

Gandouling Regulates Ferroptosis and Improves Neuroinflammation in Wilson's Disease Through the LCN2/NLRP3 Signaling Pathway

Yuya Wen¹, Chenling Zhao¹, Jie Chen¹, Liwei Tian¹, Bojin Wu¹, Wenting Xie¹, Ting Dong^{1,2}

¹Department of Neurology, The First Affiliated Hospital of Anhui University of Chinese Medicine, Hefei, 230031, People's Republic of China; ²Key Laboratory of Xin'An Medicine, Ministry of Education, The First Affiliated Hospital of Anhui University of Chinese Medicine, Hefei, 230031, People's Republic of China

Correspondence: Ting Dong, The First Affiliated Hospital of Anhui University of Chinese Medicine, Hefei, 230031, People's Republic of China, Tel +13965107566, Email dongting2002@sina.com

Purpose: Neuroinflammation is a main cause of neurological damage in Wilson's disease (WD). Ferroptosis is present in the WD pathological process, which is also closely related to the neuroinflammation. LCN2, a ferroptosis-related gene in WD, is linked with the activation of NLRP3 inflammasome. Our group has previously demonstrated that Gandouling (GDL) can effectively improve neuroinflammation in WD. This study aims to investigate the protective effect of GDL on neuroinflammation in animal and cell models of WD, and whether the pharmacological mechanism is related to the LCN2/NLRP3 signaling pathway.

Methods: Toxic milk (TX) mice and HT22 cells stimulated by copper ions were selected as models. The pathology of hippocampal tissues in TX mice were observed by HE staining and transmission electron microscopy. High-throughput sequencing analysis was conducted to screen ferroptosis-related genes in WD. The expression of LCN2 and GPX4 in hippocampus of TX mice were detected by immunohistochemical. The expression of LCN2, NLRP3, GPX4, and SLC7A11 was determined in TX mice and HT22 cells by Western blotting and RT-qPCR. The levels of Fe²⁺, inflammatory factor indicators TNF- α , IL-1 β and IL-6 and oxidative stress indicators 4-HNE, MAD, SOD, GSH and ROS were detected in each group by ELISA.

Results: The results showed that GDL ameliorated pathological and mitochondrial damages in hippocampus of TX mice. The analysis of bioinformatics showed that LCN2 was a differential gene associated with ferroptosis in WD. The results of Western blotting and RT-qPCR indicated that GDL reduced the expression of LCN2 and NLRP3, and enhanced the expression of GPX4 and SLC7A11 in TX mice and HT22 cells. The ELISA results showed that GDL decreased the expression of Fe²⁺ and inflammatory factors TNF- α , IL-1 β and IL-6 in TX mice with ferroptosis inducer intervention and copper ion-loaded HT22 cells. GDL decreased the expression of oxidative stress indicators ROS, 4-HNE and MDA, and increased the expression of oxidative stress indicators GSH and SOD in TX mice and copper ion-loaded HT22 cells.

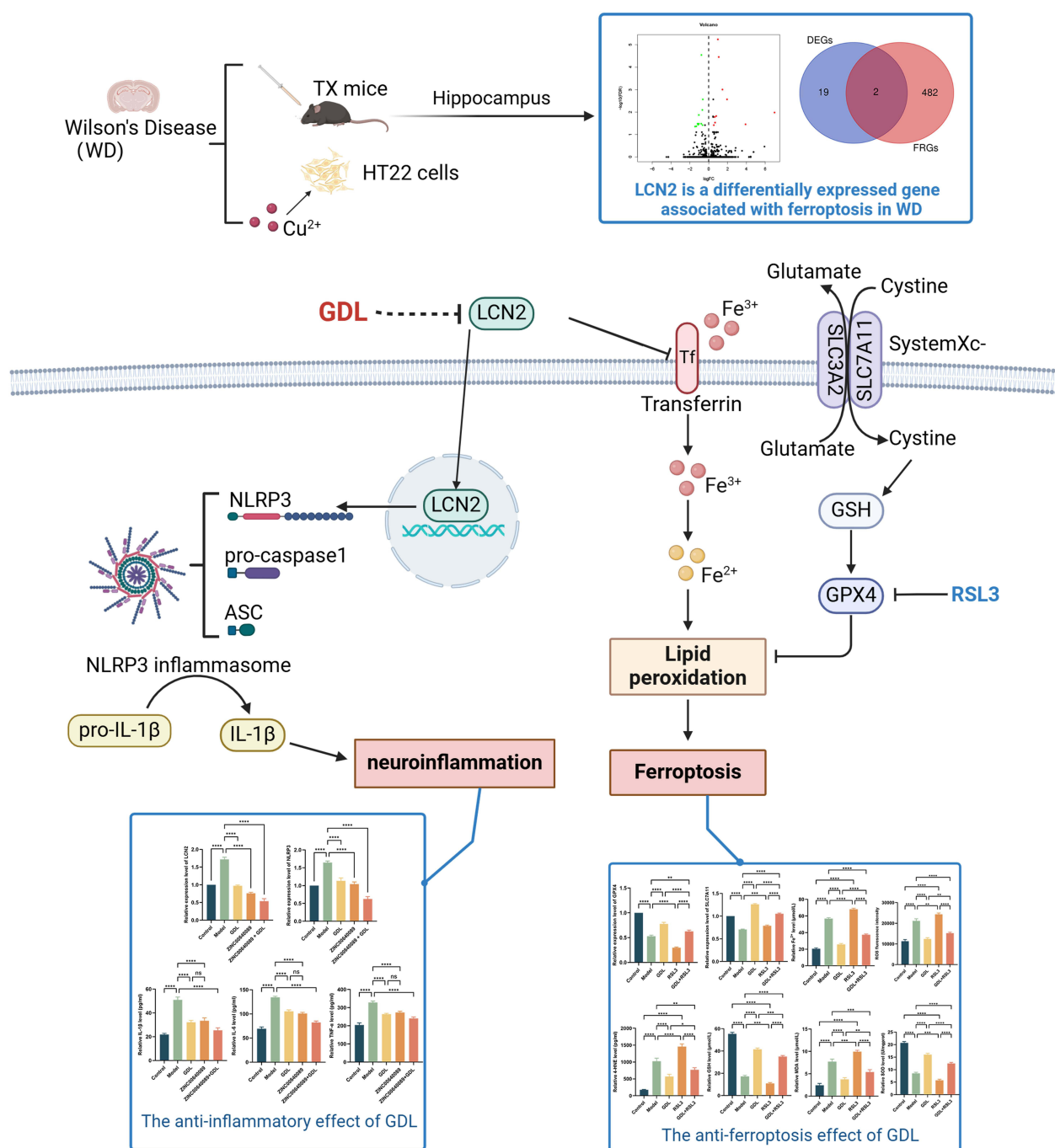
Conclusion: GDL has anti-inflammatory and antioxidant effects. LCN2 is a differential gene associated with ferroptosis in WD. GDL may alleviate ferroptosis by inhibiting the LCN2/NLRP3 signaling pathway, thereby improving neuroinflammatory responses and exerting neuroprotective effects in WD.

Keywords: neuroinflammation, ferroptosis, Wilson's disease, LCN2, NLRP3

Introduction

Wilson's disease (WD) is a genetic condition characterized by abnormal copper metabolism, inherited in an autosomal recessive manner.¹ Due to mutation of *ATP7B* in WD, ceruloplasmin in the serum decreases and elevated copper levels accumulates in the liver and brain tissue, eventually leading to liver and nerve injuries.^{2,3} Growing evidence suggests that inflammation in the nervous system is prevalent in WD.^{4,5} Metal-chelating agents such as penicillamine are of limited benefit in the clinical treatment of WD because of their many side effects. Currently, the treatment of WD using traditional Chinese medicines is widely recognized.

Graphical Abstract



Ferroptosis is a form of cell death that depends on iron and is induced by lipid peroxidation.⁶ Glutathione peroxidase 4 (GPX4), a key controller within the ferroptotic pathway,⁷ acts to regulate lipid peroxidation.^{8,9} SLC7A11 promotes glutathione synthesis by mediating the transport of cysteine and glutamic acid.^{10,11} The inactivation of GPX4 leads to the accumulation of lipid hydroperoxides, eventually inducing ferroptosis.^{12,13} Ferroptosis is observed in diverse neurodegenerative conditions, encompassing Alzheimer's disease (AD) and Parkinson's disease.¹⁴ It has been shown that

impaired iron metabolism also plays an important role in the pathogenesis of WD.¹⁵ Research has shown that penicillamine treatment for WD can ameliorate neuronal damage by inhibiting ferroptosis.¹⁶

Although ferroptosis involves lipid peroxidation, it is also associated with inflammatory reactions in various diseases.^{17–19} Studies have demonstrated that the accumulation of iron can initiate the activation of microglia and the generation of IL-1 β in AD.²⁰ Research on the relationship between ferroptosis and brain injury inflammation has shown that administration of the ferroptosis inducer RSL3 in the ventricles of rats with intracerebral hemorrhage exacerbates neuroinflammation, while upregulation of GPX4 reduces the concentrations of IL-1 β and TNF- α in both serum and cerebrospinal fluid.^{18,21} The activation of typical NLR family pyrin domain-containing 3 protein (NLRP3) inflammasomes is highly sensitive to copper accumulation in the brain and blocking the activation of NLRP3 inflammasomes can reduce IL-1 β production, thus effectively alleviating neurodegeneration in TX mice.²² Lipocalin-2 (LCN2) is a key protein in iron metabolism that maintains cellular homeostasis by chelating iron carriers.²³ Erastin and RSL3 are ferroptosis inducers that also induce LCN2 expression.²⁴ LCN2 induces the activation of NLRP3 inflammasomes through Toll-like receptor signaling pathway, thereby triggering inflammatory responses.²⁵ Studies have demonstrated that the absence of LCN2 can mitigate brain injury-induced behavioral disorder and neuroinflammation.²⁶

Gandouling (GDL) was formulated by the First Affiliated Hospital of Anhui University of Traditional Chinese Medicine, based on the characteristics of phlegm and blood stasis in WD. GDL is made up of *Coptis chinensis* Franch, *Salvia miltiorrhiza* Bunge, *Spatholobus suberectus* Dunn, *Rheum officinale* Baill, *Curcuma aeruginosa* Roxb, *Curcuma longa* L in the ratio 27:14:14:12:12:12.²⁷ The name of the plants were retrieved on December 24, 2023 (<http://www.worldfloraonline.org>). Chrysophanol, the primary constituent of *Rheum officinale* Baill, possesses the capability to diminish the accumulation of reactive oxygen species (ROS) and inhibit ferroptosis.²⁸ Extract Berberine from *Coptis chinensis* Franch can improve ferroptosis in the brain of AD mice.²⁹ Tanshinone extracted by *Salvia miltiorrhiza* Bunge significantly inhibits the activation of NF- κ B signaling pathway and the generation of pro-inflammatory cytokine.³⁰ *Coptis chinensis* Franch extract berberine and palmatine improves the survival of neurons and inhibits neuroinflammation.³¹ GDL used in the treatment of WD can delay the development of neurological symptoms in patients in multiple ways, with no toxicity or severe side effects like those of metal-chelating agents. Our preliminary research has shown that GDL demonstrates anti-inflammatory and antioxidant properties in WD by activating Nrf2 signaling and inhibiting NLRP3 inflammasomes.³²

In summary, ferroptosis and neuroinflammation are observed in WD, with ferroptosis may be involved in the pathogenesis of neuroinflammation. LCN2, as a key gene in ferroptosis, also activates the NLRP3 inflammasome. GDL exhibits significant anti-inflammatory and anti-oxidative stress. Based on these studies, we proposed the hypothesis that GDL may regulate the LCN2/NLRP3 signaling pathway to improve ferroptosis and neuroinflammation in WD. In this study, we chose TX mice and copper-loaded HT22 cells as WD models to investigate the therapeutic effect of GDL on WD and the effect of the LCN2/NLRP3 pathway. The purpose of this study is to clarify the potential regulatory mechanism of GDL on neuroinflammation at the molecular level and to provide a theoretical basis for the prevention and treatment of WD.

