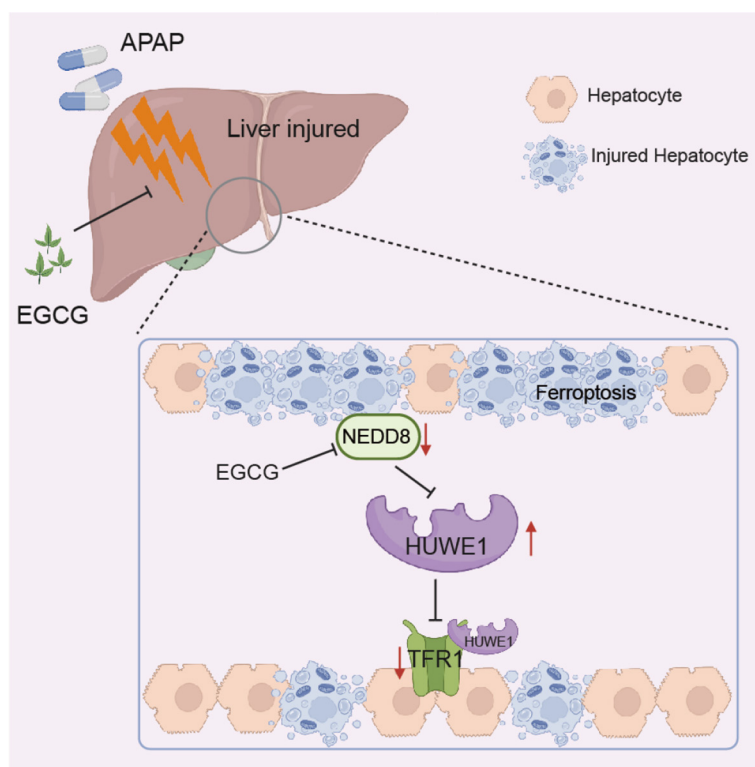


Short Communication

EGCG inhibits ferroptosis to ameliorate APAP-induced liver injury by suppressing NEDD8 and stabilizing HUWE1 *in vivo* and *in vitro*

Graphical abstract



Authors

Zijun Ouyang, Tao Zhang, Mengting Liu, Fufu Li, Hui Guo, Yanhui Li, Jieyu Chen, Feiyang Wang, Yang Sun, Dong Liu and Haiyan Sun

Correspondence

yangsun@nju.edu.cn (Y. Sun);
liudongsz@szpu.edu.cn (D. Liu);
susan@szpu.edu.cn (H. Sun)

In brief

The authors showed that EGCG alleviates acetaminophen-induced liver injury in mice by inhibiting ferroptosis in hepatocytes. EGCG protects HUWE1 from degradation by targeting and inhibiting NEDD8, thereby suppressing the expression of TFR1 and inhibiting ferroptosis.

Highlights

- EGCG protects against acetaminophen-induced liver injury.
- EGCG restrains ferroptosis in acetaminophen-induced liver injury.
- EGCG targets and inhibits NEDD8 to stabilize HUWE1.

EGCG inhibits ferroptosis to ameliorate APAP-induced liver injury by suppressing NEDD8 and stabilizing HUWE1 *in vivo* and *in vitro*

Zijun Ouyang^{a,1}, Tao Zhang^{a,b,1}, Mengting Liu^{c,1}, Fufu Li^{a,c}, Hui Guo^a, Yanhui Li^a, Jieyu Chen^a, Feiyang Wang^a, Yang Sun^{b,*}, Dong Liu^{a,*} and Haiyan Sun^{a,*}

^aSchool of Food and Drug, Shenzhen Polytechnic University, 7098 Liuxian Avenue, Shenzhen 518055, China

^bState Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Chemistry and Biomedicine Innovation Center (ChemBIC), Nanjing University, 163 Xianlin Avenue, Nanjing 210023, China

^cThe First Affiliated Hospital of Guangdong Pharmaceutical University, No. 19 NongLin Road, Guangzhou 510062, China

¹These authors contributed equally to this work.

*Correspondence: yangsun@nju.edu.cn (Y. Sun); liudongsz@szpu.edu.cn (D. Liu); susan@szpu.edu.cn (H. Sun)

Received: 5 December 2024; Revised: 29 January 2025; Accepted: 8 February 2025

Published online: 20 February 2025

DOI 10.15212/AMM-2024-0087

ABSTRACT

Excessive consumption of acetaminophen (APAP) has emerged as the primary culprit behind drug-induced liver injury (DILI), with N-acetylcysteine serving as the principal antidote. However, use of N-acetylcysteine is limited to the early stages of APAP-induced DILI and may cause adverse side effects. Consequently, it is imperative to explore alternative therapeutic approaches to alleviate APAP-induced liver toxicity. In this study the mechanisms underlying the protective role of epigallocatechin gallate (EGCG) in DILI were determined. Our findings revealed that EGCG inhibited NEDD8, thus stabilizing HUWE1, a crucial E3 ubiquitin ligase involved in protein degradation. HUWE1 binds and degrades TFR1, a protein essential for cellular iron uptake and inhibits ferroptosis. By stabilizing HUWE1 and degrading TFR1, EGCG suppressed ferroptosis and ameliorated APAP-induced liver injury. Our results highlight the therapeutic potential of EGCG in mitigating DILI through regulation of HUWE1 and ferroptosis, which offers a promising approach for the treatment of DILI.

