

Article

Boron Triggers Hepatic Ferroptosis: Unveiling the Dual-Pathogenic Nexus of Oxidative Stress and SLC7A11/GPX4 Dysregulation

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Simple Summary

Boron is essential in trace amounts but toxic in excess. Chickens in farming may ingest high boron from water or feed, yet how it damages the liver is unclear. This study examined whether boron causes liver injury through ferroptosis. We found that boron-fed chickens showed liver damage, oxidative stress, and disrupted iron balance. Boron triggered ferroptosis by blocking a key cellular defense pathway (SLC7A11/GPX4). These results first explain how boron harms the liver, offering a scientific basis to reduce its risk in poultry farming.

Abstract

Boron compounds, classified as prohibited food additives due to their high toxicity, persist in pesticides and fertilisers, industrial processes, food supply chains, and consumer goods, perpetuating multisource exposure risks. Chronic ingestion may induce fatal hepatorenal injury; however, mechanistic insights and epidemiological surveillance remain critically lacking amidst sector-wide regulatory gaps. This study employed integrated cellular and organismal models to elucidate the relationship between boron-induced hepatotoxicity and ferroptosis. We demonstrate that dietary boron accumulation in chicken livers is associated with histopathological damage, mitochondrial cristae dissolution and atrophy (a hallmark of ferroptosis), and elevated serum biomarkers AST and ALT. Boron exacerbates oxidative damage in hepatocytes by elevating malondialdehyde (MDA) production while modulating the Nrf2/ARE antioxidant signaling pathway—specifically downregulating key genes (Nrf2, HO-1, GCLM, CAT). Concurrently, it inhibits critical antioxidant enzymes (SOD, T-AOC), thereby depleting cellular antioxidant defenses. Crucially, boron disrupts iron homeostasis and induces ferroptosis by dysregulating the SLC7A11-GPX4 pathway: upregulating pro-ferroptotic genes (*ACSL4*, *TF*, *TFR*) and downregulating cytoprotective genes (*SLC7A11*, *GPX4*, *FTH1*). Co-treatment with the ferroptosis inhibitor ferrostatin-1 (Fer-1) attenuated boron-induced oxidative damage, whereas the ferroptosis inducer Erastin potentiated toxicity. Collectively, we pioneer the dual-pathogenic mechanism of boron hepatotoxicity—oxidative stress and ferroptotic cell death—establishing the SLC7A11/GPX4 axis as a novel therapeutic target against boron toxicity.

Keywords: boron; hepatocytes; ferroptosis; oxidative stress; erastin



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1. Introduction

Boron (B) is a versatile element with natural prevalence and agricultural significance, and plays an irreplaceable role in the growth and development of plants [1,2]. However, excessive application of boron fertilizer may lead to an abnormally increased boron content in the soil [3,4], causing environmental safety problems. According to the literature reports, the global background value of soil boron is 42 mg/kg, while the normal range of soil boron content is only 0.5–5 mg/kg [1,5]. With expanding industrial and agricultural use of boron, its bioaccumulation in poultry has raised concerns [6]. Poultry are exposed to elevated boron through drinking water (≥ 5 –10 mg/L in some areas), feed from boron-rich soil, direct soil ingestion, and industrial pollution. Since poultry products are a major protein source for humans, boron accumulation in tissues may pose a dietary health risk [7,8]. Multiple studies have shown that excessive boron exposure can lead to multi-organ toxicity, among which the liver, as the main target organ for heavy metal metabolism and accumulation, is particularly susceptible [9–12]. Excessive intake of boron through contaminated food chains and water sources may have significant toxic effects on organisms [13,14]. Similarly to other heavy metals, boron overload can seriously damage hepatocyte function [15–18], this highlights the necessity of in-depth research on the molecular mechanism of boron-induced hepatotoxicity. Therefore, studying boron-induced liver toxicity is vital for assessing real-world exposure in poultry and protecting public health.

The accumulation of metals/metal-like substances in organisms can disrupt the dynamic balance of the oxidation-antioxidant system and induce oxidative stress. This imbalance promotes the excessive production of Reactive Oxygen Species (ROS), which in turn leads to the damage of biological macromolecules and the dysfunction of organelles [19–21]. Among them, the accumulation of lipid ROS (especially lipid hydroperoxides) may trigger a novel type of programmed cell death characterized by significant lipid peroxidation and iron overload—Ferroptosis [22,23]. As a newly discovered mode of cell death in recent years, ferroptosis plays a “double-edged sword” role in the health of the organism [24]. Under physiological conditions, it participates in eliminating damaged cells to maintain the stability of the internal environment. Conversely, under pathological conditions, its abnormal activation is closely related to the occurrence and development of various diseases, including malignant tumors, neurodegenerative diseases and arthritis, etc. [25–28]. It is notable that ferroptosis has been confirmed to be involved in the pathological processes of liver diseases such as non-alcoholic fatty liver disease, liver fibrosis and hepatocellular carcinoma [29–31]. The latest research suggests that the toxic effects of environmental heavy metal/metal-like pollutants may have a potential association with the ferroptosis pathway [32–34]. It is particularly notable that boron exposure can interfere with the iron-dependent cell death mechanism, manifested as intracellular glutathione metabolism disorders and changes in lipid peroxidation levels [35], suggesting that boron-induced hepatotoxicity may be achieved through the ferroptosis pathway. To investigate this potential mechanism, we focused on the core regulatory axis of ferroptosis, the SLC7A11/GPX4 pathway, which serves as the primary cellular defense system against lipid peroxidation.

SLC7A11, as a key component of the reverse transporter in the Xc system, is a trans-membrane protein with multiple biological functions [36]. When the Xc system is inhibited, the uptake of cystine by cells decreases, thereby affecting the biosynthesis of Glutathione (GSH). Meanwhile, the inhibition of glutathione peroxidase 4 (GPX4) activity or the down-regulation of its expression will significantly increase the sensitivity of cells to ferroptosis and even directly induce ferroptosis [37,38]. Therefore, the SLC7A11-GPX4 signaling axis is regarded as the core pathway regulating ferroptosis [39,40]. We hypothesized that boron exposure might disrupt this protective axis, leading to glutathione depletion, lipid peroxide accumulation, and ultimately ferroptosis in hepatocytes. Existing evidence indicates

that hepatocellular toxicity caused by metal/metal-like exposure is often accompanied by increased ROS production and depletion of GSH [41].

To investigate the mechanism by which boron exposure contributes to liver damage in broiler chickens, this study adopts a combined *in vivo* and *in vitro* research strategy: By establishing live broiler models exposed to different concentrations of boron for 7 and 14 days and combining with the boron treatment experiments of primary Chicken Embryo Hepatocytes (CEHs), the accumulation characteristics of boron in broiler livers and its damaging effects on liver function and structure were comprehensively investigated. By detecting the related markers of oxidative stress and ferroptosis, the association mechanism between boron exposure and ferroptosis was deeply explored. In addition, the study also introduced the ferroptosis activator Erastin and the inhibitor Ferrostatin-1 (Fer-1). By regulating the SLC7A11-GPX4 signaling axis, it was verified whether boron exposure induced ferroptosis and exacerbated oxidative damage through this pathway, thereby providing a new theoretical basis for clarifying the molecular mechanism of boron hepatotoxicity.

2. Materials and Methods

2.1. Experimental Design and Animal Treatment

The research protocol, approved by the South China Agricultural University Ethics Committee for the Welfare of Laboratory Animals (Ethics Review Number: 2022A051), involved 60 AA broiler chickens. After a one-week acclimatization period, Boric acid (sourced from Meini Eco-Agriculture Co., Ltd, Shanghai, China) was incorporated into their daily diets. The chickens were randomly divided into three groups: the control group received a baseline meal containing 0 mg/kg of boron; the low-dose group received 120 mg/kg of boron; and the high-dose group received 240 mg/kg of boron [42]. The boron content in the drinking water of animals is less than 0.5 ppm. All groups had unrestricted access to their designated feed for 7 or 14 days. After the feeding period, liver samples were collected for examination, and the chickens were euthanized with an intravenous injection of sodium pentobarbital through the wing vein.