Materials and Methods

Materials and Reagents

GDL tablets (Z20050071) and glutathione tablets (23270071) were provided by The First Affiliated Hospital of Anhui University of Chinese Medical (Hefei, China). Ferrostatin-1 (Fer-I; HY-100579), RSL3 (ferroptosis agonist; [HY-100218A](#)) and ZINC00640089 (a specific Lipocalin-2 inhibitor; [HY-Q45780](#)) were purchased from MCE Co. (Shanghai, China). Total RNA Mini-Preps Kit (RJH09-01) was obtained from Magen Biotechnology Co. (Guangzhou, China). A reverse transcription kit (R202-02) was obtained from EnzyArtisan Biotechnology Co. (Shanghai, China). Cell Counting Kit-8 (CCK8; CT0001-A) was obtained from Sparkjade Biotechnology Co. (Jinan, China). Anti-rabbit β -actin antibody (AF0001) was purchased from Beyotime Biotechnology Co. (Shanghai, China). Anti-rabbit LCN2 antibody (PB0641) was provided by Boster Co. (Wuhan, China). Anti-rabbit NLRP3 antibody (DF15549) was purchased from Affinity Co. (Jiangsu, China). Anti-mouse GPX4 antibody (67763-1-Ig) and anti-rabbit SLC7A11 antibody (26864-1-AP) were purchased from Proteintech (Wuhan, China). TNF- α enzyme-linked immunosorbent assay (ELISA) kit (MAN0017523), IL-1 β ELISA

kit (MAN0017504) and IL-6 ELISA kit (MAN0017508) was purchased from Jiancheng Co. (Nanjing, China). 4-HNE ELISA kit (ELK8373) was obtained from Elabscience Co. (Wuhan, China). The determination kit of ferrous ion (Fe^{2+} ; E-BC-K773-M), malondialdehyde (MDA; A003-1), trace reducing glutathione (GSH; A001-3), and superoxide dismutase (SOD; A006-2-1) was purchased from Jiancheng Co. (Nanjing, China). Reactive oxygen species (ROS) determination kit (BB-460512) was purchased from Beibo Co. (Shanghai, China).

Animals and Grouping

The animal experiments in this study were conducted based on 《Guide for the Care and Use of Laboratory Animals, 8th edition》 (ISBN-13: 978-0-309-15,400-0). Seventy specific-pathogen-free male C3He-Atp7bTX-J/J (TX) mice weighing 20–25 g (6 months) were provided by the Laboratory Animal Center of Anhui University of Chinese Medicine. These mice were randomly divided into model, GDL, glutathione (Glu), Fer-1, RSL3, and RSL3+GDL groups, and ten specific-pathogen-free male wildtype (WT) mice were selected as the control group. The dosage of the administration group was converted to equivalent dose based on 9.1 times the daily dosage of an adult weighing 70 kg. Based on multiple research findings of our research group, the dosage of the GDL group was 1.16 g/kg/d by gavage.^{33–35} And the Glu group was 0.18 g/kg/d by gavage. Other groups were given equal volumes of physiological saline by gavage for 6 consecutive weeks; In the last two weeks, Fer-1 group mice were received intraperitoneal injections of Fer-1 (1 mg/kg/d, dissolved in 1% DMSO+50% PEG 300+5% Tween 80), RSL3 group mice were injected intraperitoneally with 250 mg/kg RSL3 (twice a week),³⁶ RSL3+GDL group mice were given GDL gavage at the same time, and the remaining groups were intraperitoneal injected with an equal amount of physiological saline. After the end of oral gavage and intraperitoneal injections, fasting for 12 hours, 1% pentobarbital sodium (50 mg/kg) was injected intraperitoneally for anesthesia. The hippocampal tissues of each group of mice were taken and frozen in liquid nitrogen, stored at -80°C , and fixed with 4% paraformaldehyde. All animal protocols were approved by the Institutional Animal Care and Use Committee of Anhui University of Chinese Medicine (approval number: AHUCM-mouse-2021011).

Hematoxylin and Eosin Staining

Paraffin-embedded brain tissue was cut along the coronal plane into 2- μm slices. Routine hematoxylin and eosin (HE) staining was performed under an optical microscope (BX51, OLYMPUS, JPN) after routine dewaxing and dehydration to evaluate the pathological status of the hippocampal tissue.

Transmission Electron Microscopy

Hippocampal CA3 tissue (1 mm in diameter) was quickly placed on ice and fixed sequentially with 2.5% glutaraldehyde and osmium tetroxide. The samples were immersed in acetone and embedding solution overnight. The samples were subsequently sliced into 50-nanometer sections and stained with uranyl acetate and lead citrate for a duration of 10 minutes each. Changes in mitochondrial ultrastructure and microtubules in hippocampal neurons from mice in each group were observed by TEM (FEI, Hillsboro, OR, USA).

High-Throughput Whole Transcriptome Sequencing Analysis

Experimental Method

Total RNA was extracted from mouse hippocampal tissues using MiRNeasy Mini Kit (Cat#217004, Qiagen, Germany). Then, RNA was purified using VAHTS RNA Clean Beads (N412-01, Vazyme, CN), DNase I, and RNase-free (EN401, Vazyme, CN). Quality control (QC) was achieved using NanoDrop 2100 (Thermo Fisher Scientific, United States). Small RNA (sRNA) and whole transcriptome libraries were generated using the QIAseq miRNA Library Kit (Cat#331505, Qiagen, Germany) and VAHTS Total RNA-seq (H/M/R) Library Prep Kit (NR603-01, Vazyme, China) following the manufacturers' instructions. All libraries were quantified using Agilent 2100 Bioanalyzer and Qubit[®] 3.0 fluorometer (Invitrogen; Thermo Fisher Scientific, Inc). Based on Illumina HiSeq sequencing platform, all libraries were sequenced on second generation sequencing technology (Next-Generation Sequencing, NGS).

Transcriptome Analysis Flow

HTSeq statistics were employed for the comparison of gene-specific read counts, and subsequently, fragments per kilobase of transcript per million mapped reads was utilized to normalize the expression. The analysis of gene expression variation was conducted using DESeq, applying screening conditions are $|\log_2\text{FoldChange}| > 1$ and significant P -value < 0.05 . The R package “pheatmap” was used to perform bi-directional clustering analysis. The R package “topGO” was used to perform Gene Ontology (GO) enrichment analysis on the differential genes. ClusterProfiler was employed for the implementation of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. The STRING database was used for protein interaction analysis to reveal the relationship between target genes. Ferroptosis-related genes were obtained from FerrDB and performed an intersection analysis with the differentially expressed genes.

Immunohistochemistry

Brain tissues were fixed, dehydrated, and rendered transparent with neutral formaldehyde, followed by baking at 60°C for 15 minutes. Subsequently, the samples were embedded in paraffin and cut into 4 μm sections. The slices were dewaxed with xylene and washed with an ethanol gradient for dehydration. After rewarming, the slices were incubated in secondary antibody at 20°C for 2 h and stained with DAB for 10 min while the chromogenic time was controlled under a microscope. The sections were stained with hematoxylin and differentiated using 1% hydrochloric acid–ethanol. Gradient dehydration was performed with ethanol. The sections were sealed with neutral resin, and the observations were made using a microscope.

Cell Culture and Grouping

HT22 cells (CL-0697) were provided by Procell (Wuhan, China). These cells were cultured in DMEM supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum at 37°C under 5% CO_2 . HT22 cells were divided into 5 groups: control (cultured in only DMEM), model (cultured in DMEM and optimal concentration of copper ions), GDL (cultured in DMEM and optimal concentrations of copper ions and GDL), ZINC00640089 (cultured in DMEM and optimal concentrations of copper ions and ZINC00640089), and ZINC00640089 + GDL (cultured in DMEM and optimal concentrations of copper ions, ZINC00640089, and GDL). ZINC00640089 is a specific Lipocalin-2 inhibitor.

Preparation and Preservation of GDL-Medicated Serum

Forty Sprague–Dawley (SD) rats were randomly divided into control group and GDL group, with 20 rats in each group. The dosage of the GDL group rats were gavaged with 0.4 g/kg/day of GDL tablets. The control group was given an equal amount of distilled water. Once a day for 6 days by gavage. After the last gavage and fasting for 12 h, day 7 after 1 h of gavage, 1% pentobarbital sodium was injected intraperitoneally under anesthesia, and blood was collected from the abdominal aorta under sterile conditions. The supernatant was centrifuged (4000r/min, 15min), incubated in a water bath at 56°C for 30 min, and filtered with a 0.22- μm filter. The filtrate was stored at –20°C for future use.

Cytotoxicity Assay

The optimal action time and concentration of Cu^{2+} and GDL was determined by the CCK8 method. HT22 cells were seeded at a density of $1.0 \times 10^5/\text{mL}$ in a 96-well culture plate and were incubated with 100 μL medium per well at 37°C and 5% CO_2 . Cells were grown overnight in DMEM containing 10% fetal bovine serum by volume. HT22 cells were divided into seven groups and 100 μL of different CuCl_2 concentrations in culture medium (100, 150, 200, 250, 300, 350, and 400 $\mu\text{mol/L}$) were added to each group. CuCl_2 (300 $\mu\text{mol/L}$) was added to stimulate HT22 cells and 100 μL of the different concentrations of GDL-medicated serum (15%, 20%, 25%, 30%, 35%, and 40%) were added to the wells containing copper ions. Simultaneously, a blank control group and Cu^{2+} loading group were set up. Each sample was placed in six compound wells and incubated for 12, 24, 36, or 48 h. Then, 5 μL CCK8 and 95 μL of complete culture medium were added to each well and incubated for 2 h. The solution was mixed well and the absorbance was measured at 570 nm using an enzyme-labeled instrument. After observing the effects of different concentrations of GDL-containing serum for different time periods, the IC_{50} value was calculated to determine the optimal concentration and action time of the Cu^{2+} and GDL-containing serum for subsequent experiments.

Detection of Cellular ROS Levels by Flow Cytometry

According to the above groupings, HT22 cells were gathered into a flow cytometry tube and underwent three washes using phosphate-buffered saline (PBS) centrifugation. The 2,7-DCFH-DA probe was incubated for 20 min and manually mixed once every 5 min. After incubation, the samples underwent three washes using serum-free medium and were then resuspended in 300 μ L PBS. Finally, the samples were placed on ice for further analysis.

Western Blotting

Fresh hippocampus proteins and total cellular proteins were extracted separately. After sampling and loading, gel electrophoresis and membrane transfer were performed. Membranes were exposed to the primary antibody overnight at 4°C, and subsequently, the secondary antibody was applied at room temperature for 2 hours. Electrochemiluminescent imaging was performed, and β -actin was used as a reference to calculate LCN2, NLRP3, GPX4, and SLC7A11 protein levels.

RT-qPCR

Total RNA was extracted from the hippocampus or HT22 cells using Trizol (Thermo Fisher Scientific) for subsequent reverse transcription. The total reaction volume was 20 μ L and was heated to 42°C in a water bath for 1 h, and for reverse-transcription quantitative PCR (RT-qPCR), single-stranded cDNA was generated. Each qPCR reaction volume was 20 μ L. Using *GADPH* as the reference, the expression levels of *LCN2*, *NLRP3*, *GPX4*, and *SLC7A11* were analyzed by calculating the relative expression level using the $2^{-\Delta\Delta CT}$ method. The following primers were used: *GADPH*, forward GTGTTCTACCCCAATGTG, reverse GTCATTGAGAGCAATGCCAG; *LCN2*: forward TGTCATGTGTCTG GGCCTTG, reverse AACTGATCGCTCCGGAAGTC; *NLRP3*: forward TCACAACTCGCCCAAGGAGGAA, reverse AAGAGACCACGGCAGAAGCTAG; *GPX4*: forward GACGCCAAAGTCCTAGGAAAC, reverse CCGGGTTG AAAGGTTTCAGGA; *SLC7A11*: forward GGTGTGTAATGATAGGGCAGCA, reverse TTTGCTATCACCGACTGGCT.

Determination of Fe²⁺, ROS, 4-HNE, GSH, MDA and SOD Levels

To determine the levels of Fe²⁺, ROS, 4-HNE, GSH, MDA and SOD in mice hippocampus or HT22 cells, we used Fe²⁺, ROS, GSH, MDA and SOD assay kits according to the manufacturer's instructions. To assess the level of the lipid peroxidation marker 4-HNE, we used the ELISA kit of 4-HNE according to the manufacturer's instructions.

Statistical Analysis

SPSS 25.0 (IBM, Armonk, NY) was used for statistical analysis, and single-factor analysis of variance was used for comparison between multiple groups. Tukey's test was involved in correcting for multiple comparisons. Differences were considered statistically significant if $P < 0.05$.