Keywords: EGCG, liver injury, NEDD8, HUWE1, ferroptosis

1. INTRODUCTION

Drug-induced liver injury (DILI) refers to a type of damage to the liver or biliary system caused by the ingestion of hepatotoxic drugs, such as acetaminophen (APAP), and is a frequent cause of acute liver failure [1, 2]. APAP is metabolically cleared by forming N-acetyl-p-benzoquinone imine (NAPQI) [3]. However, when APAP is ingested in excessive amounts, NAPQI accumulates in the liver, which leads to hepatotoxicity. Currently, the clinical treatment for DILI involves administration N-acetylcysteine (NAC). However, the therapeutic window for NAC is limited to the early stages of APAP ingestion, emphasizing the urgent need for novel treatment strategies [4].

Ferroptosis is a newly recognized form of regulated cell death that is characterized by iron-dependent lipid

peroxidation [5]. This process has a pivotal role in a variety of physiologic conditions, including cancers, neurodegenerative diseases, and tissue damage [6]. Epigallocatechin gallate (EGCG), a primary constituent of tea polyphenols, has been reported to exhibit beneficial properties in a variety of diseases, including liver injury and fibrosis [7, 8]. However, the mechanism by which EGCG improves drug-induced liver injury remains unclear. This study aimed to determine the potential protective effects of EGCG and the underlying mechanisms in a mouse model of APAP-induced liver injury.

2. MATERIALS AND METHODS

2.1 Animals

Male C57BL/6 mice (7 weeks old) were purchased from Jiangsu Jichui Pharmaceutical Biotechnology Co., Ltd.

Short Communication

(Jiangsu, China). The mice were housed at $22 \pm 3^\circ\text{C}$ with 12-h light and dark cycles. Mice in the EGCG group were administered 60 or 120 mg/kg of EGCG orally once daily for 7 d. The APAP and EGCG treatment groups were injected with 300 mg/kg of APAP intraperitoneally to establish an acute liver injury model. The mice were euthanized 6 h later. The animal procedures were approved by the Animal Ethical Committee of Shenzhen Polytechnic University [Shenzhen, China] (AEC-20241105-0001).

2.2 Cell lines and cell cultures

L02 cells were obtained from FuHeng BioLogY (city, China). L02 cells were cultured in RPMI-1640 medium (VivaCell, city, China) containing 10% fetal bovine serum at 37°C in 5% CO_2 . Cells were challenged with 5 mM APAP and 20 or 40 μM EGCG for 24 h.

2.3 DIA proteomics

The proteins were digested enzymatically after protein extraction from the samples. The resulting peptides

were then enriched and separated before analysis with LC-MS/MS, which generated mass spectrometry data. Identification and analysis were performed using DIA-NN software.

2.4 Biochemical indicators

The alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactic dehydrogenase (LDH) levels were measured to assess liver injury. Assay kits were used to determine the contents in the serum. Cell contents of lactoperoxidase (LPO), malondialdehyde (MDA), glutathione (GSH), and superoxide dismutase (SOD) were measured to assess ferroptosis. Assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (<http://www.njjcbio.com/>). All steps were carried out according to the manufacturer's instructions.

2.5 Immunocytochemistry and immunofluorescence staining

The sections were antibody-retrieved in sodium citrate buffer for 5 min, followed by incubation with goat serum.