2.2. CEH Isolation and Culture

CEHs were isolated and cultured using established methods [43]. In brief, livers from 12-day-old chick embryos were aseptically dissected, rinsed with PBS, and minced. The minced tissue was digested with type IV collagenase (Sigma, St. Louis, MI, USA) at 37 °C for 15 min. Digestion was halted with DMEM culture medium, and the cell suspension was filtered through 200 and 400 mesh sieves, with three washes and centrifugation-resuspension cycles. The isolated CEHs were then resuspended in fresh media and cultured until 70% hepatocyte coverage was reached.

2.3. CEH Viability Assay

The effects of various concentrations of boron (1, 2, 4, 8, 16, 32, 64, 128, and 256 mM), Fer-1 (1, 2, 3, and 4 µM), and Erastin (1.25, 2.5, 5, 10, and 30 µM) on cell viability were assessed using the CCK-8 assay. Cell suspensions were prepared and planted in 96-well plates. To determine optimal dosages, these concentrations of boron, Fer-1, and Erastin were used. Each group included five replicate wells, with a control containing 0 mM boron and a blank containing only medium (no cells). In rescue experiments, Fer-1 and Erastin were pre-treated for varying durations before B application. After adding CCK-8 solution to the plates, they were incubated at 37 °C for two hours. Finally, the absorbance at 450 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader (Thermo, Multiskan SkyHigh, Singapore).

2.4. CEH Treatment

The dose and timing of subsequent drug treatments were determined by the CCK-8 assay results. In the boron-induced cell injury study, cells were treated with 0 mM, 20 mM, 40 mM, and 80 mM (IC₅₀ concentration) of boron for 24 h. For the rescue experiments, cells were pre-treated with either 3 µM Fer-1 for 6 h or 5 µM Erastin for 4 h before exposure to 80 mM (IC₅₀ concentration) of boron for 24 h.

2.5. Detection of Boron in Broiler Liver

The boron content in liver tissue of each broiler was determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES; PerkinElmer, Norwalk, CT, USA, model Optima 8000) following a protocol by Ballentine & Burford. Liver samples (50 mg) and serum samples (100 µL) were digested in a mixture of 30% hydrogen peroxide and concentrated nitric acid at 100 °C for about three hours. The digested samples were then filtered, and the resulting solutions were analyzed for boron content.

2.6. Biochemical Tests for Liver Function Indicators

After centrifuging the whole blood samples and allowing them to stand at 26 °C for three hours, the upper serum layer was separated into three equal parts. One part was used for biochemical analysis. The blood concentrations of alkaline phosphatase (ALP) and aspartate transaminase (AST) were measured using an automated biochemical analyzer (BS-380, Mindray, Shenzhen, China) according to the manufacturer's instructions. All serum samples were tested immediately to ensure the accuracy of the results.

2.7. Histopathological Examination

H&E staining was conducted on fixed liver tissue blocks according to established protocols [44] to evaluate morphological changes in chicken livers. The blocks were trimmed, rinsed overnight, dehydrated, embedded, sectioned, deparaffinized, and stained with H&E. The stained sections were then examined using a Leica light microscope (Leica, Wetzlar, Germany).

2.8. Ultrastructural Observation of Liver Tissue

Liver tissues were trimmed into 1 mm³ pieces, and 200 µL of 2.5% glutaraldehyde was added to each sample, incubated at 26 °C for 2 h. The glutaraldehyde was then removed, and the samples were rinsed four times with 1.5 mL of 0.1 M PBS buffer for 20 min each to clear impurities. Next, 1% osmium acid was added and the samples were fixed for 3 h to preserve tissue morphology. The tissue blocks turned dark coal-colored, indicating effective osmium acid fixation. The osmium acid was then removed, and the tissue blocks were rinsed four times with double-distilled water for 20 min each to eliminate residual osmium acid. Each sample was stained with 0.2 mL of uranyl acetate overnight at 4 °C. The uranyl acetate was recovered, and the tissues were rinsed with double-distilled water before being dehydrated with a gradient ethanol series and treated with 200 µL of acetone. The tissues were further processed with acetone-resin mixtures in varying ratios. Ultrathin sections were stained with uranyl acetate and lead citrate. Finally, the nuclei and organelles, particularly mitochondria, of the hepatocytes were observed using transmission electron microscopy (TEM, FEI/Talos L120, Thermo Fisher Scientific, Brno, Czech Republic).

2.9. Whole-Transcriptome Sequencing (RNA-Seq) Data Analysis

Novogene, based in Beijing, China, conducted comprehensive whole-transcriptome sequencing on liver tissue samples. RNA was extracted using standard techniques, and its quality was verified with an Agilent 2100 bioanalyzer (Santa Clara, CA, USA). Total RNA was used for library preparation. mRNA with polyA tails was isolated using Oligo (dT)

magnetic beads, and the RNA was fragmented in NEB Fragmentation Buffer with divalent cations. The fragmented RNA was reverse-transcribed into cDNA, which was then cleaned, mended, and A-tailed. The double-stranded cDNA was ligated to sequencing adapters. cDNA fragments of approximately 370–420 bp were selected for PCR amplification, and the PCR products were purified using AMPure XP beads (Beijing, China) to create the first library. The library was initially quantified using a Qubit2.0 Fluorometer, followed by quality control of the insert size and final quantification by qRT-PCR. Sequencing was performed on the Illumina NovaSeq 6000 (Illumina, Inc., San Diego, CA, USA), generating 150 bp paired-end reads.

Transcriptomics analysis was conducted on the 14-day control and experimental groups. To compare differential expression, DESeq2 R4.1.2 software was used. DESeq2 provided the statistical model, and after calculating the p -value for the negative binomial distribution, the corrected p -value was adjusted using the Benjamini–Hochberg method for multiple hypothesis testing to control the false discovery rate and reduce false positives. Gene Ontology (GO) enrichment analysis was performed using the clusterProfiler function in R, after identifying differentially expressed genes (DEGs). The analysis adjusted for gene length bias to account for potential artifacts. GO terms were considered significantly enriched if the Benjamini–Hochberg corrected p -value (FDR) was less than 0.05. Additionally, the Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to understand complex biological functions and systems at various scales, including cellular, organismal, and ecological levels. KEGG uses molecular data to elucidate these functions, particularly from large-scale datasets generated by high-throughput techniques like genome sequencing. In this study, the statistical enrichment of DEGs in KEGG pathways was assessed using the R clusterProfiler 4.0 software.

2.10. Immunohistochemistry and Immunofluorescence Staining Analyses

Protein distribution and expression levels within liver tissues were thoroughly assessed using immunohistochemistry and immunofluorescence staining techniques. These staining procedures were carried out in compliance with accepted practices that have been documented in the literature [45]. Briefly, for immunohistochemistry, antigen retrieval was performed in sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 3% H₂O₂. After blocking with normal goat serum, sections were incubated with primary antibodies, followed by a Goat Anti-Rabbit IgG, HRP Conjugated (1:200, CWBio, Taizhou, Jiangsu, China). DAB (Beyotime, Shanghai, China) as the chromogen. For immunofluorescence, appropriate fluorescent dye-conjugated secondary antibodies (Cy3-labeled Goat Anti-Rabbit IgG (H+L), 1:500, Beyotime, Shanghai, China; FITC-labeled Goat Anti-Mouse IgG (H+L), 1:500, Beyotime, Shanghai, China) were used. Antifade Mounting Medium with DAPI (Beyotime, Shanghai, China). ImageJ software version 1.54p (National Institutes of Health, USA) was then utilized to quantify the positivity rate and fluorescence intensity of the target proteins. The primary antibodies listed below were employed: Nrf2 (1:500 dilution, Proteintech, Wuhan, China), GPX4 (1:200 dilution, Bioworld, NanJing, China), HO-1 (1:200 dilution, Affinity, Changzhou, Jiangsu, China), and SLC7A11 (1:200 dilution, Bioss, Beijing, China).