Result

GDL Improves Pathological Damage in Hippocampus of TX Mice

The control mice had more neurons in hippocampus with plump morphology and regular arrangement. The cells were lightly stained, without obvious nuclear pyknosis, exhibiting intact cell structure, distinct nucleoli, and no pathological damage. Compared to the control group, the model group showed significant pathological damage to neurons, with loose cell arrangement and disordered tissues. The cells were deeply stained with irregular cell morphology, nuclear pyknosis, decreased neuronal density, and neuronal atrophy. Compared to the model group, the pathological damage in the hippocampal tissue of the GDL, Glu, and Fer-1 groups was significantly reduced (Figure 1).

GDL Improves Mitochondrial Damage in TX Mice

The control mice had many intact mitochondria in the hippocampus. In contrast, the hippocampi of the model mice had significantly fewer mitochondria; some mitochondria showed swelling and vacuoles, and the mitochondrial cristae had dissolved and disappeared. The mitochondrial damage in the GDL, Glu, and Fer-1 groups was significantly improved (Figure 2).

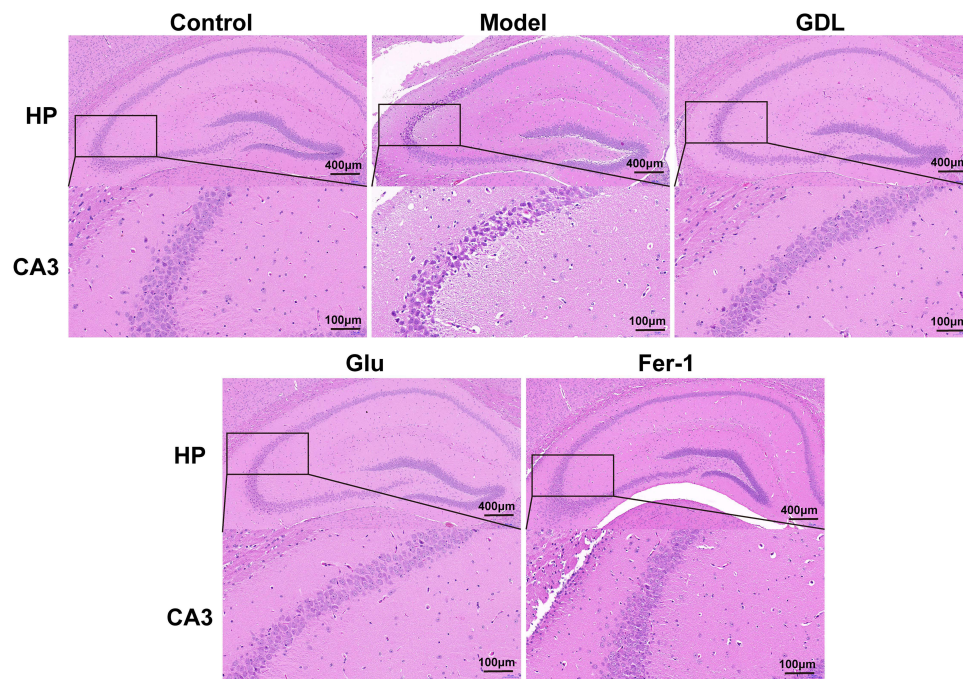


Figure 1 GDL improves pathological damage in TX mouse hippocampal tissue ($n = 3$ mice/group). HE staining in HP and CA3 region of mice hippocampus (100x, bar = 400 μ m; 400x, bar = 100 μ m).

LCN2 is a Differential Gene Involved with Ferroptosis in WD

First, we identified 18,065 protein-coding genes from six samples. Further, 21 DEMs were filtered from the hippocampus of WD mice, including 10 upregulated and 11 downregulated DEMs ($|\log_2\text{FoldChange}| > 0.5$, $P < 0.05$). The results were visualized via volcano and hierarchical clustering plots (Figure 3A). The higher the Pearson correlation coefficient approaches 1, the more pronounced the similarity in expression patterns between samples. An extremely strong correlation was indicated by a coefficient ranging from 0.8 to 1, as illustrated in Figure 3B. Eleven KEGG signaling pathways were identified, with the top pathway being the MAPK signaling pathway (Figure 3C). To further identify the 21 DEPs, GO enrichment analysis was performed, which revealed that 514 GO terms were significantly enriched. GO biological process items included “skeletal muscle cell differentiation”, “skeletal muscle tissue development”, and “regulation of cysteine-type endopeptidase activity involved in the apoptotic process”. Cell component terms included “apical plasma membrane”, “hemoglobin complex”, and “haptoglobin-hemoglobin complex”. Finally, molecular function terms included “RNA polymerase II-specific DNA-binding”, “transcription factor binding”, and “iron ion binding” (Figure 3D). To understand the interactions between proteins translated from mRNAs, the PPI network of DEGs was constructed according to the information on the STRING database. The screening criterion was a minimum required interaction score > 0.4 , and free points were hidden (Figure 3E). To delve deeper into the connection between differentially expressed WD genes and ferroptosis, we obtained 484 ferroptosis-related genes from FerrDB and performed an intersection analysis with the differentially expressed WD genes. We identified two ferroptosis-related genes in the differentially expressed WD genes: *NR4A1* and *LCN2*. The Venn plot is shown in Figure 3.

GDL Regulates the Expression of LCN2 and Ferroptosis-Related Indicator GPX4

The immunohistochemical results of the mouse frontal and temporal cortices showed a significant increase in LCN2 staining in the model group compared to control, while a decrease in the GDL group (Figure 4A and B). In addition, GPX4 staining was decreased in the model group and increased in the GDL group (Figure 4C and D).

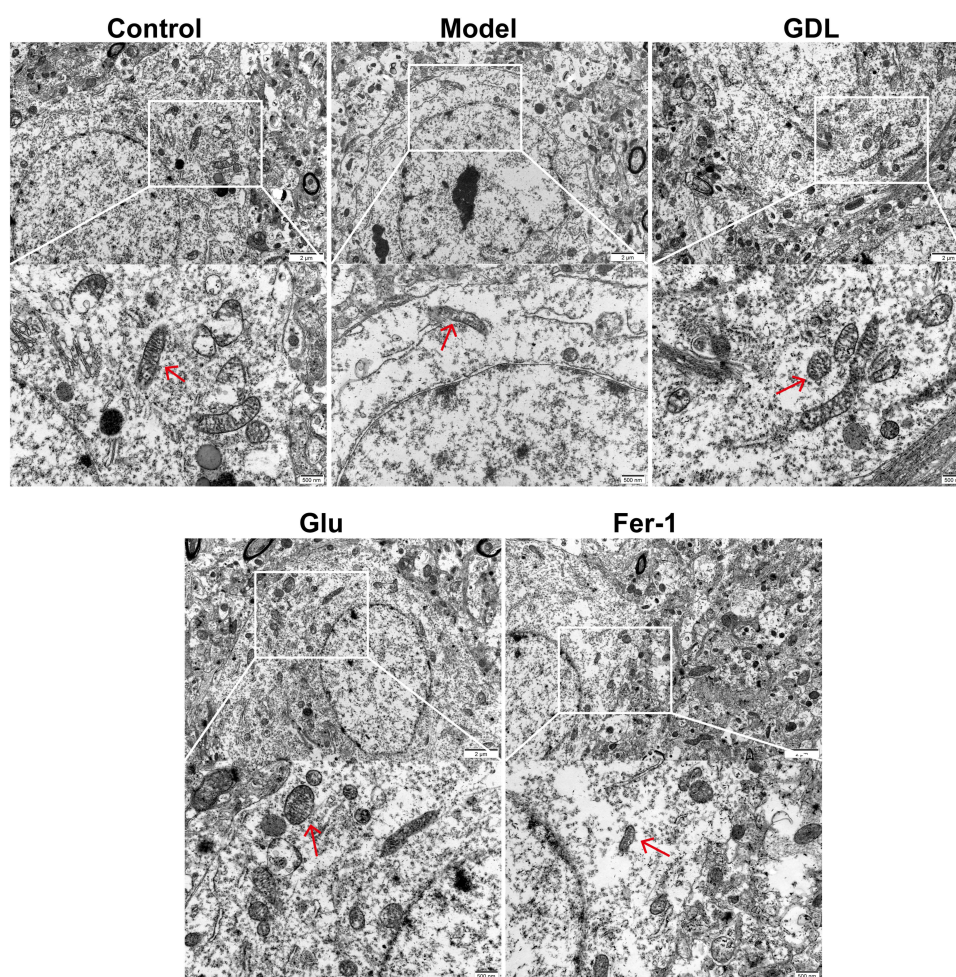


Figure 2 GDL improves mitochondrial damage in TX mice ($n = 3$ mice/group). Ultrastructure of hippocampus observed under TEM, with red arrows pointing to mitochondria (scale bar = 2 μ m and 500 nm).

GDL Improves Ferroptosis and Neuroinflammation in TX Mice Through the LCN2/NLRP3 Signaling Pathway

Protein expression levels of the signaling pathway indicators LCN2 and NLRP3 and ferroptosis indicators GPX4 and SLC7A11 in the hippocampus of mice in each group were detected using Western blotting (Figure 5A). The protein expression levels of LCN2 and NLRP3 in the model group were increased (Figure 5B and C), while those of GPX4 and SLC7A11 were reduced (Figure 5D and E). Compared with the model group, the expression levels of LCN2 and NLRP3 in the GDL, Glu, and Fer-1 groups were reduced, while ferroptosis indicators GPX4 and SLC7A11 were increased. Finally, the RT-qPCR results of the above indicators showed that the changes of the mRNA expression were consistent with the results of Western blotting (Figure 5F–I). The above results indicate that inhibiting LCN2 and ferroptosis may be the mechanism by which GDL improves WD neuroinflammation.

GDL Improves Ferroptosis and Neuroinflammation in RSL3-Induced TX Mice Through the LCN2/NLRP3 Signaling Pathway

RSL3 is a specific inducer of ferroptosis which promotes ferroptosis by inhibiting GPX4 expression. This study established *in vivo* models of ferroptosis by injecting RSL3 into TX mice.

The results of Western blotting revealed that increased protein expression of LCN2 and NLRP3 and decreased protein expression of GPX4 and SLC7A11 in the WD model and RSL3 group compared with the control group. Compared with

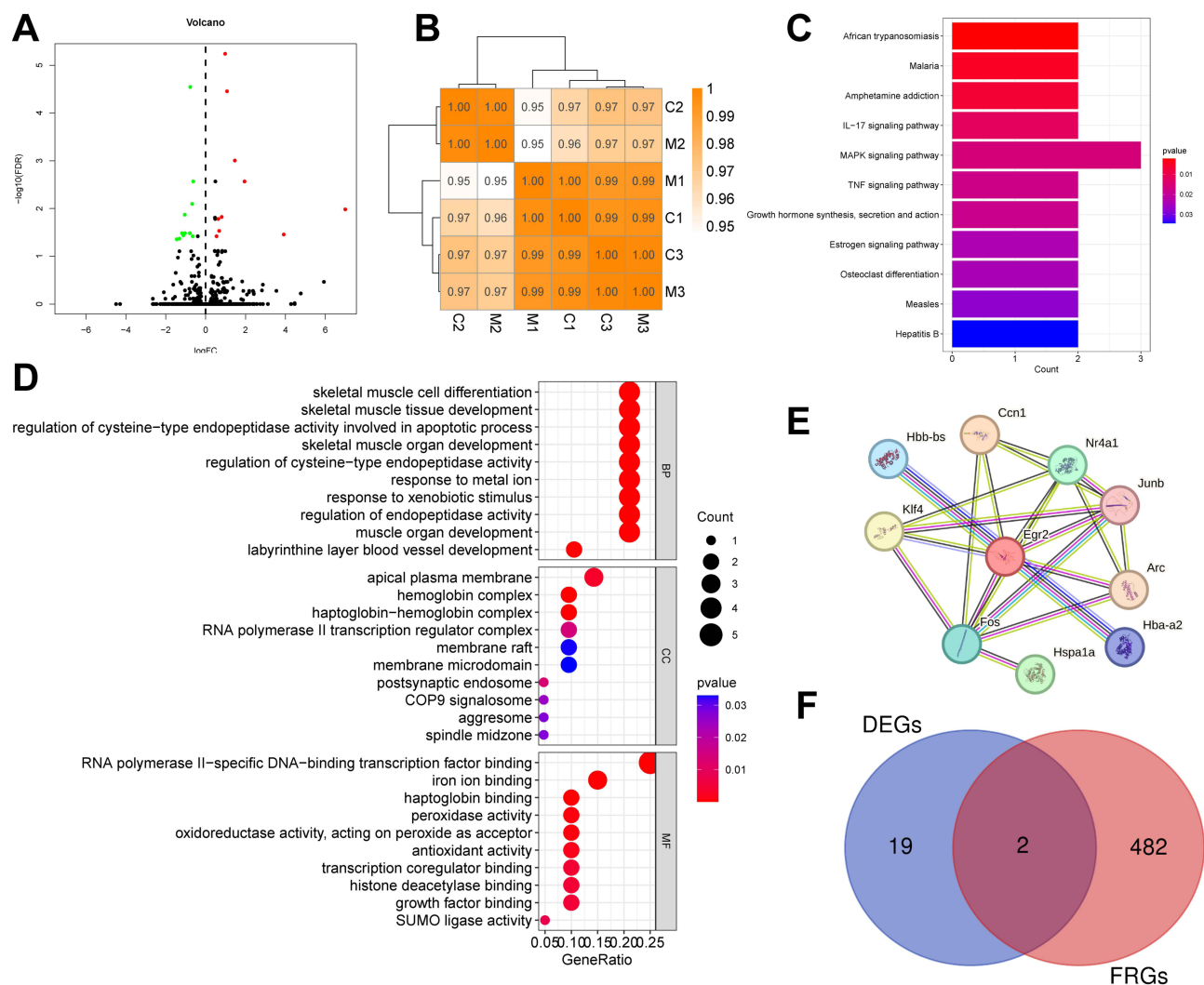


Figure 3 LCN2 is a differential gene involved with ferroptosis in WD. **(A)** Volcano plot of differentially expressed genes between wildtype and WD mice. The selection criteria were $|\log_2\text{FoldChange}| > 0.5$, $P < 0.05$. **(B)** Correlation of gene expression levels between samples. **(C)** Kyoto Encyclopedia of Genes and Genomes enrichment analysis. **(D)** Gene Ontology enrichment analysis. **(E)** Protein-protein interaction network **(F)** Venn diagram of intersection between differentially expressed WD genes and ferroptosis-related genes.