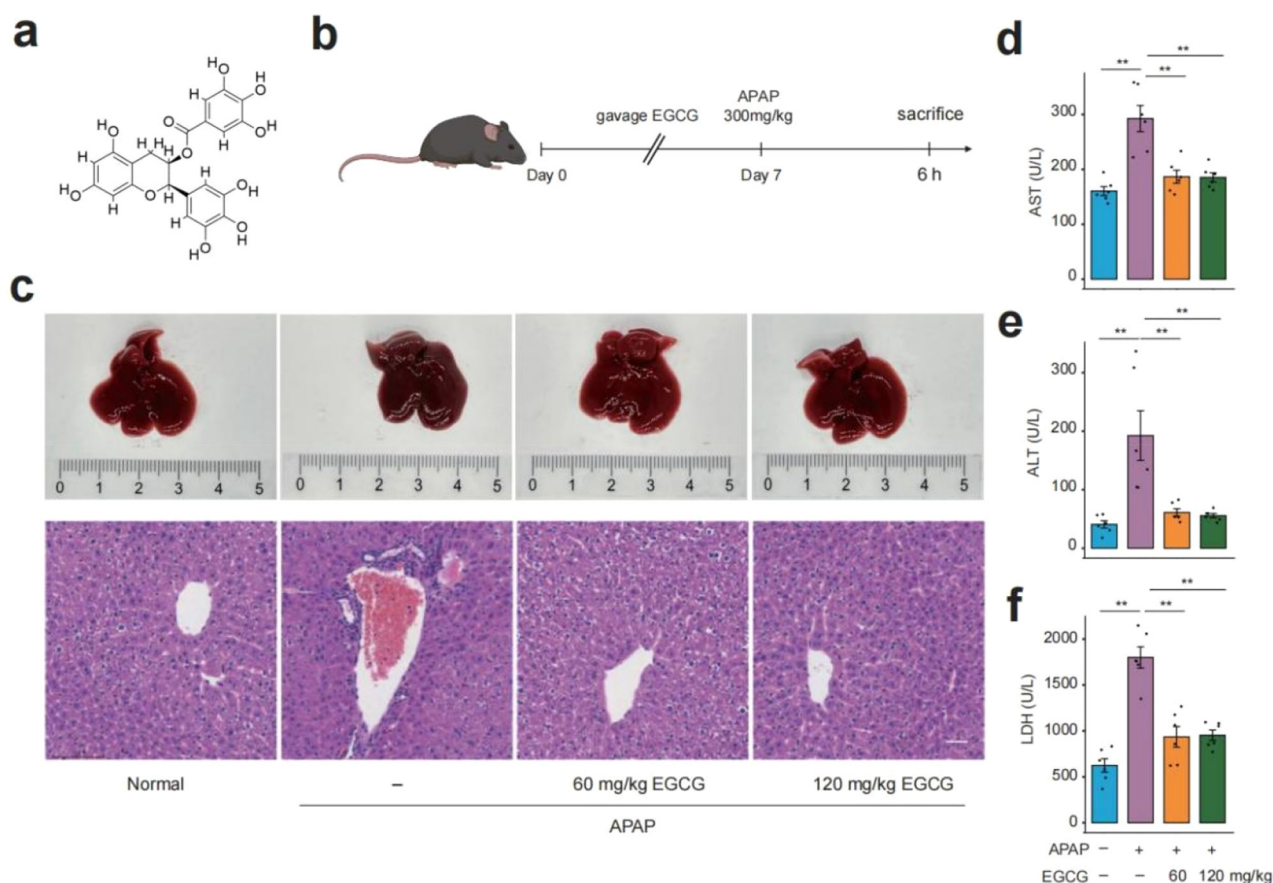


Figure 1 | EGCG protects mice from APAP-induced liver injury.

(a) The structure of EGCG. (b) Schematic overview of mouse model design. (c) Representative macrophotographs and H&E staining of liver tissues from mice. Scale bar = 100 μm . (d-f) AST, ALT, and LDH in serum from mice (n = 10). *P* values are determined by two-tailed Student's *t* test. **P* < 0.05, ***P* < 0.01.

Primary antibodies were then incubated overnight at 4°C, succeeded by incubation with streptavidin-HRP or lexa Fluor secondary antibody for 2 h. Subsequently, the tissues were stained with diaminobenzidine and hematoxylin or DAPI.

2.6 FerroOrange detection

L02 cells were treated with APAP or EGCG, FerroOrange (Dojindo, city, China) was added and incubated for 0.5 h. Finally, the cells were observed under a fluorescence microscope or quantified using flow cytometry.

2.7 Data analysis and statistics

Statistical differences were assessed using one-way analysis of variance (ANOVA) followed by Tukey's

multiple comparison test. Differences were considered statistically significant as follows: * $P < 0.05$; and ** $P < 0.01$.

3. RESULTS

3.1 EGCG attenuates APAP-induced DILI in mice

The structure of EGCG is depicted in [Figure 1a](#). An APAP-induced DILI murine model was established to determine the effect of EGCG on DILI ([Figure 1b](#)). EGCG effectively alleviated APAP-induced hepatotoxicity in mice ([Figure 1c](#)). Furthermore, EGCG reduced the elevated ALT, AST, and LDH levels following APAP ingestion ([Figure 1d-f](#)). Therefore, EGCG protects against APAP-induced DILI by mitigating liver toxicity.