2.11. Measurement of Oxidative Stress Indicators

The first step involved pre-treating liver samples from broiler chickens. One gram of liver tissue was homogenized in nine milliliters of pre-cooled saline on ice. The homogenate was centrifuged, and the supernatant was prepared for further experiments. Using the assay kit according to the manufacturer's instructions, MDA, T-AOC, GSSG content, and

SOD activity in liver tissue were rapidly and accurately measured. Absorbance readings were taken with an ELISA reader.

2.12. Iron Assay

The concentrations of total iron, ferrous iron, and ferric iron in liver tissue and cells were quantified using an iron assay kit (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. First, 10 μL of 100 mM iron standard was mixed with 990 μL of double-distilled water to create a 1 mM iron standard solution. In a 96-well plate, 0, 2, 4, 6, 8, and 10 nM of this solution were added to wells, with the total volume adjusted to 100 μL using double-distilled water. Then, 5 μL of iron reducing agent was added to each well, mixed gently, and incubated at 25 °C for 30 min. Absorbance was read at 593 nm to generate the Fe^{2+} standard curve. Next, 100 μL of iron detection probe was added to each well, and after 60 min of incubation at 25 °C in the dark, the total iron standard curve was plotted by reading absorbance at 593 nm. For sample analysis, 10 mg of liver tissue was homogenized in 100 μL of iron buffer, centrifuged, and the supernatant collected. Cultivate the cells to a density of 2×10^6 , then digest them with trypsin. Centrifuge the cells, discard the trypsin, and re-suspend them in PBS. Repeat this process three times. A 50 μL aliquot of the sample was mixed with an equal volume of iron detection reagent in a 96-well plate, bringing the total volume to 100 μL . After adding 5 μL of iron reducing agent and mixing, the solution was incubated for 30 min, and absorbance was measured at 593 nm. The iron probe was then added, and the Fe^{2+} , Fe^{3+} , and total iron contents of the liver and CEH samples were calculated using the standard curves.

2.13. Intracellular ROS Assay

Established methods were used to treat cultured CEHs with boron, Fer-1, and Erastin. After a 24-h incubation, DCFH-DA diluted in serum-free medium was added to the cells. The cells were then incubated in the dark at 37 °C for 20 min. Following this, the cells were washed three times with PBS and resuspended in fresh, serum-free medium. The final assessment was conducted using a fluorescence microscope (Leica, Wetzlar, Germany).

2.14. Quantitative Real-Time PCR (RT-qPCR) Analysis

For the quantitative real-time PCR (RT-qPCR) analysis, total RNA was extracted following the Takara kit's manufacturer's instructions. The RNA quantity and quality were assessed using the NanoDrop-2000 microneucleic acid analyzer (Waltham, MA, USA). Reverse transcription (RT) was performed according to the kit's protocol. Subsequently, RT-qPCR was performed on the complementary DNA (cDNA) with a 10 μL reaction system, following the reagent instructions for the reaction setup and thermal cycling conditions. This procedure aimed to measure the relative mRNA expression levels of *GPX4*, *Nrf2*, *SLC7A11*, *Keap1*, *FTH1*, *HO-1*, *GCLM*, *TF*, *TFR1*, and *ACSL4*, with β -actin used as the reference gene for normalization. Table S1 lists the specific primer sequences used, which were designed with Primer Express 5.0 software and synthesized by Sangon Bioengineering (Shanghai, China) Co., Ltd.

2.15. Western Blot Analysis

Quantitative protein expression analysis was performed following established protocols [46]. Protein lysates, isolated from tissues or cells, were quantified using a loading buffer. After electrophoresis-based separation and membrane transfer, the membrane was blocked and incubated with specific primary antibodies overnight. The following day, protein bands were visualized and photographed using imaging software after adding secondary antibodies. Protein expression levels were then quantitatively analyzed using ImageJ. The primary antibodies used included β -actin (1:1500, Servicebio, Wuhan, China),

GAPDH (1:20,000, Proteintech, Wuhan, China), Nrf2 (1:2000, Proteintech, Wuhan, China), Keap1 (1:5000, Proteintech, Wuhan, China), ACSL4 (1:30,000, Proteintech, Wuhan, China), HO-1 (1:3000, Affinity, Jiangsu, China), CAT (1:1500, Affinity, Jiangsu, China), GPX4 (1:1000, Bioworld, Nanjing, China), SLC7A11 (1:1000, Bioss, Beijing, China), FTH1 (1:800, Wanleibio, Shenyang, China), TF (1:1000, Wanleibio, Shenyang, China), TFR1 (1:800, Wanleibio, Shenyang, China), and FTL (1:2000, Abmart, Shanghai, China).

2.16. Statistical Analysis

Data were presented as means $\pm$ standard deviation (SD). Statistical analyses were conducted using GraphPad Prism 9.0 (GraphPad Software Inc., Boston, MA, USA). Significant differences between groups were examined using one-way ANOVA followed by Tukey's multiple comparison test. Student's *t*-test was used exclusively for comparisons between two groups. An asterisk (*) or pound (#) indicated $p < 0.05$, two signs indicated $p < 0.01$, three signs indicated $p < 0.001$, and four signs indicated $p < 0.0001$. * represented the difference between the control group and treatment group, and # represented the pairwise comparisons difference between the treatment groups. A p -value < 0.05 was considered statistically significant.

3. Results

3.1. Effects of Boron Exposure and Accumulation in Broiler Liver and Liver Function

Concentrations of boron in chicken liver and serum are depicted in Figure 1A,B. Boron levels in all treatment groups were significantly higher than in the control group ($p < 0.05$). As the boron concentration in the feed rises, the weight of the broilers tends to decrease. Notably, after 12 days on boron-supplemented feed, the broilers in the treatment group weighed significantly less than those in the control group ($p < 0.05$) (Figure 1E,F). To assess liver damage, serum biochemical indices were analyzed, revealing that ALT and AST activity were significantly elevated in all boron-treated groups compared to the control group (Figure 1C,D).

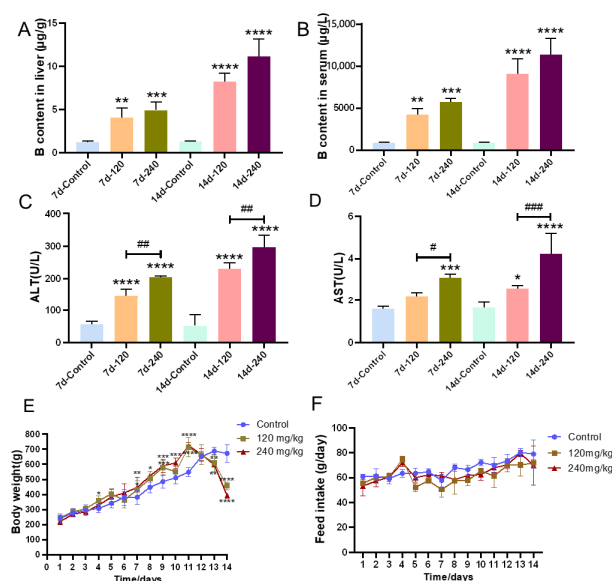


Figure 1. Effects of B exposure and accumulation in broiler liver and liver function. (A) The boron amounts in the liver. (B) The boron amounts in blood. (C,D) The ALT and AST levels. (E) The impact of varying levels of dietary boron supplementation on the body weight of broiler chickens. (F) The variations in feed intake among broilers in various treatment groups. Data for each group ($n = 5$) are presented as mean $\pm$ SD. Statistical significance compared to the control group is denoted by “*” ($p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Statistical significance comparison between the treatment groups is indicated by “#” (# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$). The same as below.