the model and RSL3 groups, the protein expression of LCN2 and NLRP3 decreased, while the protein expression of GPX4 and SLC7A11 increased after using GDL intervention (Figure 6A–E). In this study, RT-qPCR results of the above indicators showed that the changes of the mRNA expression were consistent with the results of Western blotting (Figure 6F–I). The above results displayed that GDL regulated ferroptosis induced by RSL3, suggesting that GDL has the potential to inhibit ferroptosis and inflammatory response.

GDL Improves Neuroinflammation and Oxidative Stress in TX Mice

Firstly, ELISA method was used to detect the content of inflammatory factors in the hippocampus of TX mice in each group. These results showed that the expression of TNF- α , IL-1 β and IL-6 in the model and RSL3 groups was increased compared with the control group, while the expression decreased after GDL intervention (Figure 7A–C). These results indicated that GDL inhibited inflammatory responses in ferroptosis models. In addition, compared with the control group, the levels of Fe²⁺, ROS, 4-HNE, and MDA were increased in the model and RSL3 groups, while the levels of GSH and SOD were significantly decreased. Compared with the model and RSL3 groups, the levels of Fe²⁺, ROS, 4-HNE, and MDA in the GDL and RSL3+GDL groups were significantly reduced, while the levels of GSH and SOD were

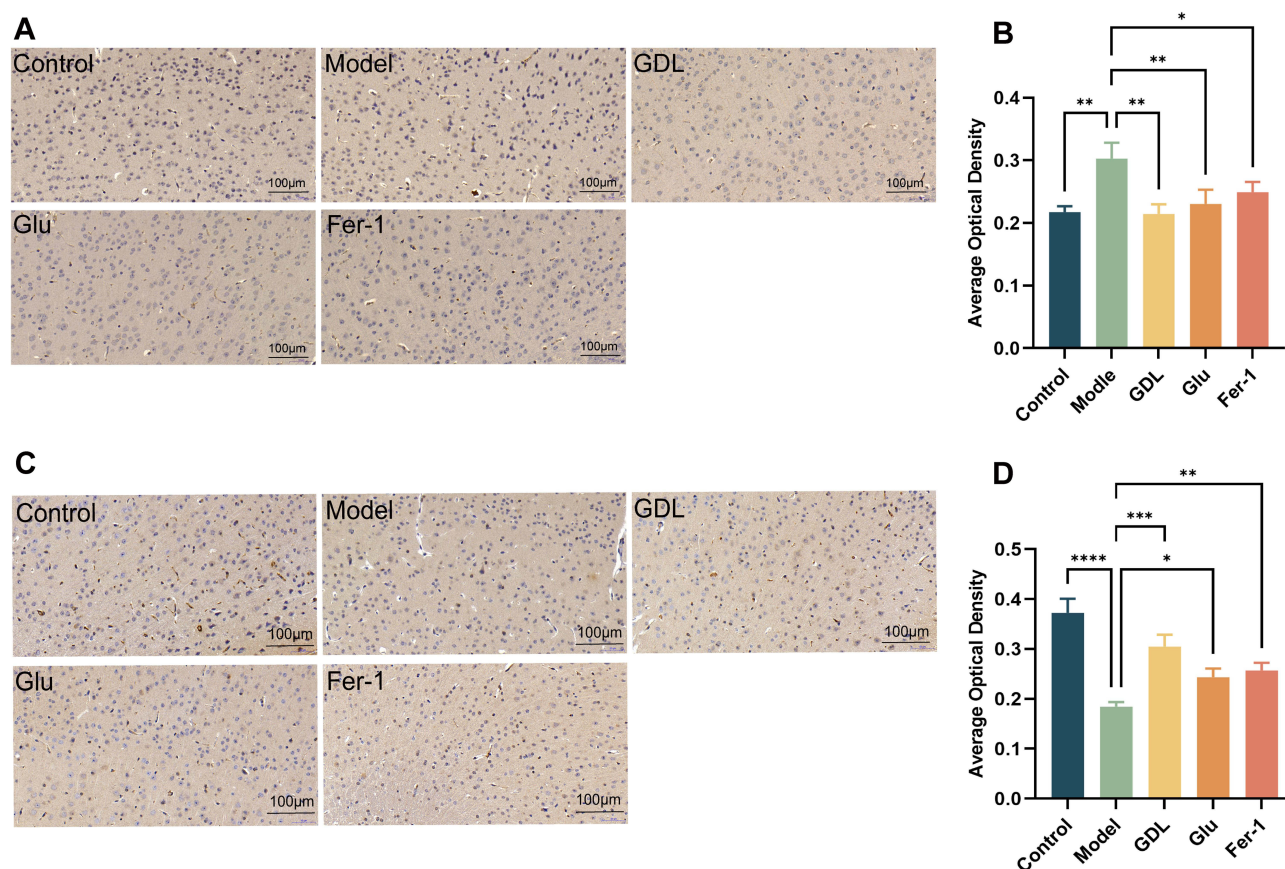


Figure 4 GDL regulates the expression of LCN2 and ferroptosis-related indicator GPX4. **(A)** Immunohistochemistry of hippocampus: GDL treatment decreased the expression of LCN2. **(B)** The quantification of average optical density of LCN2. **(C)** Immunohistochemistry of hippocampus: GDL treatment increased the expression of GPX4. **(D)** The quantification of average optical density of GPX4. Data are shown as the mean $\pm$ SD ($n=3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.001$.

significantly increased. (Figure 7D–I). These results suggested that the effect of GDL can reverse the changes in lipid peroxidation-related indicators and rescue ferroptosis in WD model.

GDL Improves Ferroptosis and Neuroinflammation in Copper Ion-Induced HT22 Cells Through the LCN2/NLRP3 Signaling Pathway

Firstly, the CCK8 method was used to screen for the optimal action time and concentration of Cu^{2+} and GDL-containing serum in HT22 cells. The higher the concentration of Cu^{2+} , the lower the cell survival rate. The cell survival rate was unstable when the Cu^{2+} acted for 12 h. When calculating the Cu^{2+} load for 24 h, the IC_{50} was 325.5 $\mu\text{mol/L}$, which was close to 300 $\mu\text{mol/L}$. At this point, the cells were found well adhering to the wall, and growth state was more stable. When using GDL-mediated serum to treat HT22 cells, cell viability began to decrease when the concentration of GDL-mediated serum reached 30% after 12, 24, and 36 h, and the highest cell viability was observed with 24 h of intervention. When the intervention lasted for 48 h, cell viability began to decrease after the GDL serum concentration reached 35%; however, cell viability was lower than that at other times. Therefore, 24 h and 30% GDL-mediated serum were chosen as the optimal action time and concentration, respectively (Figure 8A and B).

The protein and mRNA levels of the signaling pathway indicators and ferroptosis indicators in HT22 cells in each group were detected. The results are the expression of inflammatory indicators LCN2 and NLRP3 in the model group was significantly increased, and GDL and ZINC00640089 could reverse this increase. The expression of GPX4 and SLC7A11 in the model group significantly decreased. And the expression in the GDL, ZINC00640089, and ZINC00640089 + GDL groups increased compared to the model group. These results demonstrate that GDL could improve ferroptosis and inflammatory response in WD through the LCN2 pathway and play a role in protecting neurons (Figure 8C–K).

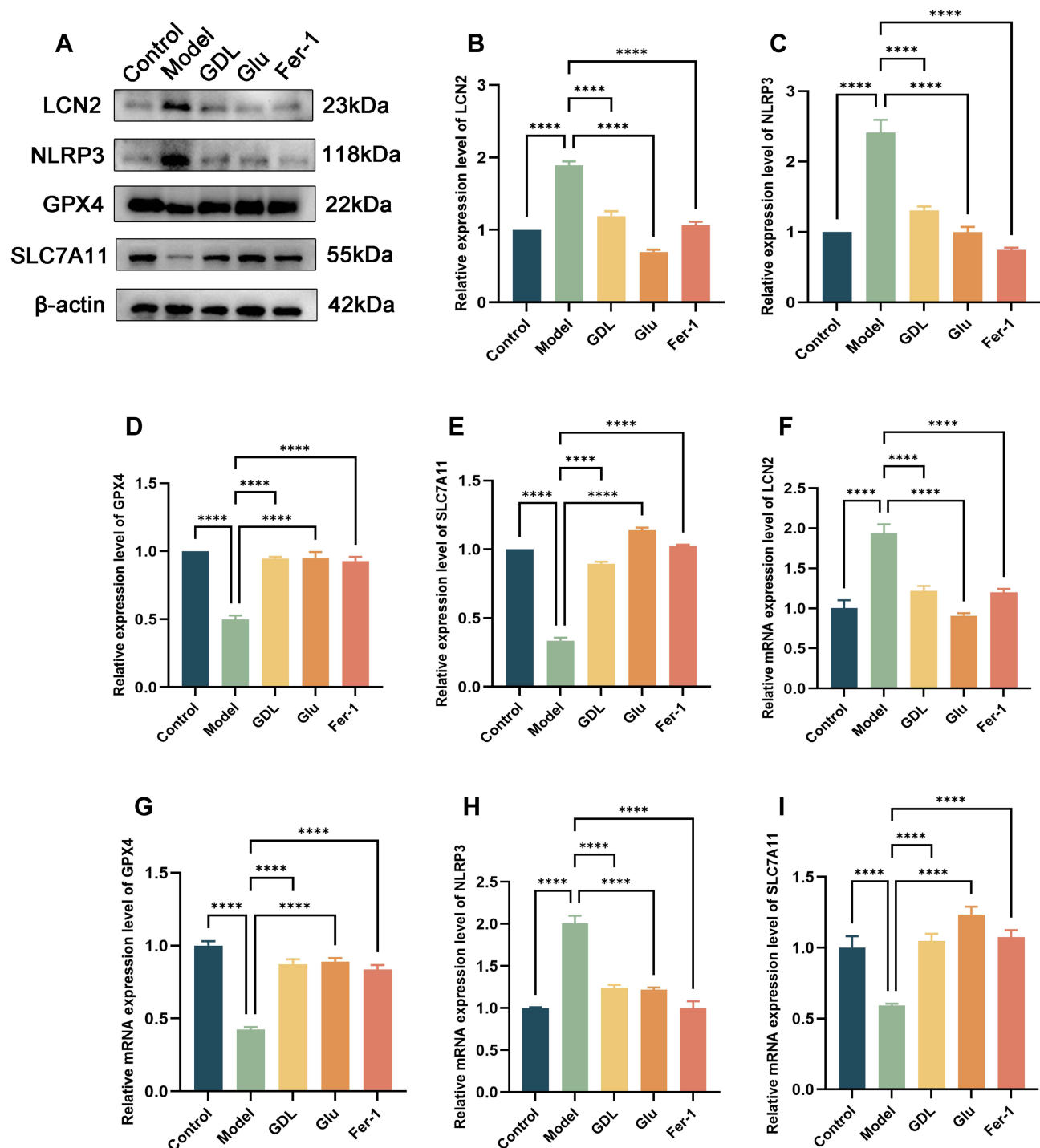


Figure 5 GDL improves ferroptosis and neuroinflammation in TX mice through the LCN2/NLRP3 signaling pathway. (A) Representative Western blots of LCN2, NLRP3, GPX4, and SLC7A11 in the hippocampus. (B–E) the protein expression levels of LCN2, NLRP3, GPX4, and SLC7A11. (F–I). RT-qPCR analysis of the mRNA expression levels of *Lcn2*, *Nlrp3*, *Gpx4*, and *Slc7a11*. Data are shown as the mean $\pm$ SD ($n=3$). **** $P < 0.001$, *** $P < 0.001$.