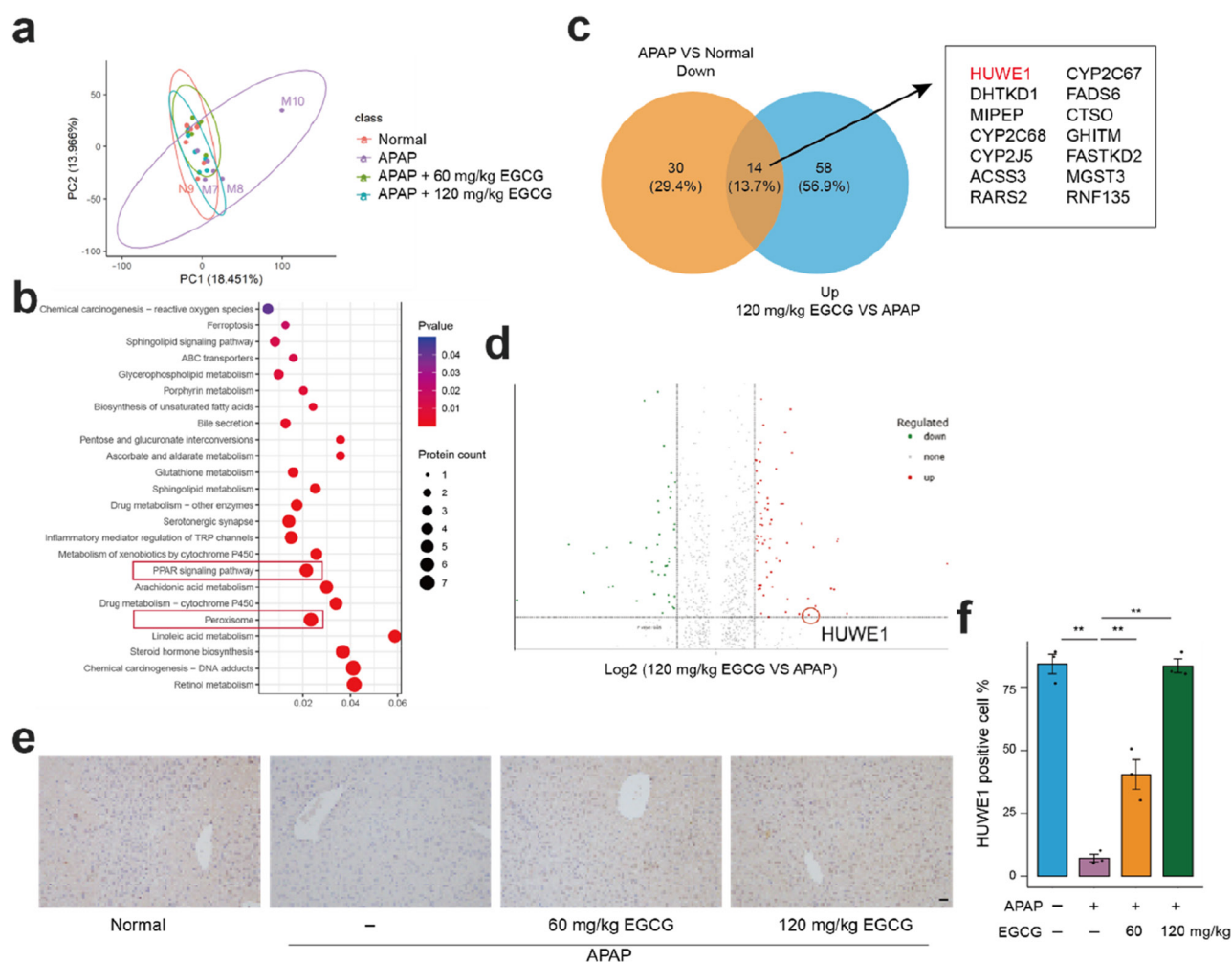


Figure 2 | EGCG-mediated upregulation of HUWE1.

(a) The PCA analysis of proteomic. (b) KEGG enrichment analysis of the proteins about APAP-induced DILI. (c) Venn diagram showing the numbers of various proteins in different groups treated as indicated. (d) Volcano plot showing protein expression in different groups. (e-f) Immunostaining for HUWE1 (e) and the statistics of HUWE1-positive cells (f) in different groups. Scale bars = 50 μ m. P values are determined by two-tailed Student's t test. * $P < 0.05$, ** $P < 0.01$.

Short Communication

3.2 Proteomics reveals EGCG-induced upregulation of HUWE1

Data-independent acquisition proteomic analysis was performed to investigate the impact of EGCG treatment in mice. Principal component analysis revealed significant differences in the protein level between the normal and APAP groups, which were reversed with EGCG treatment (Figure 2a). Kyoto Encyclopedia of Genes and Genomes analysis indicated that pathways related to peroxisomes, PPAR signaling pathway, glutathione metabolism, and ferroptosis underscores

the well-known antioxidant properties of EGCG and its potential, yet unexplored ability to protect against ferroptosis (Figure 2b). APAP-treated mice had 30 downregulated proteins compared to normal mice. Further analysis showed that 14 proteins were upregulated by EGCG treatment (Figure 2c). Notably, EGCG upregulated HUWE1, a protective factor against liver issues and ferroptosis [9] (Figure 2c, d). EGCG treatment significantly upregulated the expression of HUWE1, which is consistent with the proteomic results (Figure 2e, f).