3.2. Effects of Boron Exposure on the Physiological Structure of the Liver

H&E staining was employed to evaluate the impact of boron on chicken liver tissue (Figure 2A,C). In the control groups (7-day and 14-day), hepatocytes exhibited intact structure, with well-organized hepatic cords, clear hepatic sinusoids, and nucleated erythrocytes in the portal areas and sinusoids. Conversely, after 7 days of boron treatment, the 120 mg/kg group (7d-120) displayed initial signs of cellular degeneration, manifested as granule degeneration. The 240 mg/kg group (7d-240) showed more severe lesions, including pale-staining cytoplasm in hepatocytes, indicative of edema, and increased cellular size. Notably, these pathological changes worsened after 14 days of boron treatment. Both the 120 mg/kg group (14d-120) and the 240 mg/kg group (14d-240) displayed hepatocellular edema, inflammatory cell infiltration, and lysed cell nuclei.

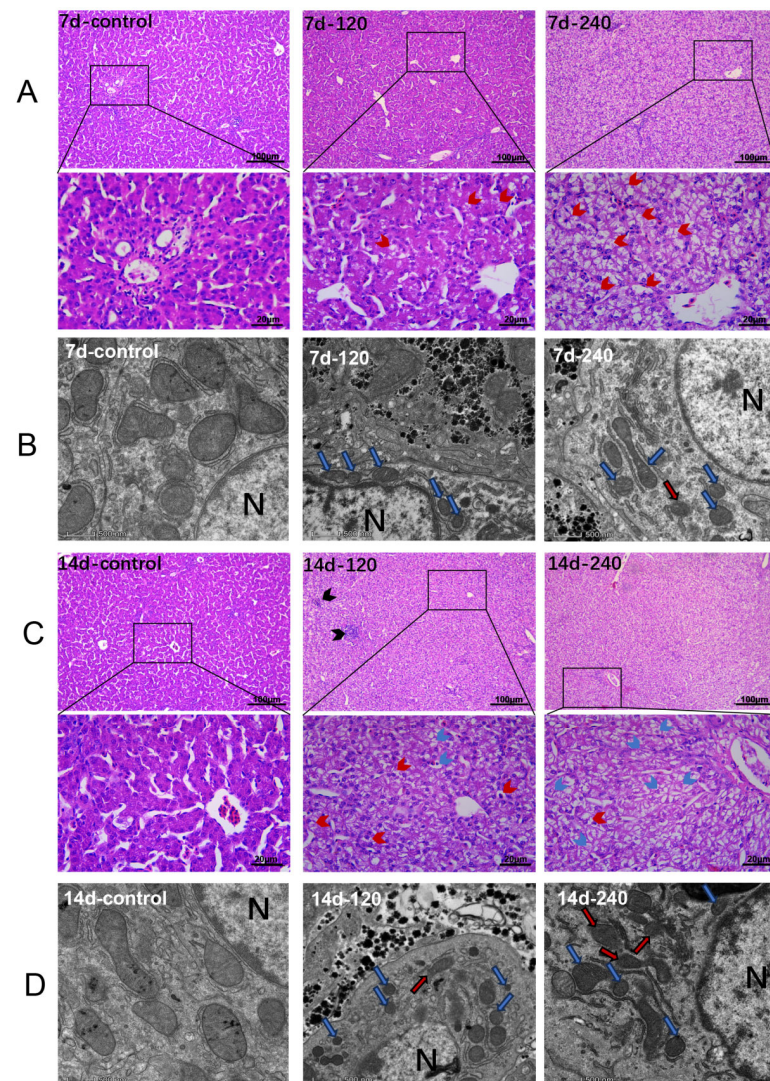


Figure 2. Effects of B exposure on the physiological structure and ultrastructure of the liver. (A,C) Observation of liver tissues using histopathology ($\times 100$, scale: 100 μm). Histopathological observation of liver tissues ($\times 400$, scale: 20 μm). Red arrow: hepatocyte edema; Black arrow: inflammasomes; Blue arrow: nucleus lysis. (B,D) Alterations to the chicken livers' ultrastructure in each group (13,500 $\times$, scale: 500 nm). N: nucleus; Blue arrow: mitochondrial atrophy, increased matrix electron density; Red arrow: mitochondrial membrane rupture.

3.3. Effects of Boron Exposure on Liver Ultrastructure

TEM was utilized to investigate ultrastructural changes in chicken liver tissue (Figure 2B,D). Boron treatment at various concentrations and durations resulted in a dose- and time-dependent increase in mitochondrial damage. After 7 days of exposure, the 120 mg/kg boron-treated group (7d-120) displayed a decrease in mitochondrial volume and an increase in electronic matrix density. This damage escalated to mitochondrial membrane rupture at the higher treatment dose of 240 mg/kg (7d-240). Notably, mitochondrial injury became significantly more severe in the 14-day boron treatment groups. The 120 mg/kg boron-treated group (14d-120) exhibited pronounced mitochondrial membrane rupture, while the 240 mg/kg boron-treated group (14d-240) displayed extensive mitochondrial membrane rupture, mitochondrial shrinkage, and further increase in matrix electron density. These results indicate a direct relationship between the total dose and duration of boron exposure and the extent of mitochondrial damage.

3.4. Differential Expression Analysis of the mRNA-Seq

After applying a Benjamini–Hochberg corrected p -value threshold of 0.05 and an absolute fold change threshold of 2, differentially expressed genes (DEGs) were identified. RNA sequencing results revealed a total of 10,446 genes commonly expressed across the control, 120 mg/kg boron, and 240 mg/kg boron groups. Additionally, the control group, 120 mg/kg boron group, and 240 mg/kg boron group harbored 920, 211, and 205 unique genes, respectively (Figure 3A). A volcano plot (Figure 3B,C) was generated to visualize the DEGs. Compared to the control group, the 120 mg/kg boron group demonstrated 3183 DEGs (1429 upregulated and 1754 downregulated), and the 240 mg/kg boron group presented 3018 DEGs (1400 upregulated and 1618 downregulated) based on transcripts per million (TPM) with a p -value less than 0.05. To explore the potential functions of these target genes, KEGG and GO enrichment analyses were performed. GO analysis revealed an enrichment of genes mainly associated with cellular and metabolic processes (Figure 3D,E). Scatter plots representing the top 20 important KEGG pathways are displayed, highlighting pathways related to ferroptosis, steroid biosynthesis, and fatty acid elongation (Figure 3F,G). Furthermore, qPCR was employed to validate the expression levels of four randomly selected DEGs (Figure 3H). The qPCR results confirmed dysregulation patterns consistent with the mRNA-sequencing data.

3.5. Boron-Induced Oxidative Stress in Chicken Livers

To investigate whether boron exposure induces oxidative stress in chicken livers, T-AOC, the amount of lipid oxidation end products, and the activity of antioxidant enzymes were assessed. boron exposure caused significant increases in MDA content ($p < 0.05$) and decreases in T-AOC, SOD, and GSSG activity ($p < 0.05$) compared to the control group (Figure 4A–D), indicating the induction of oxidative stress. RT-qPCR analysis revealed a significant downregulation of *Nrf2*, *HO-1*, and *GCLM* mRNA expression levels ($p < 0.05$) and upregulation of *Keap1* mRNA ($p < 0.05$) in the boron-exposed group (Figure 4E–H). Western blot analysis confirmed decreased protein expression of Nrf2, HO-1, and CAT ($p < 0.05$) (Figure 4I,J). Furthermore, immunohistochemical labeling showed reduced localized HO-1 expression in boron-exposed livers ($p < 0.05$) (Figure 4K,L). Immunofluorescence staining results revealed that with increasing boron exposure duration and dose, Nrf2 expression decreased in the liver of the boron-exposed group, accompanied by reduced nuclear localization of Nrf2 protein (Figure 4M–O). The 240 mg/kg boron-treated group exhibited more pronounced oxidative damage at both mRNA and protein levels ($p < 0.05$).

