death (e.g., apoptosis, necroptosis, autophagy and ferroptosis) can converge in pathogenic environment [47]. These modalities of regulated cell death mirror as the "backup" dying strategies to maintain internal

homeostasis in the circumstance that the cellular death-triggering threshold is reached, since they may share overlapping mechanisms. The interplay between different forms of regulated cell death appears to be complicated and still under debate. For instance, autophagy can facilitate ferroptosis due to iron overloaded suppression of xCT, and erastin-induced ferroptosis was inhibited by removing Atg5/Atg7 [48, 49]. Conversely, a latest report showed that astaxanthin can protect against APAP-induced liver injury through inhibiting ferroptosis and promoting autophagy via the Nrf2/HO-1 pathway [50]. In conjunction with other reports, wherein the medicinal effects pertinent to genipin encompassed the induction of autophagy and activation of GPX4-Nrf2 axis, as key defensive systems against liver injury in the context of ferroptosis, we suppose it is operational to investigate the reciprocal relations between divergent forms of regulated cell death in the future studies [51].

Conclusion

In conclusion, our findings demonstrated that genipin treatment protects against CCl_4-triggered acute liver injury by abrogating ferroptosis-dictated hepatocyte death, and pharmacological modification of dysregulated GPX4 and ALOX15-launched lipid peroxidation partially accounted for its medicinal effects as underlying molecular basis.

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Data Availability The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval All animal studies were approved by the Institutional Animal Care and Use Committee at Tianjin Medical University General Hospital (IRB2021-DWFL-142).

Competing interest All authors declare no conflict of interest.

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