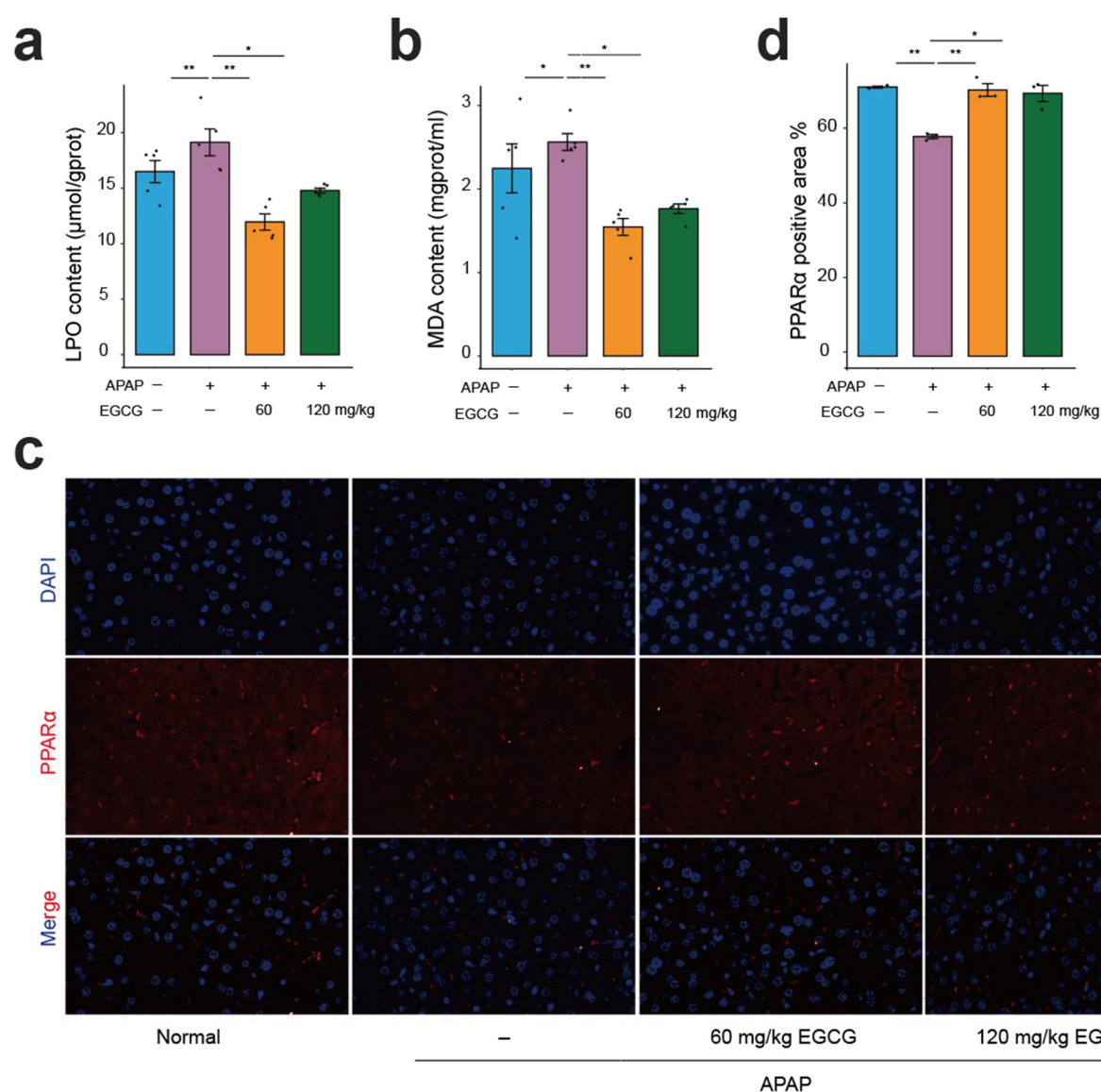


Figure 3 | EGCG suppressed ferroptosis in APAP-induced DILI mice.

(a-b) The content levels of LPO and MDA in different groups. (c-d) IF analysis for PPARα in liver tissues (c) and proportion of PPARα-positive cells (d) in different groups. Scale bars = 50 μm. *P* values are determined by two-tailed Student's *t* test. **P* < 0.05, ***P* < 0.01.