3.6. Boron-Induced Ferroptosis and Ferritinophagy in Chicken Livers

Given the established role of oxidative stress in inducing ferroptosis, we investigated its potential activation in chicken livers after boron exposure. Liver iron levels, including total iron, Fe^{2+} , and Fe^{3+} , were significantly higher in the boron-exposed group compared to the control group ($p < 0.05$) (Figure 5A). This suggests possible iron accumulation, a key factor in ferroptosis. RT-qPCR analysis showed that the boron-exposed group significantly overexpressed ferroptosis-related genes, such as *ACSL4*, *TF*, and *TFR1*, compared to the control group ($p < 0.05$) (Figure 5B–G). Conversely, the expression levels of *FTH1*, *GPX4*, and *SLC7A11* were significantly downregulated. This trend was more pronounced in the 240 mg/kg boron-treated group. Western blot analysis confirmed these changes in gene expression (Figure 5H–J). Protein levels of ferroptosis markers, including FTHL, TF, and TFR1, were significantly elevated ($p < 0.05$) in the boron-exposed group, whereas SLC7A11, GPX4, and FTH1 protein levels were significantly reduced. Immunohistochemical staining of SLC7A11 protein revealed a diminished distribution and lower positive staining rate in the livers of the boron-exposed group compared to the control group (Figure 6A,B).

Similarly, ferroptosis marker GPX4 immunofluorescence staining showed a notable decrease in the boron-exposed group relative to the control group ($p < 0.05$) (Figure 6C–E). A dose-dependent reduction in GPX4 and SLC7A11 protein expression was observed, with the 240 mg/kg boron treatment group exhibiting the most significant effects.

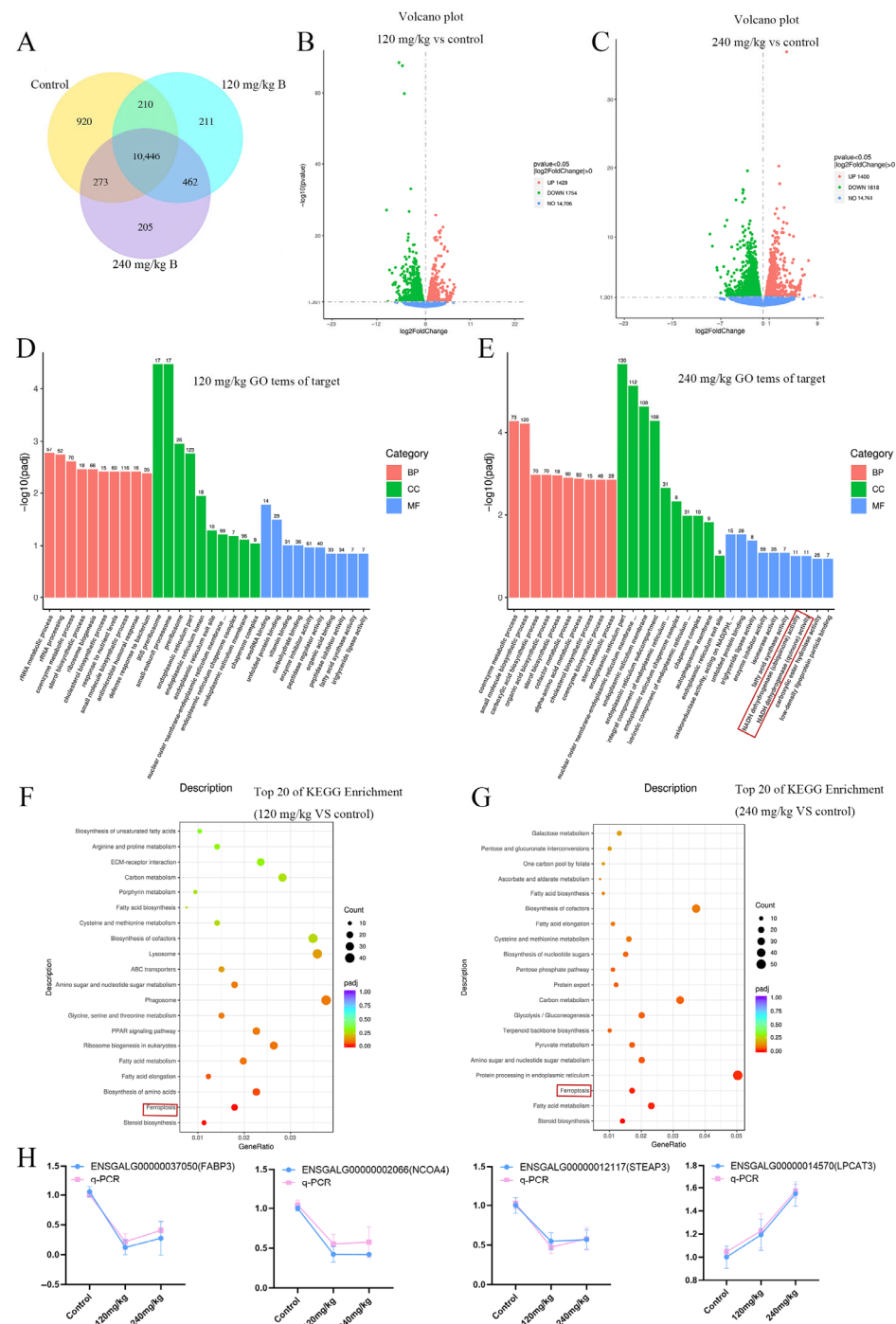


Figure 3. Differential expression analysis of the mRNA-Seq. (A) Venn diagram among control, 120 mg/kg, and 240 mg/kg B group. The same as below. (B,C) Volcano plots of DEGs; The green dot indicates down-regulation, and the red dot indicates up-regulation. (D,E) Bar diagram of GO. (F,G) Analysis of KEGG pathway enrichment. (H) Transcript expression validation using qPCR.