GDL Reduces the Content of ROS in Copper Ion-Induced HT22 Cells

The ROS content was detected by flow cytometry. The average fluorescence intensity of ROS increased in the model group stimulated by copper ions. The intervention of GDL and ZINC00640089 can reduce the content of ROS and improve oxidative stress (Figure 9A and B).

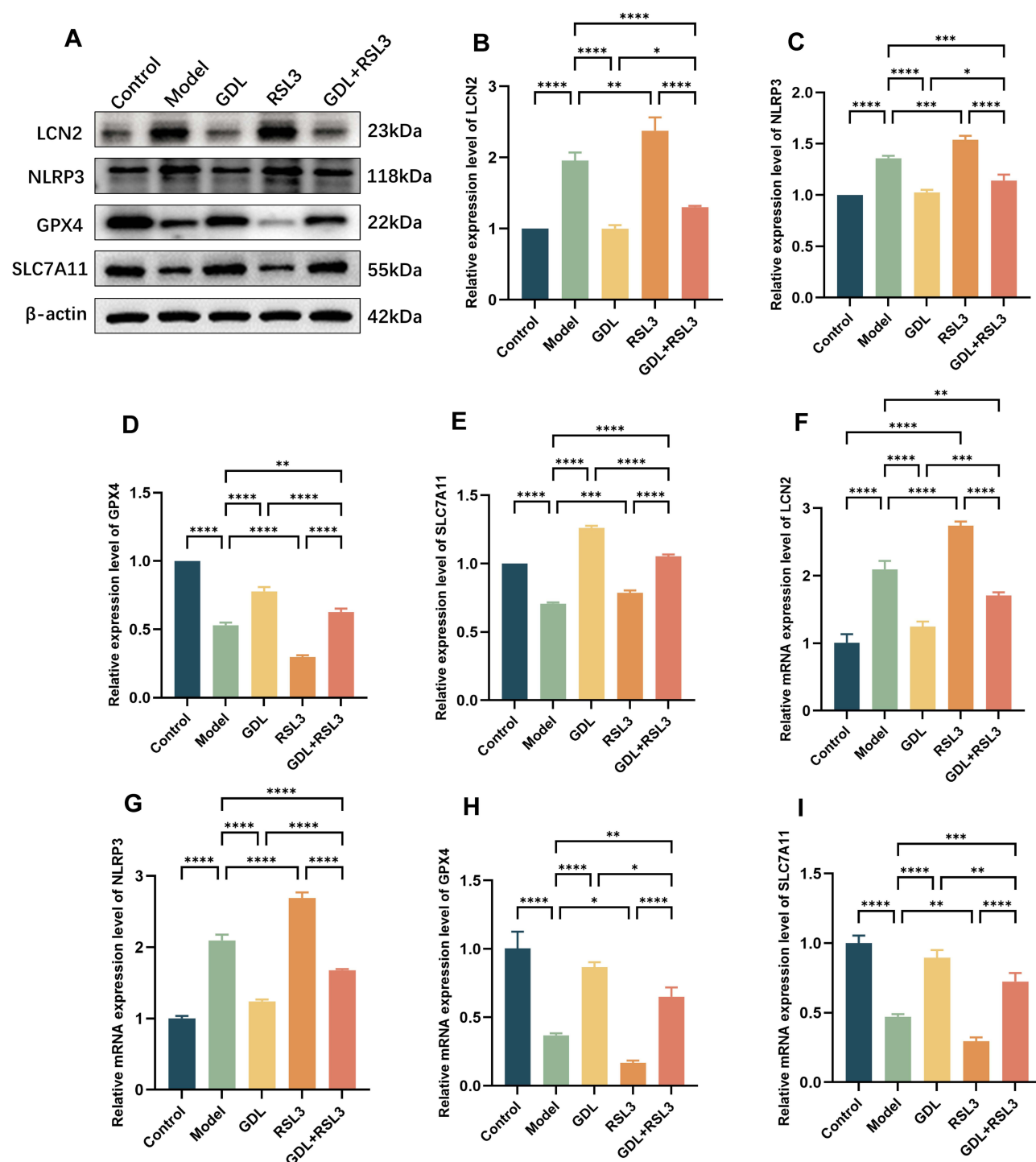


Figure 6 GDL improves ferroptosis and neuroinflammation in RSL3-induced TX mice through the LCN2/NLRP3 signaling pathway. (A) Representative Western blots of LCN2, NLRP3, GPX4, and SLC7A11 in the hippocampus. (B–E) the protein expression levels of LCN2, NLRP3, GPX4, and SLC7A11. (F–I). RT-qPCR analysis of the mRNA expression levels of *Lcn2*, *Nlrp3*, *Gpx4*, and *Slc7a11*. Data are shown as the mean $\pm$ SD ($n=3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

GDL Improves Neuroinflammation and Oxidative Stress in Copper Ion-Induced HT22 Cells

In vitro experiments, ZINC00640089, a specific inhibitors of LCN2, and GDL were used to intervene in copper ion-induced HT22 cells. ELISA method was used to detect the content of inflammatory factors and oxidative stress indicators

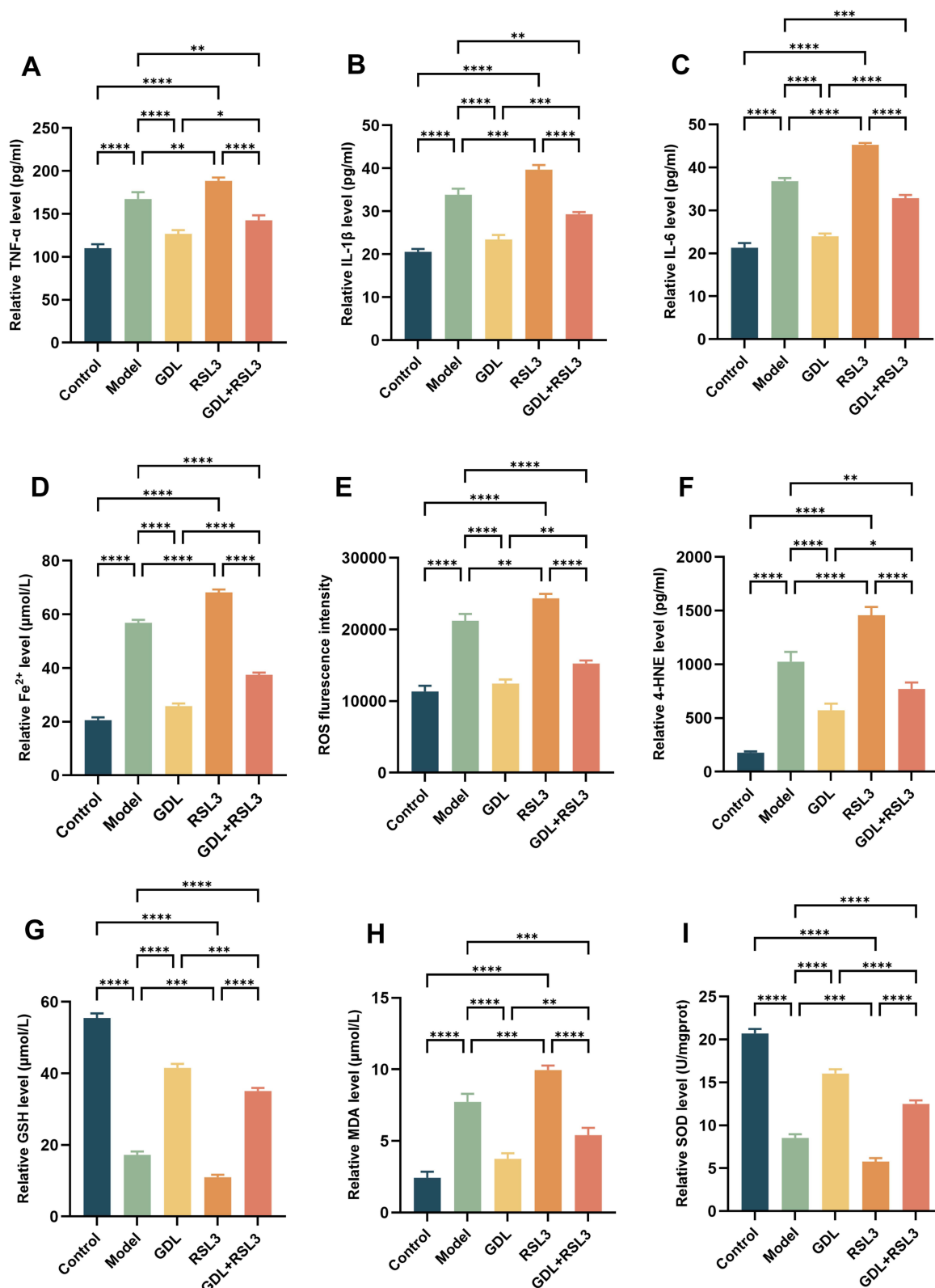


Figure 7 GDL improves neuroinflammation and oxidative stress in TX mice. (A–C) Determination of TNF- α , IL-1 β and IL-6 levels in mice hippocampus. (D–I) Determination of Fe²⁺, ROS, 4HNE, GSH, MDA and SOD levels in mice hippocampus. Data are shown as the mean $\pm$ SD (n=3). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