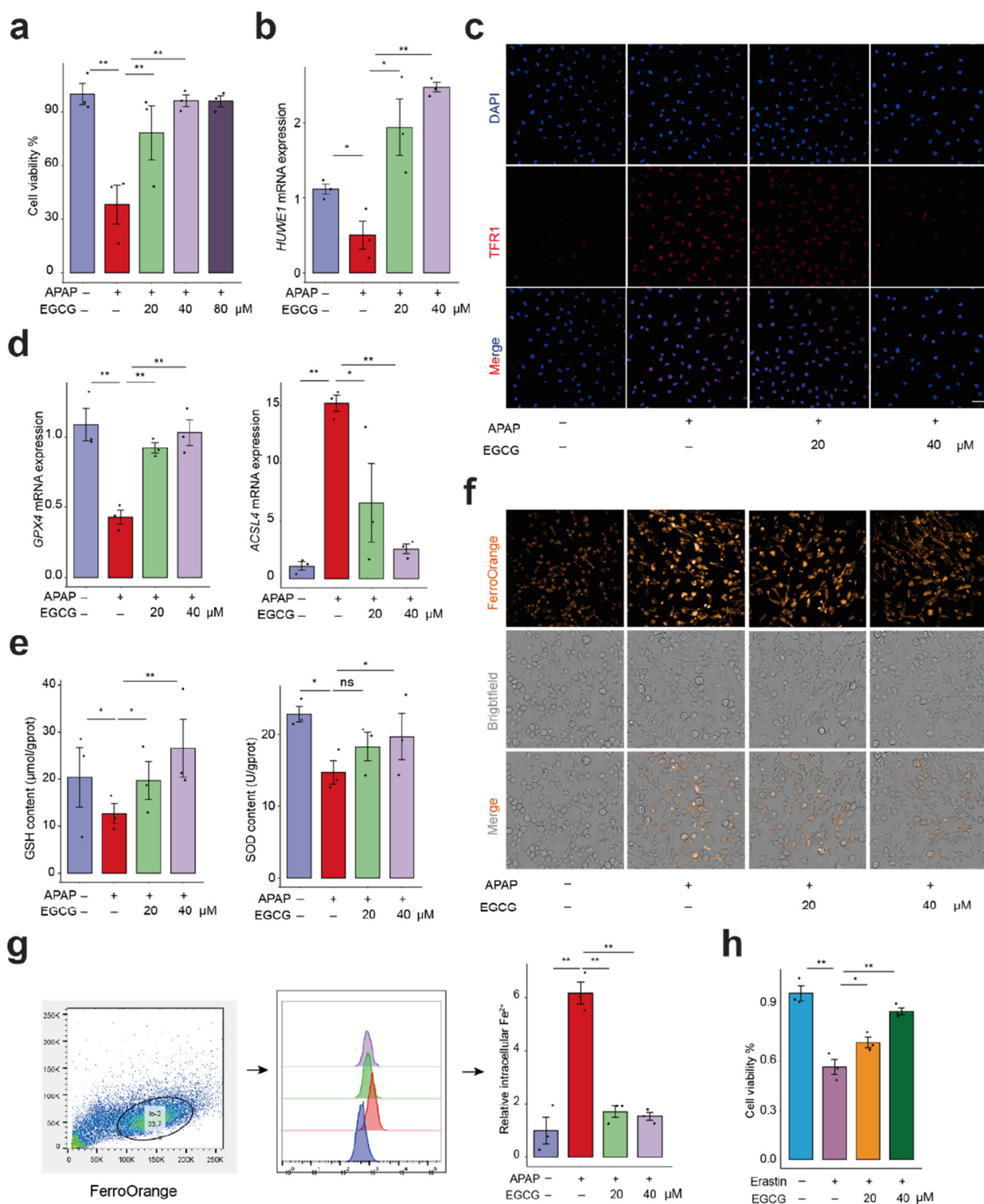


Figure 4 | EGCG suppressed APAP-induced ferroptosis.

L02 cells were challenged with indicated concentration of EGCG and APAP (5 μ M) for 24 h. (a) The cell proliferation levels were measured by CCK-8. (b, d) *HUWE1*, *GPX4* and *ACSL4* mRNA levels in L02 cells. (c) IF analysis for TFR1 in L02 cells. (e) The intracellular levels of GSH and SOD in L02 cells. (f-g) The intracellular Fe^{2+} were detected by fluorescence imaging (f) and flow cytometry (g). (h) L02 cells were challenged with indicated concentration of EGCG and Erastin (10 μ M). Cell proliferation levels were measured by CCK-8. Scale bars = 25 μ m. *P* values are determined by two-tailed Student's *t* test. **P* < 0.05, ***P* < 0.01.

Short Communication

3.3 EGCG restrains ferroptosis in mice with APAP-induced DILI

We hypothesized that ferroptosis may engage the mechanism by which EGCG exerts its effects. The results indicated that the LPO and MDA contents were significantly reduced with treated with 60 and 120 mg/kg of EGCG (Figure 3a-b). EGCG administration increased PPAR α expression, a nuclear receptor crucial for lipid metabolism and antioxidant processes (Figure 3c-d).

3.4 EGCG rescues APAP-induced ferroptosis in L02 cells

L02 cells were processed with EGCG during APAP challenge to evaluate the role of ferroptosis in EGCG treatment. (40 μ M) effectively reduced the death of L02

cells (Figure 4a). Consistent with previous results, EGCG increased *HUWE1* expression (Figure 4b), which was consistent with previous results. Inhibition of HUWE1 reduces ubiquitination and stabilization of TFR1, which leads to dysregulation of iron metabolism [9]. EGCG (40 μ M) effectively reduced the expression of TFR1 (Figure 4c), which suggested that EGCG may promote degradation of TFR1 by upregulating HUWE1, thereby providing a mechanism to resist ferroptosis. *GPX4* and *ACSL4*, two key regulatory genes involved in ferroptosis, had reversed expression following EGCG treatment (Figure 4d). Furthermore, the intracellular GSH and SOD levels increased after EGCG treatment. APAP treatment led to increased Fe²⁺ production and this effect was restored by EGCG treatment (Figure 4e-g). Furthermore,