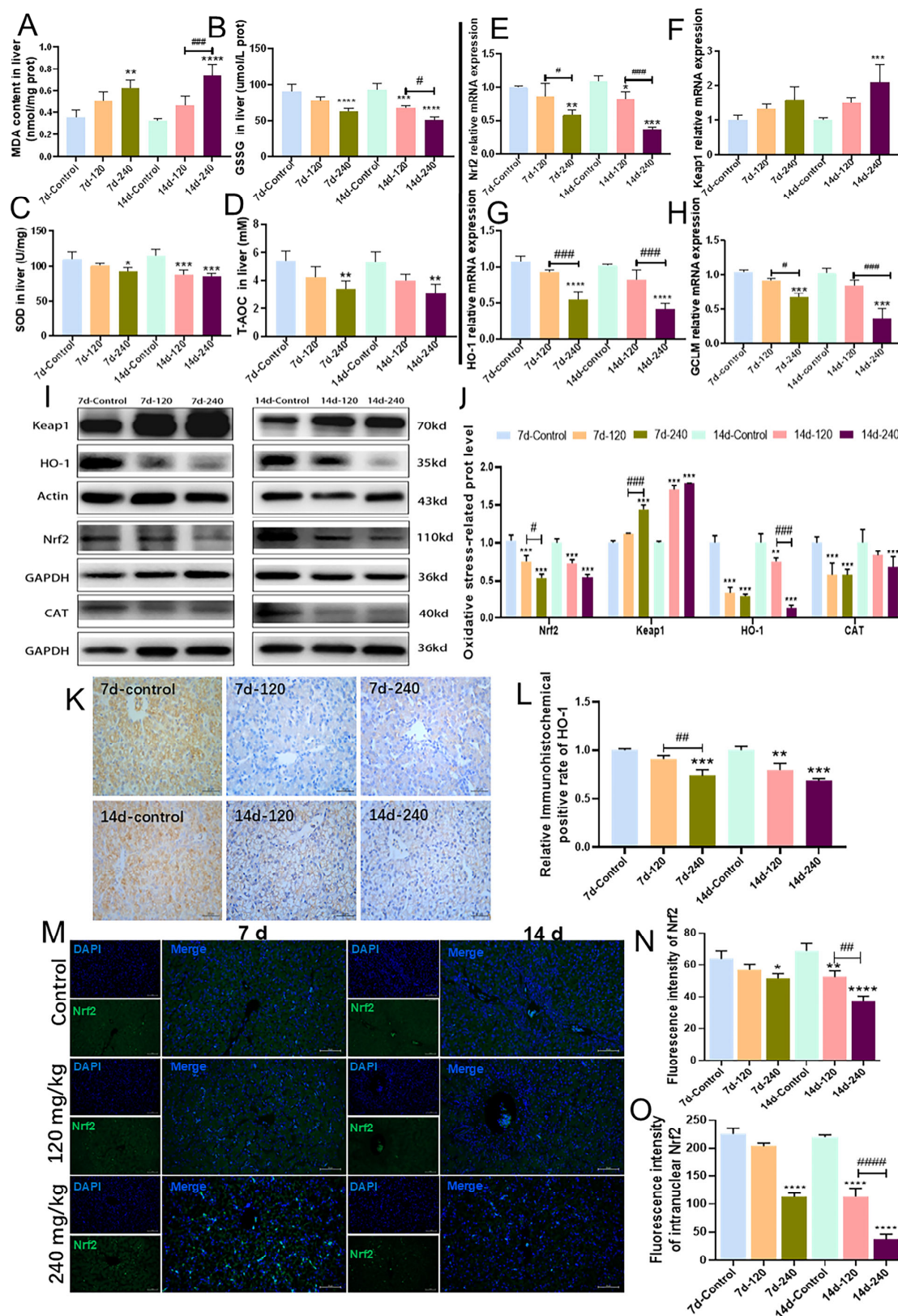


Figure 4. B-induced oxidative stress in chicken livers. (A–D) The content of MDA, GSSG, SOD, and T-AOC. (E–H) The relative expression of *HO-1*, *GCLM*, *Keap1*, *Nrf2*, and *Keap1* mRNA in liver. (I,J) Quantification of Western blots and representative Western blotting of *Nrf2*, *Keap1*, *HO-1*, and *CAT* in liver of chicken of B treatment. (K,L) Immunohistochemistry staining examination of the liver's *HO-1* protein expression ($\times 400$, scale: 20 μm). (M,N) The outcomes of the *Nrf2* Immunofluorescent staining Image J analysis ($\times 400$, scale: 50 μm). (O) The fluorescence intensity of

intracellular Nrf2. Data for each group ($n = 5$) are presented as mean $\pm$ SD. Statistical significance compared to the control group is denoted by “*” ($p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Statistical significance comparison between the treatment groups is indicated by “#” (# $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$). The same as below.

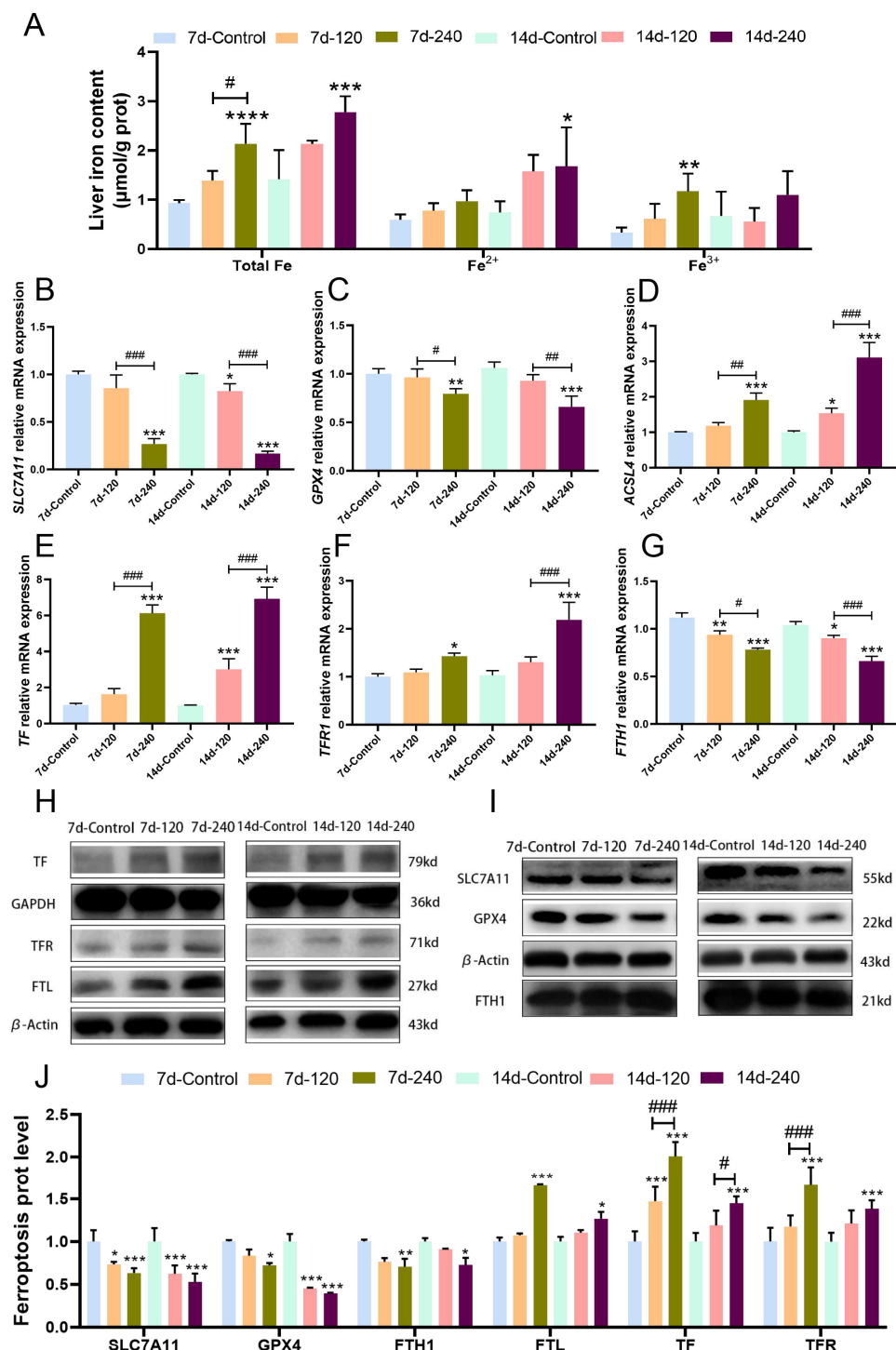


Figure 5. B-induced ferroptosis and ferritinophagy in chicken livers. (A) Hepatic total iron, Fe^{2+} , and Fe^{3+} content. (B–G) The relative mRNA expression of *SLC7A11*, *GPX4*, *ACSL4*, *TF*, *TFR*, and *FTH1* in liver. (H–J) Quantification of Western blots and representative Western blotting of *SLC7A11*, *GPX4*, *FTH1*, *FTL*, *TF*, and *TFR* in liver of chicken of B treatment.