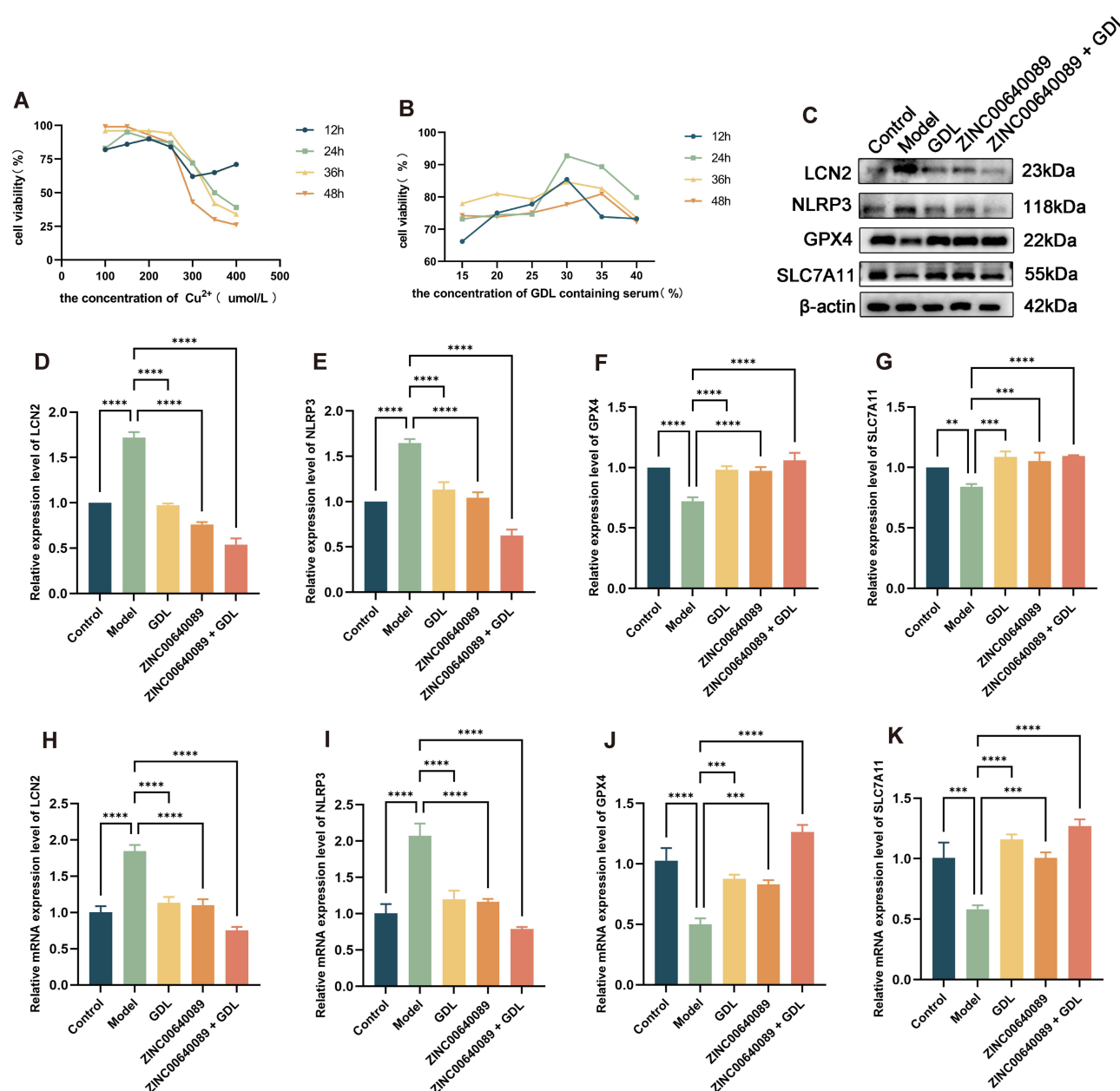


Figure 8 GDL improves ferroptosis and neuroinflammation in copper ion-induced HT22 cells through the LCN2/NLRP3 signaling pathway. **(A)** The viability rate of HT22 cells under the action of different concentrations of Cu^{2+} for different periods of time. **(B)** The viability rate of HT22 cells under the action of different concentrations of GDL containing serum for different periods of time. **(C)** Representative Western blots of LCN2, NLRP3, GPX4, and SLC7A11 in the HT22 cells. **(D–G)** The protein expression levels of LCN2, NLRP3, GPX4, and SLC7A11. **(H–K)** RT-qPCR analysis of the mRNA expression levels of *Lcn2*, *Nlrp3*, *Gpx4*, and *Slc7a11*. Data are shown as the mean $\pm$ SD ($n=3$). ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

in the HT22 cells. These results showed that the expression of $\text{TNF-}\alpha$, $\text{IL-1}\beta$ and IL-6 in the model groups was increased compared with the control group, while the expression decreased after GDL or ZINC00640089 intervention (Figure 10A–C). Then, compared with the control group, the levels of Fe^{2+} , 4-HNE, and MDA were increased in the model group, while the levels of GSH and SOD were decreased. Compared with the model group, the levels of Fe^{2+} , 4-HNE, and MDA in the GDL and ZINC00640089 groups were reduced, while the levels of GSH and SOD were increased. (Figure 10D–H). These results suggested that GDL can inhibit oxidative stress and inflammatory response and may be related to the LCN2 signaling pathway.

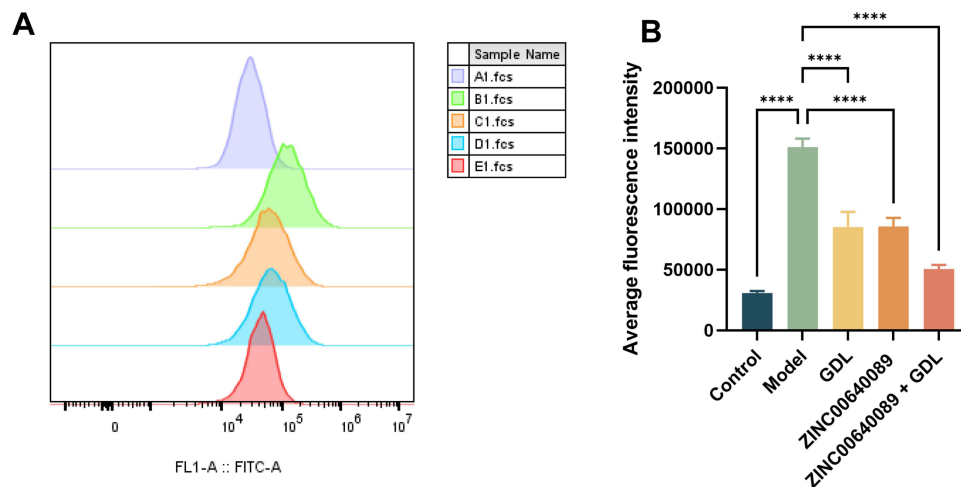


Figure 9 GDL reduces the content of ROS in the HT22 cells stimulated by copper ions. **(A)** Flow cytometry detected the content of reactive oxygen species (ROS) (A1: control group; B1: model group; C1: GDL group; D1: ZINC00640089; E1: ZINC00640089 + GDL group). **(B)** average fluorescence intensity of ROS. Data are shown as the mean $\pm$ SD (n=3), ****P < 0.001.

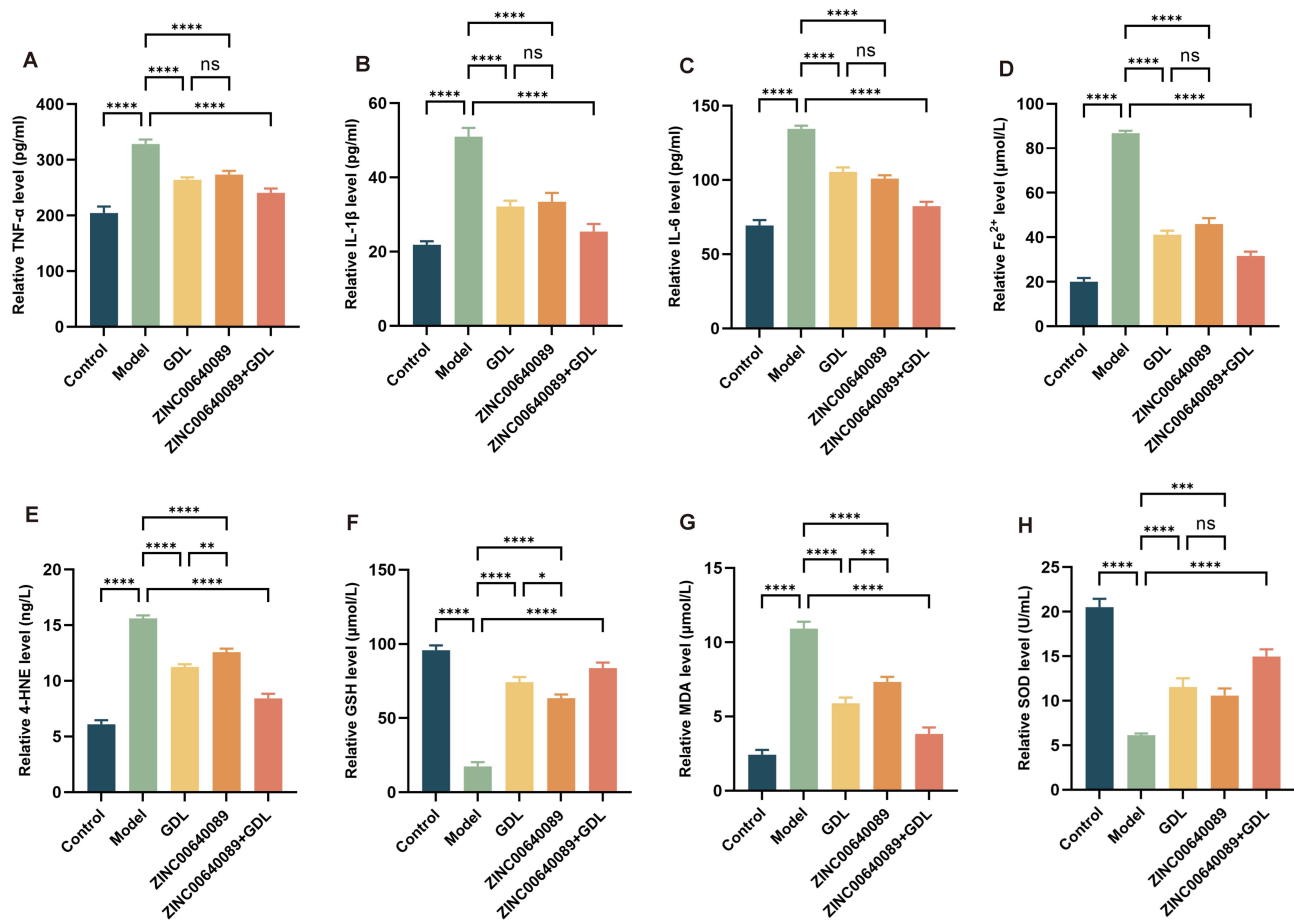


Figure 10 GDL improves neuroinflammation and oxidative stress in copper-loaded HT22 cells. **(A–C)** Determination of TNF- α , IL-1 β and IL-6 levels in HT22 cells. **(D–H)** Determination of Fe²⁺, 4-HNE, GSH, MDA and SOD levels in mice hippocampus. Data are shown as the mean $\pm$ SD (n=3). *P < 0.05, **P < 0.01, ****P < 0.001.

Discussion

GDL, a specific drug developed by our hospital for the treatment of WD, has been used clinically for nearly 20 years with significant therapeutic benefits. Modern pharmacological studies have shown that *Coptis chinensis Franch* reduces ROS production and fights inflammatory.³³ Tanshinone also exhibits anti-inflammatory and antioxidant effects.³⁴ Recent studies have indicated that curcumin has the potential to suppress ferroptosis by activating GPX4, facilitating copper excretion, and providing protection against WD.³⁵ While copper overload occurs in WD, a disorder in iron metabolism is also present. Studies in China and elsewhere have shown the presence of ferroptosis in WD.^{36–38} In the initial phases of WD, substantial research has been undertaken, which has proven the presence of neuroinflammation in WD and that GDL can effectively alleviate neuroinflammation through the NLRP3 or Nrf2 pathways.^{32,39}

WD is an autosomal recessive disorder characterized by copper accumulating in the liver, brain and kidneys. Neurological symptoms are one of the common clinical symptoms. Our previous study found that neuroinflammation is an important cause of nerve injury in WD, and the mechanism may be related to the NLRP3 pathway.^{22,35} NLRP3 is expressed in neurons and plays an important role in various neurological diseases such as AD and PD.³⁷ By capturing danger signals and recruiting downstream molecules, NLRP3 mediates the maturation and release of IL-1 β .³⁷ As shown in the results of this study, the expression of NLRP3 and inflammatory factors TNF - α , IL-1 β , and IL-6 were increased in both TX mice and copper ion-loaded HT22 cells. GDL, a specific drug developed by our hospital for the treatment of WD, has been used clinically for nearly 20 years with significant therapeutic benefits. Modern pharmacological studies have shown that Curcumin inhibits the pro-inflammatory response by suppressing the activation of the NF - κ B pathway.³⁸ Tanshinone also exhibits anti-inflammatory and antioxidant effects.³⁹ In the initial phases of WD, substantial research has been undertaken, which has proven the presence of neuroinflammation in WD and that GDL can effectively alleviate neuroinflammation through the NLRP3 or Nrf2 pathways.^{35,40} While copper overload occurs in WD, a disorder in iron metabolism is also present. Ferroptosis is an iron-dependent cell death form driven by lipid peroxidation. Studies in China and elsewhere have shown the presence of ferroptosis in WD.^{15,41,42} Recent studies have indicated that curcumin has the potential to suppress ferroptosis by activating GPX4, facilitating copper excretion, and providing protection against WD.⁴³ As anticipated, the pathological results and ultrastructure of the hippocampus in vivo experiments showed that GDL and ferroptosis inhibitor Fer-1 effectively ameliorate neuronal and mitochondrial damage. Additionally, GDL decreased the expression of inflammatory factors and thus reduced inflammatory responses in both in vitro and in vivo experiments. The results of ELISA indicated that GDL enhances the expression of GSH and SOD, while reducing the expression of 4-HNE and MDA, and improving the accumulation of Fe²⁺. These results suggested that GDL can mitigate neuronal oxidative stress and ferroptosis.

Our study first performed high-throughput full-transcriptome sequencing of hippocampal tissues of TX and WT mice. Ten upregulated and eleven downregulated genes were identified, which can be used to further investigate the pathogenesis of WD. The results of GO and KEGG enrichment analyses indicated that pathways related to oxidative stress and inflammatory responses are highly enriched in WD. In addition, an analysis of intersection was performed between differentially expressed genes and ferroptosis-related genes, identifying LCN2 as a gene associated with ferroptosis in WD. These findings establish a foundation for understanding the mechanism through which GDL operates in the context of ferroptosis and indicate that LCN2 could potentially serve as a crucial target for treating WD. Subsequently, the effect of GDL on ferroptosis in WD through LCN2 signaling was further explored.