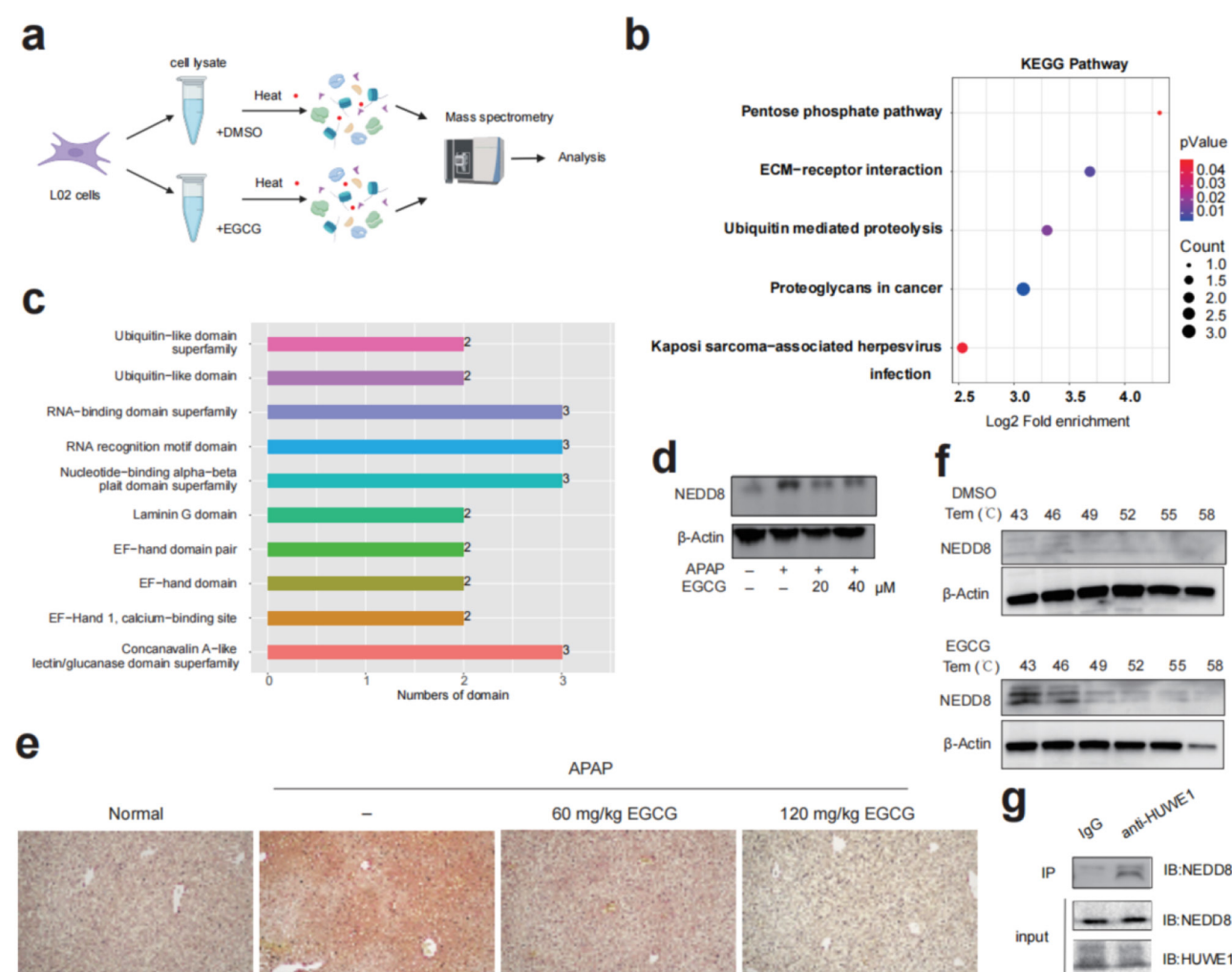


Figure 5 | EGCG binds to NEDD8 to rescue HUWE1 degradation.

(a) Schematic overview of proteomic thermal stability analysis *in vitro*. (b) KEGG enrichment analysis and (c) domain structures analysis of the identification proteins in Figure 5a. (d) The expression of NEDD8 were detected by Western blot. (e) Sirius red staining of liver tissues. (f) CETSA analyzed the thermal stabilization of NEDD8 at indicated temperatures. (g) Coimmunoprecipitation verified that the binding of NEDD8 and HUWE1 in L02 cells.

EGCG mitigated cell death induced by erastin (Figure 4h). Taken together, these studies showed that EGCG rescues APAP-induced ferroptosis in L02 cells.

3.5 EGCG inhibits NEDD8 to stabilize HUWE1

To elucidate the molecular mechanism by which EGCG improves APAP-induced DILI, potential EGCG binding proteins were obtained by proteomic thermal stability analysis (Figure 5a). This resulted in the identification of 23 proteins that exhibited significantly improved stability after EGCG treatment. KEGG functional analysis of these proteins revealed enrichment in the ubiquitin-mediated proteolysis pathway (Figure 5b). Additionally, the domain structures of the differential protein sequences were predicted. The results indicated the presence of ubiquitin-like domains (Figure 5c). Among the 23 proteins identified, the NEDD8 protein exhibited a significant decrease in intensity. Neural cell expressed developmentally down-regulated protein 8 (NEDD8) plays a crucial role in regulating protein degradation, including HUWE1 [10-12]. Therefore, we hypothesized that EGCG rescues HUWE1 degradation by targeting inhibition of NEDD8. We found EGCG could inhibit NEDD8 expression (Figure 5d). Given that neddylation inhibition reduced liver fibrosis, EGCG was also shown to attenuate collagen deposition (Figure 5e). The engagement between EGCG and NEDD8 by CETSA was then confirmed (Figure 5f). Furthermore, NEDD8 was shown to bind to HUWE1 in L02 cells (Figure 5g). Taken together, these results suggested that EGCG binds to NEDD8 to rescue HUWE1 degradation.

4. DISCUSSION

Unraveling the mechanisms underlying APAP-induced liver toxicity and developing corresponding therapeutic interventions is crucial. Herein EGCG was shown to protect HUWE1 from degradation by targeting and inhibiting NEDD8, thereby suppressing ferroptosis in hepatocytes and ultimately improving APAP-induced liver injury in mice.

Recent studies have revealed that abnormal expression or activity of HUWE1 is related to liver diseases, including acute liver injury, non-alcoholic fatty liver disease, and hepatic steatosis [13]. HUWE1 binds specific proteins, such as TFR1 and WIP1, for ubiquitination and subsequent proteasomal degradation [14]. TFR1 facilitates cellular iron uptake and initiates ferroptosis, a process crucial for maintaining hepatic homeostasis [15]. While the current study suggested that EGCG inhibits TFR1 expression via the NEDD8/HUWE1 pathway, the exact mechanism underlying TFR1 reduction (e.g., degradation) and the role of HUWE1 or NEDD8 in this process has not been established. Additional experiments are needed to validate the role of TFR1 in EGCG-mediated hepatoprotection. HUWE1 expression varies significantly across different tumor types, suggesting that HUWE1 may have cell-specific functions depending

on the diverse targets. This finding highlights the importance of understanding the novel mechanisms involved in this process [16]. Recent research has shown that CUL4B is essential for the proteasomal degradation of HUWE1 in response to DNA damage. CUL4B is activated in an NEDD8-dependent manner and catalyzes HUWE1 ubiquitination [12]. EGCG inhibits NEDD8 in L02 cells, suggesting a potential strategy for treating DILI by stabilizing HUWE1.