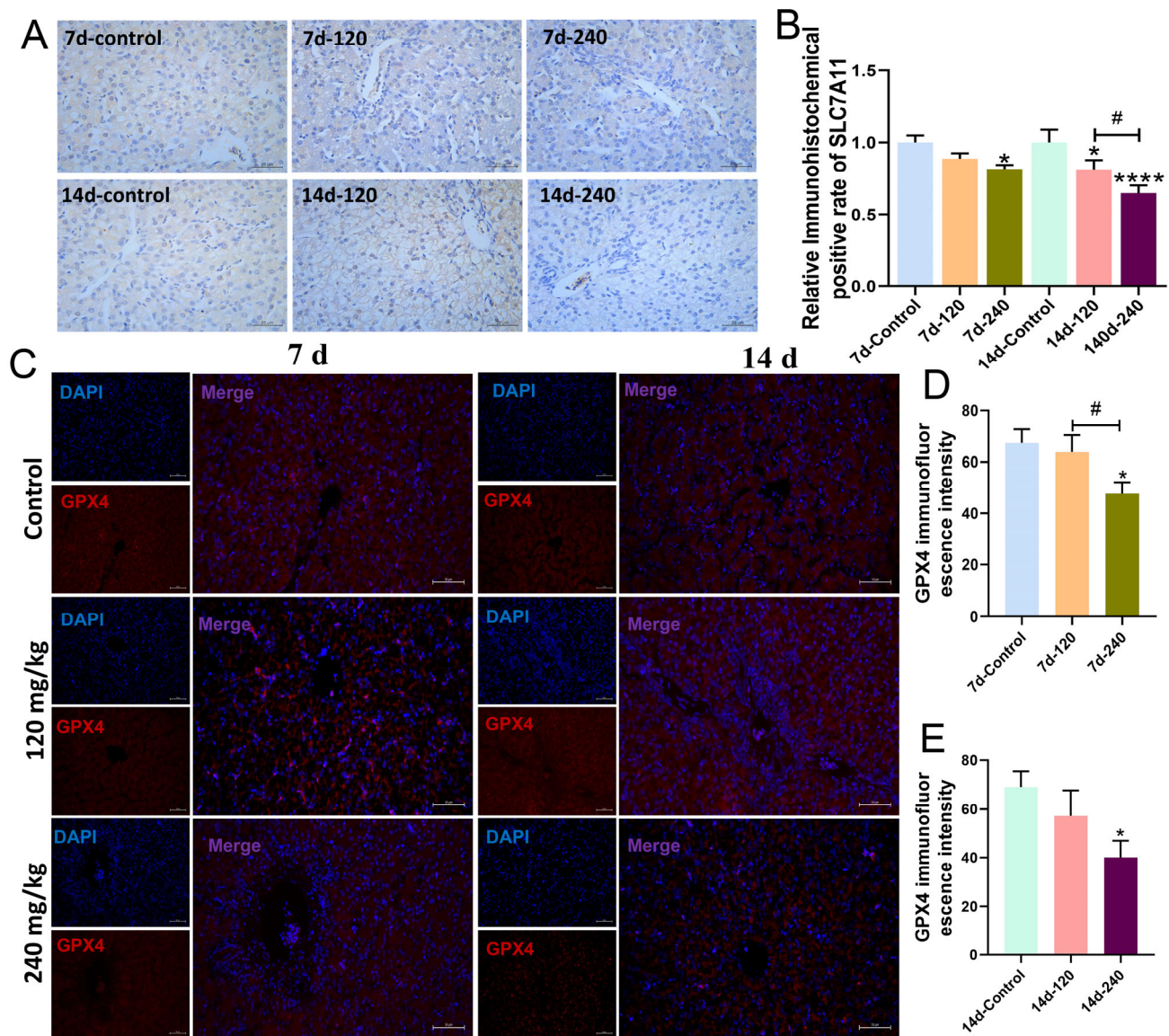


Figure 6. B-induced ferroptosis and ferritinophagy in chicken livers. (A,B) Immunohistochemistry staining examination of the liver's SLC7A11 protein expression ($\times 400$, scale: 20 μm). (C–E) The outcomes of the GPX4 Immunofluorescent staining Image J analysis ($\times 400$, scale: 50 μm).

3.7. Boron Exposure Triggers Ferroptosis and Oxidative Stress in Hepatocytes

While ferroptosis is a recognized form of cell death, susceptibility varies among cell types. To investigate if ferroptosis can be induced in CEHs after boron exposure, we measured cellular iron content. As depicted in Figure S1 treatment with boron exhibited a significant dose-dependent cytotoxic effect on CEHs. Additionally, we calculated the IC₅₀ based on the cell proliferation activity and determined the subsequent cell treatment concentrations for grouping: control, 20 mM, 40 mM, 80 mM. There was also a marked increase in intracellular Fe^{2+} , Fe^{3+} , and total iron levels following boron exposure ($p < 0.05$) (Figure 7A–C), indicating potential iron accumulation. Additionally, ROS levels were significantly elevated with increasing boron treatment concentrations (Figure 7N,O). RT-qPCR analysis demonstrated a significant dose-dependent downregulation of key ferroptosis-related genes ($p < 0.05$) in boron-treated CEHs compared to controls (Figure 7D–G). These genes included *GPX4*, *SLC7A11*, *Nrf2*, and *HO-1*. Western blot analysis corroborated the observed changes in gene expression, revealing dose-dependent alterations in protein levels of *GPX4*, *SLC7A11*, *Nrf2*, and *HO-1* following boron treatment (Figure 7H–M).