LCN2 is an adipokine expressed in both adipose tissue and neurons in the brain,^{44,45} and is notably linked with diverse neurological conditions, including ischemic stroke, encephalomyelitis, and dementia.^{46–48} Knockdown of LCN2 improves neurological deficits in the brain tissue of mice with cerebral infarction and reduces inflammation and apoptosis in HT22 cells induced by oxygen–glucose deprivation.⁴⁹ Multiple studies have shown that LCN2 triggers inflammation by activating the NLRP3 inflammasome.^{50,51} Studies have indicated that LCN2 regulates the assembly of NLRP3 inflammasomes by activating the NF- κ B pathway. NLRP3 promotes the activation of caspase-1 through the recruitment of adapter protein ASC, thereby leading to the production and maturation of inflammatory factors such as IL-1 β .⁵² In addition, LCN2 can induce mitochondrial dysfunction, leading to an increase in ROS or the initiation and activation of NLRP3 inflammasomes through mitochondrial DNA.⁵³ Our findings indicated that the expression of LCN2, NLRP3 and

inflammatory factors such as TNF- α , IL-1 β , and IL-6 was upregulated in WD models. The levels of these inflammation indicators were downregulated after intervention of GDL and LCN2 inhibitor. Pathological examination of TX mice also confirmed the presence of mitochondrial damage in WD mice. Electron microscopy also confirmed that the rupture and dissolution of mitochondria were significantly reduced after GDL treatment, and mitochondrial cristae were increased. In addition, we intervened in copper-loaded HT22 cells by GDL and LCN2 inhibitors in this experiment. The results showed that both LCN2 inhibitors and GDL reduced the expression of NLRP3 and the release of inflammatory factors in HT22 cells, and the effects were more obvious when they were used together. The above results show that GDL improves neuroinflammation through the LCN2/NLRP3 signaling pathway, providing a new molecular mechanism for the treatment of WD.

Disturbance of iron homeostasis exists in neurodegenerative diseases such as AD and WD, and a ferroptosis inhibitor can effectively improve neuronal damage induced by amyloid β .⁵⁴ GPX4 plays a master role in blocking ferroptosis by eliminating phospholipid hydroperoxides, and copper amplifies ferroptosis by promoting autophagic degradation of GPX4.⁵⁵ SLC7A11, a multi-pass transmembrane protein, mediates the cystine/glutamate antiporter activity in the system xc⁻, thereby regulating the biosynthesis of glutathione and exerting antioxidant defenses.⁵⁶ Through the detection of oxidation indicators and iron metabolism, this study demonstrated the presence of ferroptosis in WD and showed that GDL can effectively inhibit ferroptosis in WD. The expression levels of GPX4 and SLC7A11 also supported this conclusion. In both in vitro and in vivo experiments, the protein and mRNA expression for GPX4 and SLC7A11 in the model group were reduced compared to the control group. After intervention with GDL, the expression of GPX4 and SLC7A11 was upregulated. Subsequently, we constructed the ferroptosis models by injecting the ferroptosis inducer RSL3 into the WD model TX mice. The results revealed that the expression of GPX4 and SLC7A11 in both the model and RSL3 groups was significantly lower than in the control group. Compare with the model and RSL3 groups, the expression of GPX4 and SLC7A11 were increased in the GDL and GDL+RSL3 groups, suggesting that GDL enhances the expression of specific markers in the ferroptosis model. Additionally, the ELISA results indicated elevated Fe²⁺ levels in the model and RSL3 groups of TX mice, accompanied by reduced expression of the oxidative stress markers GSH and SOD, and increased expression of ROS, 4-HNE, and MDA. In addition, the results of ELISA indicated that the expression of Fe²⁺, ROS, 4-HNE and MDA were increased in the model and RSL3 groups, while the expression of oxidative stress indicators GSH and SOD were decreased. Following GDL intervention, the level of Fe²⁺, ROS, 4-HNE and MDA were reduced, while the expression of GSH and SOD were increased compare with the model and RSL3 groups. These results suggested that GDL can alleviate oxidative stress and the accumulation of iron ions, thereby ameliorating ferroptosis in neurons of TX mice. Therefore, we proposed that mitigating ferroptosis may represent a promising strategy for treating WD.

Ferroptosis is intricately linked with neuroinflammation in central neurological disorders.⁵⁷ In neurodegenerative conditions, an excess of iron induces the transformation of microglia into the pro-inflammatory M1 type through the elevation of ROS.⁵⁸ The interplay of inflammatory factors with iron amplifies the generation of ROS, culminating in neuronal ferroptosis.⁵⁸ Deactivation of GPX4 activates NF- κ B signal transduction, which activates NLRP3.⁵⁹ Previous research has shown that Forsythoside A regulates ferroptosis-mediated neuroinflammation by regulating NF- κ B signal transduction.⁶⁰ LCN2, a key protein in iron metabolism, maintains cellular homeostasis by chelating iron carriers.²³ Erastin and RSL3, as ferroptosis inducers, also induce the expression of LCN2.²⁴ An increase in LCN2 expression in retinal pigmented epithelial cells can disrupt iron homeostasis and inhibit autophagy flux, resulting in the activation of inflammasomes and triggering ferroptosis.⁶¹ Silencing *LCN2* inhibits lipopolysaccharide-induced inflammation and oxidative stress.⁶² The increased expression of LCN2 in reactive astrocytes of mice treated with kainic acid is associated with neuronal ferroptosis.⁶³ The results of this study indicate that, in addition to GDL, the use of glutathione and the Fer-1 can reduce the expression levels of LCN2 and NLRP3 in a WD model. In addition, in vivo experiments revealed that following treatment with the ferroptosis inducer RSL3, there was an elevation in the protein expression of LCN2 and NLRP3, accompanied by increased expression of inflammatory factors. GDL mitigated the upregulation of LCN2 and NLRP3 triggered by RSL3 and the release of inflammatory factors, and consequently alleviate the inflammatory response. After intervention with GDL or LCN2 inhibitors in copper ion-induced HT22 cells, the expression of GPX4, SLC7A11, GSH and SOD were upregulated, and the expression of Fe²⁺, ROS, 4-HNE, and MDA was decreased,

suggesting that both GDL and inhibition of LCN2 improved the level of oxidative stress, thereby reducing ferroptosis. And the combination of GDL and LCN2 inhibitor made this change more significant. In conclusion, these findings suggest that GDL has a protective effect on neuroinflammation associated with ferroptosis.

Above all, our current study suggested that GDL may confer protection against ferroptosis and neuroinflammation in TX mice and copper ion-induced HT22 cells by downregulating LCN2/NLRP3 signaling Pathway. In the future, we will further explore the regulatory mechanism of ferroptosis and its upstream and downstream signaling pathways involved in WD, so as to provide a basis for the development of new therapies for WD neuroinflammation.

Conclusion

In the present research, we displayed for the first time that LCN2 is a key regulatory factor for neuronal ferroptosis in WD. The present study revealed that GDL may improve hippocampus damage in TX mice and alleviate neuronal ferroptosis and inflammatory response in WD models by regulating the LCN2/NLRP3 signaling pathway. In conclusion, our researches discovered that LCN2 may be a new target for treating neuroinflammation of WD, and GDL is a potential drug for treating ferroptosis and neuroinflammation in WD.

Acknowledgments

This study was supported by the Key Laboratory of Xin'An Medicine, Ministry of Education; the Natural Science Foundation of Anhui Province (2208085MH270, 2108085QH368); the Key Research and Development Plan Projects of Anhui Province (202204295107020043); the Higher Education Science Research Project Anhui Province (KJ2021A0547); National Excellent Talent Training Program of Western Medicine Studying Traditional Chinese Medicine (2019qgxxzggrcpxxm20220104); National Administration of Traditional Chinese Medicine: 2019 Project of building evidence based practice capacity for TCM (2019XZZX-NB001); and Plans for Major Provincial Science&Technology Projects (202303a07020004).

Disclosure

The authors report no conflicts of interest in this work.

References

1. Bandmann O, Weiss KH, Kaler SG. Wilson's disease and other neurological copper disorders. *Lancet Neurol*. 2015;14(1):103–113. doi:10.1016/S1474-4422(14)70190-5
2. Czlonkowska A, Litwin T, Dusek P, et al. Wilson disease. *Nat Rev Dis Primers*. 2018;4(1):21. doi:10.1038/s41572-018-0018-3
3. Dong T, Yang WM, Wu MC, et al. Microstructure changes in whiter matter relate to cognitive impairment in Wilson's disease. *Biosci Rep*. 2019;39(3):BSR20181651. doi:10.1042/BSR20181651
4. Terwel D, Löschmann YN, Schmidt HH, Schöler HR, Cantz T, Heneka MT. Neuroinflammatory and behavioural changes in the Atp7B mutant mouse model of Wilson's disease. *J Neurochem*. 2011;118(1):105–112. doi:10.1111/j.1471-4159.2011.07278.x
5. Wu P, Dong J, Cheng N, Yang R, Han Y, Han Y. Inflammatory cytokines expression in Wilson's disease. *Neurol Sci*. 2019;40(5):1059–1066. doi:10.1007/s10072-018-3680-z
6. Tang D, Chen X, Kang R, Kroemer G. Ferroptosis: molecular mechanisms and health implications. *Cell Res*. 2021;31(2):107–125. doi:10.1038/s41422-020-00441-1
7. Seibt TM, Proneth B, Conrad M. Role of GPX4 in ferroptosis and its pharmacological implication. *Free Radic Biol Med*. 2019;133:144–152. doi:10.1016/j.freeradbiomed.2018.09.014
8. Ingold I, Berndt C, Schmitt S, et al. Selenium utilization by GPX4 is required to prevent hydroperoxide-induced ferroptosis. *Cell*. 2018;172(3):409–422.e21. doi:10.1016/j.cell.2017.11.048
9. Yang WS, SriRamaratnam R, Welsch ME, et al. Regulation of ferroptotic cancer cell death by GPX4. *Cell*. 2014;156(1–2):317–331. doi:10.1016/j.cell.2013.12.010
10. Koppula P, Zhang Y, Zhuang L, Gan B. Amino acid transporter SLC7A11/xCT at the crossroads of regulating redox homeostasis and nutrient dependency of cancer. *Cancer Commun*. 2018;38(1):12. doi:10.1186/s40880-018-0288-x
11. Ye Y, Chen A, Li L, et al. Repression of the antiporter SLC7A11/glutathione/glutathione peroxidase 4 axis drives ferroptosis of vascular smooth muscle cells to facilitate vascular calcification. *Kidney Int*. 2022;102(6):1259–1275. doi:10.1016/j.kint.2022.07.034
12. Gaschler MM, Andia AA, Liu H, et al. FINO2 initiates ferroptosis through GPX4 inactivation and iron oxidation. *Nat Chem Biol*. 2018;14(5):507–515. doi:10.1038/s41589-018-0031-6
13. Homma T, Kobayashi S, Fujii J. Cysteine preservation confers resistance to glutathione-depleted cells against ferroptosis via CDGSH iron sulphur domain-containing proteins (CISDs). *Free Radic Res*. 2020;54(6):397–407. doi:10.1080/10715762.2020.1780229
14. Stockwell BR. Ferroptosis turns 10: emerging mechanisms, physiological functions, and therapeutic applications. *Cell*. 2022;185(14):2401–2421. doi:10.1016/j.cell.2022.06.003