In conclusion, the findings in the current study indicated that EGCG inhibits NEDD8 in hepatocytes, stabilize sHUWE1, and subsequently degraded TFR1, thereby inhibiting ferroptosis and improving APAP-induced liver injury. This approach provides a safe and effective strategy for the clinical treatment and prevention of DILI. Additionally, the current study provides a novel management approach for the safe use or combined administration of APAP in clinical practice.

ACKNOWLEDGEMENTS

This work was financially supported by the Shenzhen Stability Support Project for Colleges and Universities (Nos. 20220814205518001 and 20231126130044001), Shenzhen Sustainable Development Project (KCFZ20230731094501002), Heparin and its Derivative Novel Drug R&D Center (602431004PQ), the Lihu Talent Cultivation Program of Shenzhen Polytechnic University (No. LHRC20220408), and the National Natural Science Foundation of China (No.82104684).

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

REFERENCES

- [1] Ostapowicz G, Fontana RJ, Schiødt FV, Larson A, Davern TJ, Han SH, et al.: Results of a Prospective Study of Acute Liver Failure at 17 Tertiary Care Centers in the United States. *Annals of Internal Medicine* 2002, 137:947-954.
- [2] Fontana RJ: Pathogenesis of Idiosyncratic Drug-induced Liver Injury and Clinical Perspectives. *Gastroenterology* 2014, 146:914-928.
- [3] Bajt ML, Ramachandran A, Yan HM, Lebofsky M, Farhood A, Lemasters JJ, et al.: Apoptosis-inducing Factor Modulates Mitochondrial Oxidant Stress in Acetaminophen Hepatotoxicity. *Toxicological Sciences* 2011, 122:598-605.
- [4] Heard KJ: Acetylcysteine for Acetaminophen Poisoning. *The New England Journal of Medicine* 2008, 359:285-292.
- [5] Dixon SJ, Lemberg KM, Lamprecht MR, Skouta R, Zaitsev EM, Gleason CE, et al.: Ferroptosis: An Iron-dependent form of Nonapoptotic Cell Death. *Cell* 2012, 149:1060-1072.
- [6] Zhang M, Zhang T, Song C, Qu J, Gu Y, Liu S, et al.: Guizhi Fuling Capsule Ameliorates Endometrial Hyperplasia through Promoting p62-Keap1-NRF2-mediated Ferroptosis. *Journal of Ethnopharmacology* 2021, 274:114064.
- [7] Zaveri NT: Green Tea and Its Polyphenolic Catechins: Medicinal Uses in Cancer and Noncancer Applications. *Life Sciences* 2006, 78:2073-2080.
- [8] Winiarska-Mieczan A, Jachimowicz-Rogowska K, Kwiecień M, Borsuk-Stanulewicz M, Tomczyk-Warunek A,

Short Communication

- Stamirowska-Krzaczek E, et al.: Regular Consumption of Green Tea as an Element of Diet Therapy in Drug-Induced Liver Injury (DILI). *Nutrients* 2024, 16:2837.
- [9] Wu Y, Jiao H, Yue Y, He K, Jin Y, Zhang J, et al.: Ubiquitin Ligase E3 HUWE1/MULE Targets Transferrin Receptor for Degradation and Suppresses Ferroptosis in Acute Liver Injury. *Cell Death and Differentiation* 2022, 29:1705–1718.
- [10] Baek K, Krist DT, Prabu JR, Hill S, Klügel M, Neumaier LM, et al.: NEDD8 Nucleates a Multivalent Cullin-RING-UBE2D Ubiquitin Ligation Assembly. *Nature* 2020, 578:461–466.
- [11] Watson IR, Irwin MS, Ohh M: NEDD8 Pathways in Cancer, Sine Quibus Non. *Cancer Cell* 2011, 19:168–176.
- [12] Yi J, Lu G, Li L, Wang X, Cao L, Lin M, et al.: DNA Damage-induced Activation of CUL4B Targets HUWE1 for Proteasomal Degradation. *Nucleic Acids Research* 2015, 43:4579–4590.
- [13] Feng WW, Bang S, Takacs EM, Day C, Crawford KJ, Al-Sheyab R, et al.: Hepatic *Huwe1* Loss Protects Mice from Non-alcoholic Fatty Liver Disease through Lipid Metabolic Rewiring. *iScience*. 2023, 26:108405.
- [14] Wan W, You Z, Zhou L, Xu Y, Peng C, Zhou T, et al.: mTORC1-Regulated and HUWE1-Mediated WIPI2 Degradation Controls Autophagy Flux. *Molecular Cell* 2018, 72:303–315.e6.
- [15] Tang LJ, Zhou YJ, Xiong XM, Li NS, Zhang JJ, Luo XJ, et al.: Ubiquitin-specific Protease 7 Promotes Ferroptosis via Activation of the p53/TfR1 Pathway in the Rat Hearts After Ischemia/Reperfusion. *Free Radical Biology & Medicine* 2021, 162:339–352.
- [16] Kao SH, Wu HT, Wu KJ: Ubiquitination by HUWE1 in Tumorigenesis and Beyond. *Journal of Biomedical Science* 2018, 25:67.