15. Hao W, Yang W, Wei T, et al. Based on bioinformatics and cell experiments, explore the role of gandoufumu decoction in regulating ferroptosis in the treatment of hepatolenticular degeneration. *Chinese Traditional Patent Med.* **2023**;45(10):3461–3468.
16. Yang N, Zhang K, Guan QW, Wang ZJ, Chen KN, Mao XY. D-penicillamine reveals the amelioration of seizure-induced neuronal injury via inhibiting Aqp11-dependent ferroptosis. *Antioxidants.* **2022**;11(8):1602. doi:10.3390/antiox11081602
17. Li Y, Feng D, Wang Z, et al. Ischemia-induced ACSL4 activation contributes to ferroptosis-mediated tissue injury in intestinal ischemia/reperfusion. *Cell Death Differ.* **2019**;26(11):2284–2299. doi:10.1038/s41418-019-0299-4
18. Tsurusaki S, Tsuchiya Y, Koumura T, et al. Hepatic ferroptosis plays an important role as the trigger for initiating inflammation in nonalcoholic steatohepatitis. *Cell Death Dis.* **2019**;10(6):449. doi:10.1038/s41419-019-1678-y
19. Zhang Z, Wu Y, Yuan S, et al. Glutathione peroxidase 4 participates in secondary brain injury through mediating ferroptosis in a rat model of intracerebral hemorrhage. *Brain Res.* **2018**;1701:112–125. doi:10.1016/j.brainres.2018.09.012
20. Ayton S, Portbury S, Kalinowski P, et al. Regional brain iron associated with deterioration in Alzheimer's disease: a large cohort study and theoretical significance. *Alzheimers Dement.* **2021**;17(7):1244–1256. doi:10.1002/alz.12282
21. Cao Y, Li Y, He C, et al. Selective ferroptosis inhibitor liproxstatin-1 attenuates neurological deficits and neuroinflammation after subarachnoid hemorrhage. *Neurosci Bull.* **2021**;37(4):535–549. doi:10.1007/s12264-020-00620-5
22. Dong J, Wang X, Xu C, et al. Inhibiting NLRP3 inflammasome activation prevents copper-induced neuropathology in a murine model of Wilson's disease. *Cell Death Dis.* **2021**;12(1):87. doi:10.1038/s41419-021-03397-1
23. Golonka R, Yeoh BS, Vijay-Kumar M. The iron tug-of-war between bacterial siderophores and innate immunity. *J Innate Immun.* **2019**;11(3):249–262. doi:10.1159/000494627
24. Liu J, Song X, Kuang F, et al. NUPR1 is a critical repressor of ferroptosis. *Nat Commun.* **2021**;12(1):647. doi:10.1038/s41467-021-20904-2
25. Adler O, Zait Y, Cohen N, et al. Reciprocal interactions between innate immune cells and astrocytes facilitate neuroinflammation and brain metastasis via lipocalin-2. *Nat Cancer.* **2023**;4(3):401–418. doi:10.1038/s43018-023-00519-w
26. Kim JH, Kang RJ, Hyeon SJ, et al. Lipocalin-2 is a key regulator of neuroinflammation in secondary traumatic and ischemic brain injury. *Neurotherapeutics.* **2023**;20(3):803–821. doi:10.1007/s13311-022-01333-5
27. Cheng C, Wang Q, Huang Y, et al. Gandouling inhibits hepatic fibrosis in Wilson's disease through Wnt-1/ β -catenin signaling pathway. *J Ethnopharmacol.* **2023**;311:116445. doi:10.1016/j.jep.2023.116445
28. Lin CH, Tseng HF, Hsieh PC, et al. Nephroprotective role of chrysophanol in hypoxia/reoxygenation-induced renal cell damage via apoptosis er stress, and ferroptosis. *Biomedicines.* **2021**;9(9):1283. doi:10.3390/biomedicines9091283
29. Li X, Chen J, Feng W, et al. Berberine ameliorates iron levels and ferroptosis in the brain of 3 $\times$ Tg-AD mice. *Phytomedicine.* **2023**;118:154962. doi:10.1016/j.phymed.2023.154962
30. Ding B, Lin C, Liu Q, et al. Tanshinone IIA attenuates neuroinflammation via inhibiting RAGE/NF- κ B signaling pathway in vivo and in vitro. *J Neuroinflammation.* **2020**;17(1):302. doi:10.1186/s12974-020-01981-4
31. Qi Y, Ni S, Heng X, et al. Uncovering the potential mechanisms of *coptis chinensis* franch. for serious mental illness by network pharmacology and pharmacology-based analysis. *Drug Des Devel Ther.* **2022**;16:325–342. doi:10.2147/DDDT.S342028
32. Dong T, Wu MC, Tang LL, et al. Gandouling promotes proliferation and differentiation of neural stem cells in the mouse model of Wilson's disease. *Biosci Rep.* **2021**;41(1):BSR20202717. doi:10.1042/BSR20202717
33. Jiang H, Wu Y-Q, Ni W-J, Shen A-Z, Liu S. Study on the regulation and mechanism of Gandouling on neuroinflammation in Wilson disease model mice and inflammatory response of copper loaded microglia based on NF- κ B signal pathway. *Anhui Univ Tradit Chin Med.* **2020**;15. doi:10.26922/d.cnki.ganzc.2020.000002.
34. Wang X, Xie D, Du S, et al. The mechanism of action of Gandouling tablets on autophagy of hippocampal neurons in a cognitive impairment model of hepatolenticular degeneration in mice. *Shanxi J Tradit Chin Med.* **2023**;44(07):833–838.
35. Wen Y, Dong T, Jiang Z, et al. The mechanism of Gandouling tablet alleviating hepatolenticular degeneration neuroinflammation by regulating TLR4/NF- κ B/NLRP3 signaling pathway. *Shandong Sci.* **2023**;36(04):42–51.
36. Chu J, Li H, Yuan Z, et al. Acetaminophen impairs ferroptosis in the hippocampus of septic mice by regulating glutathione peroxidase 4 and ferroptosis suppressor protein 1 pathways. *Brain Behav.* **2023**;13(8):e3145. doi:10.1002/brb3.3145
37. Fu J, Wu H. Structural mechanisms of NLRP3 inflammasome assembly and activation. *Annu Rev Immunol.* **2023**;41(1):301–316. doi:10.1146/annurev-immunol-081022-021207
38. Ran Y, Su W, Gao F, et al. Curcumin ameliorates white matter injury after ischemic stroke by inhibiting microglia/macrophage pyroptosis through NF- κ B suppression and NLRP3 inflammasome inhibition. *Oxid Med Cell Longev.* **2021**;2021(1):1552127. doi:10.1155/2021/1552127
39. Wang X, Yang Y, Liu X, Gao X. Pharmacological properties of tanshinones, the natural products from *Salvia miltiorrhiza*. *Adv Pharmacol.* **2020**;87:43–70. doi:10.1016/bs.apha.2019.10.001
40. Jiang Z, Dong T, Wang Y, et al. Gandouling alleviates cognitive dysfunction by regulates the p62/Nrf2 signaling pathway to reduce oxidative stress and autophagy in mice models of Wilson's disease. *Arabian J. Chem.* **2023**;16(2):104477. doi:10.1016/j.arabjc.2022.104477
41. Gromadzka G, Wierzbicka D, Litwin T, Przybyłkowski A. Iron metabolism is disturbed and anti-copper treatment improves but does not normalize iron metabolism in Wilson's disease. *Biomaterials.* **2021**;34(2):407–414. doi:10.1007/s10534-021-00289-x
42. Wang X, Shao N, Zhang X, et al. Ferulic acid activates SIRT1-mediated ferroptosis signaling pathway to improve cognition dysfunction in Wilson's disease. *Neuropsychiatr Dis Treat.* **2023**;19:2681–2696. doi:10.2147/NDT.S443278
43. Sun X, Zhang X, Yan H, et al. Protective effect of curcumin on hepatolenticular degeneration through copper excretion and inhibition of ferroptosis. *Phytomedicine.* **2023**;113:154539. doi:10.1016/j.phymed.2022.154539
44. Jin Z, Kim KE, Shin HJ, et al. Hippocampal Lipocalin 2 is associated with neuroinflammation and iron-related oxidative stress in ob/ob mice. *J Neuropathol Exp Neurol.* **2020**;79(5):530–541. doi:10.1093/jnen/nlaa017
45. Kim KE, Jeong EA, Lee JY, et al. Myeloid sirtuin1 deficiency aggravates hippocampal inflammation in mice fed high-fat diets. *Biochem Biophys Res Commun.* **2018**;499(4):1025–1031. doi:10.1016/j.bbrc.2018.04.044
46. Jin M, Kim JH, Jang E, et al. Lipocalin-2 deficiency attenuates neuroinflammation and brain injury after transient middle cerebral artery occlusion in mice. *J Cereb Blood Flow Metab.* **2014**;34(8):1306–1314. doi:10.1038/jcbfm.2014.83
47. Kim JH, Ko PW, Lee HW, et al. Astrocyte-derived lipocalin-2 mediates hippocampal damage and cognitive deficits in experimental models of vascular dementia. *Glia.* **2017**;65(9):1471–1490. doi:10.1002/glia.23174

48. Nam Y, Kim JH, Seo M, et al. Lipocalin-2 protein deficiency ameliorates experimental autoimmune encephalomyelitis: the pathogenic role of lipocalin-2 in the central nervous system and peripheral lymphoid tissues. *J Biol Chem.* **2014**;289(24):16773–16789. doi:10.1074/jbc.M113.542282
49. Wang J, Jin J, Li G. NR3C2 activates LCN2 transcription to promote endoplasmic reticulum stress and cell apoptosis in ischemic cerebral infarction. *Brain Res.* **2024**;1822:148632. doi:10.1016/j.brainres.2023.148632
50. Jeong EA, Lee J, Shin HJ, et al. Tonicity-responsive enhancer-binding protein promotes diabetic neuroinflammation and cognitive impairment via upregulation of lipocalin-2. *J Neuroinflammation.* **2021**;18(1):278. doi:10.1186/s12974-021-02331-8
51. Kim SL, Shin MW, Kim SW. Lipocalin 2 activates the NLRP3 inflammasome via LPS-induced NF- κ B signaling and plays a role as a pro-inflammatory regulator in murine macrophages. *Mol Med Rep.* **2022**;26(6):358. doi:10.3892/mmr.2022.12875
52. Li J, Xu P, Hong Y, et al. Lipocalin-2-mediated astrocyte pyroptosis promotes neuroinflammatory injury via NLRP3 inflammasome activation in cerebral ischemia/reperfusion injury. *J Neuroinflammation.* **2023**;20(1):148. doi:10.1186/s12974-023-02819-5
53. Song E, Jahng JW, Chong LP, et al. Lipocalin-2 induces NLRP3 inflammasome activation via HMGB1 induced TLR4 signaling in heart tissue of mice under pressure overload challenge. *Am J Transl Res.* **2017**;9(6):2723–2735.
54. Bao WD, Pang P, Zhou XT, et al. Loss of ferroportin induces memory impairment by promoting ferroptosis in Alzheimer's disease. *Cell Death Differ.* **2021**;28(5):1548–1562. doi:10.1038/s41418-020-00685-9
55. Xue Q, Yan D, Chen X, et al. Copper-dependent autophagic degradation of GPX4 drives ferroptosis. *Autophagy.* **2023**;19(7):1982–1996. doi:10.1080/15548627.2023.2165323
56. Koppula P, Zhuang L, Gan B. Cystine transporter SLC7A11/xCT in cancer: ferroptosis, nutrient dependency, and cancer therapy. *Protein Cell.* **2021**;12(8):599–620. doi:10.1007/s13238-020-00789-5
57. Xu Y, Jia B, Li J, Li Q, Luo C. The interplay between ferroptosis and neuroinflammation in central neurological disorders. *Antioxidants.* **2024**;13(4):395. doi:10.3390/antiox13040395
58. Urrutia PJ, Bórquez DA, Núñez MT. Inflaming the Brain with Iron. *Antioxidants.* **2021**;10(1):61. doi:10.3390/antiox10010061
59. Li C, Deng X, Xie X, Liu Y, Friedmann Angeli JP, Lai L. Activation of glutathione peroxidase 4 as a novel anti-inflammatory strategy. *Front Pharmacol.* **2018**;9:1120. doi:10.3389/fphar.2018.01120
60. Wang C, Chen S, Guo H, et al. Forsythoside A mitigates Alzheimer's-like pathology by inhibiting ferroptosis-mediated neuroinflammation via Nrf2/GPX4 axis activation. *Int J Biol Sci.* **2022**;18(5):2075–2090. doi:10.7150/ijbs.69714
61. Gupta U, Ghosh S, Wallace CT, et al. Increased LCN2 (lipocalin 2) in the RPE decreases autophagy and activates inflammasome-ferroptosis processes in a mouse model of dry AMD. *Autophagy.* **2023**;19(1):92–111. doi:10.1080/15548627.2022.2062887
62. Wang X, Zhang C, Zou N, et al. Lipocalin-2 silencing suppresses inflammation and oxidative stress of acute respiratory distress syndrome by ferroptosis via inhibition of MAPK/ERK pathway in neonatal mice. *Bioengineered.* **2022**;13(1):508–520. doi:10.1080/21655979.2021.2009970
63. Shin HJ, Jeong EA, Lee JY, et al. Lipocalin-2 deficiency reduces oxidative stress and neuroinflammation and results in attenuation of kainic acid-induced hippocampal cell death. *Antioxidants.* **2021**;10(1):100. doi:10.3390/antiox10010100