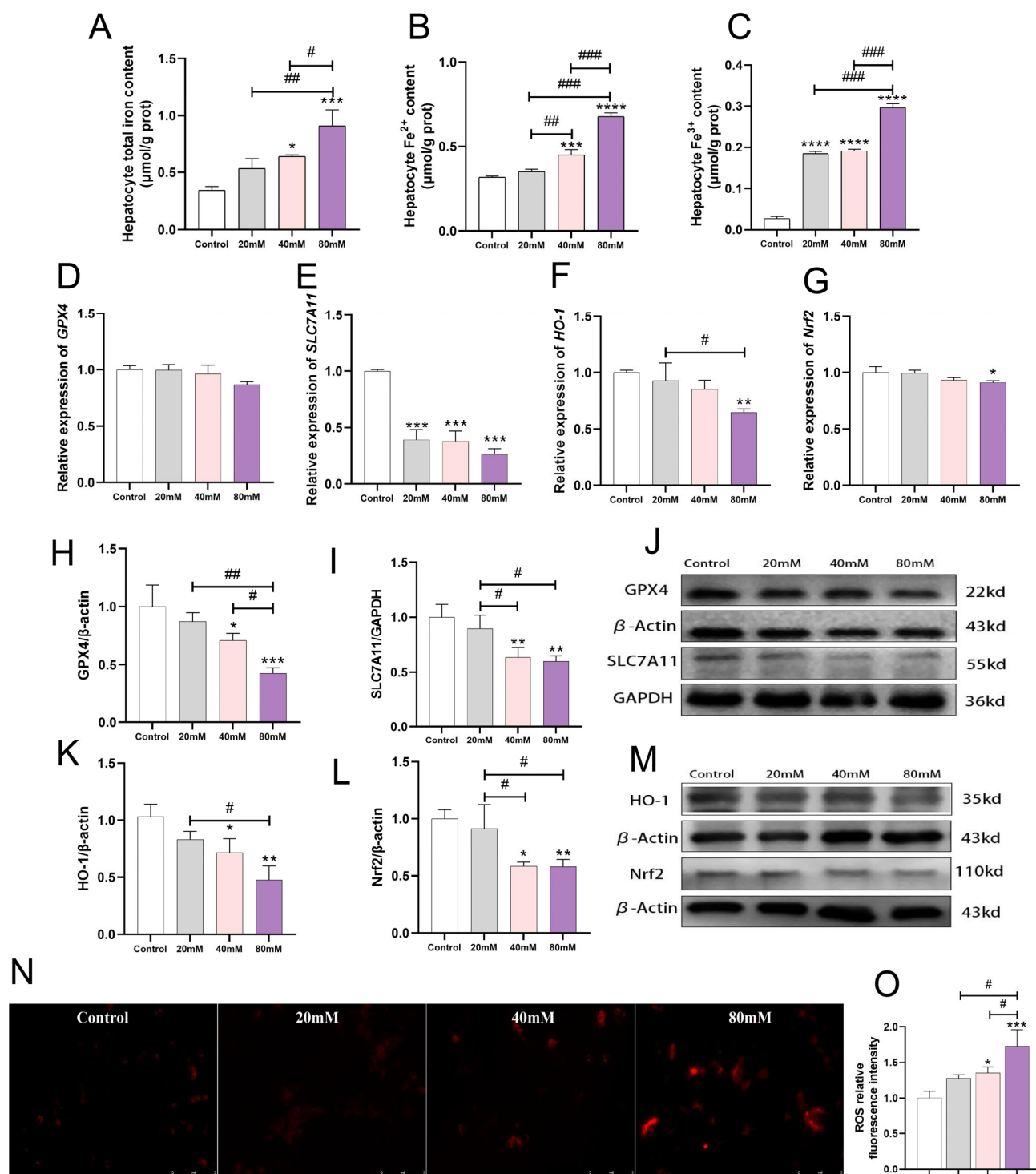


Figure 7. B exposure induces ferroptosis and oxidative stress in hepatocytes. (A–C) Hepatocyte total iron, Fe²⁺, and Fe³⁺ content ($n = 6$). (D–G) The relative mRNA expression of ($n = 6$). (H–M) The relative amounts of GPX4, HO-1, SLC7A11, and Nrf2 proteins ($n = 6$). (N,O) DCFH-DA staining was used to determine where ROS had accumulated ($\times 400$).

3.8. Regulators of Ferroptosis in the Context of Boron-Induced CEH Oxidative Stress

Using ferroptosis inhibitors Fer-1 and activators Erastin, we investigated ferroptosis's role in the boron-induced cell death of CEHs. The optimal Fer-1 and Erastin concentration were determined by the CCK-8 assay results (Figures S2 and S3). As shown in Figures S2 and S3, Fer-1 pretreatment significantly alleviated the decrease in cell viability

caused by boron ($p < 0.05$), but Erastin significantly exacerbated cellular damage and markedly reduced cell viability. Furthermore, Fer-1 treatment markedly inhibited the boron-induced increase in intracellular total iron, Fe^{2+} , and Fe^{3+} levels and ROS levels, but co-treatment with Erastin led to a notable rise (Figure 8A,C,D). Notably, Fer-1 treatment reversed the boron-mediated downregulation of Nrf2, HO-1, GPX4 and SLC7A11 mRNA and protein expression levels, while Erastin further enhanced the boron-induced upregulation of Nrf2, HO-1, GPX4 and SLC7A11 mRNA and protein expression levels (Figure 8B,E–J).

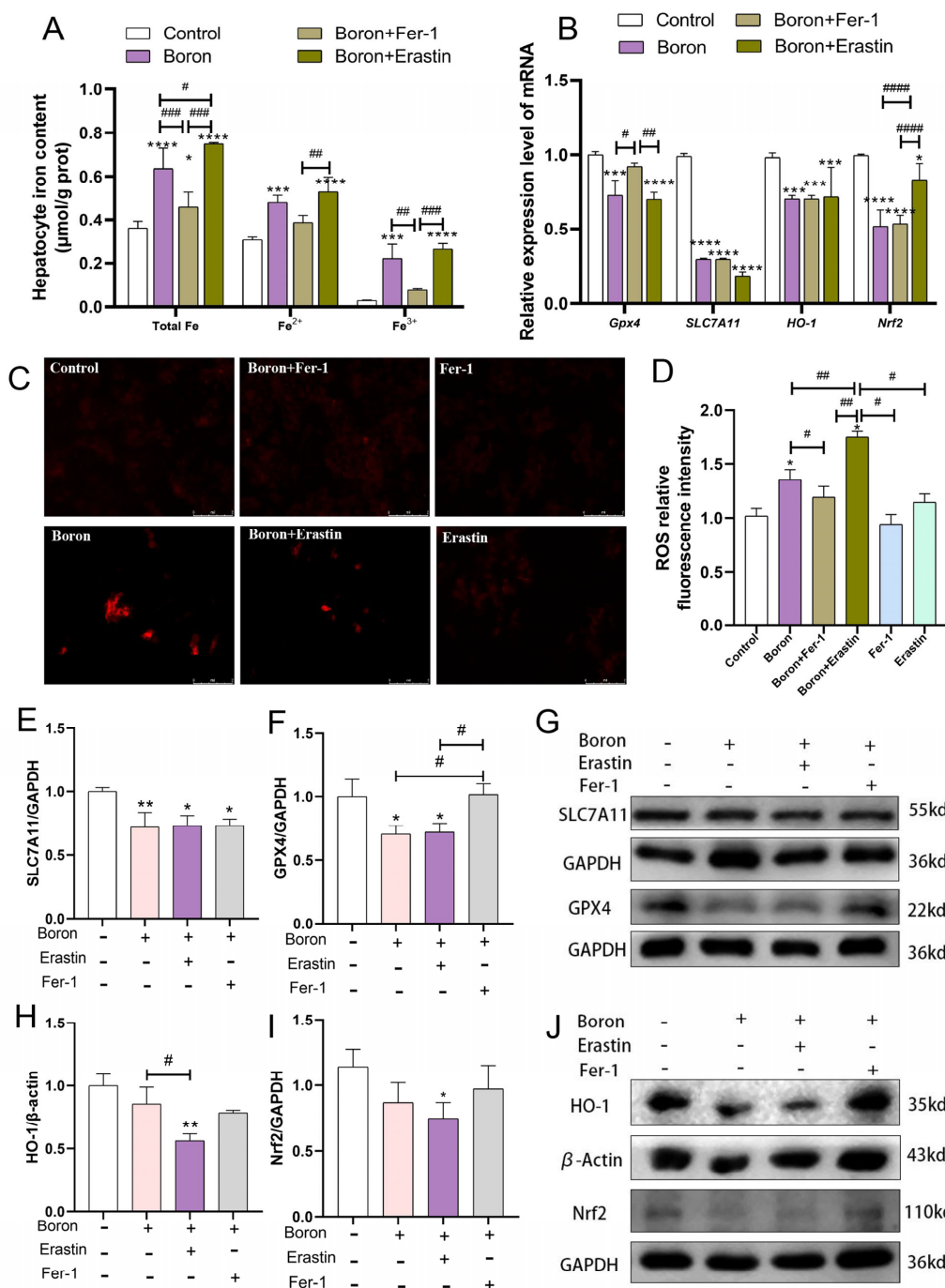


Figure 8. Regulators of ferroptosis in the context of B-induced CEH oxidative stress. (A) Total iron, Fe^{2+} , and Fe^{3+} content inside cells ($n = 6$). (B) The relative mRNA expression of *GPX4*, *SLC7A11*, *HO-1*, and *Nrf2* ($n = 6$). (C,D) DCFH-DA staining was used to determine where ROS had accumulated ($\times 400$). (E–J) The relative amounts of SLC7A11, GPX4, Nrf2, and HO-1 proteins ($n = 6$).

4. Discussion

Boron is a trace element essential for all living organisms, playing a key role in maintaining normal physiological processes in humans and animals [3,47,48]. Although boron is an essential trace element for organisms, its excessive intake—particularly through environmental or feed-borne exposure encountered in livestock production—can cause significant hepatotoxicity, the molecular mechanisms of which remain incompletely elucidated [49,50]. Elucidating the underlying mechanisms of boron-induced hepatotoxicity is vital for developing strategies to prevent and treat boron toxicity. This study investigated the effects of boron exposure on chicken livers. Our findings delineate a clear progression from boron accumulation to mitochondrial dysfunction and subsequent oxidative injury. The elevated serum and hepatic boron levels were directly correlated with markers of hepatocyte damage (AST, ALT) and ultrastructural lesions, most notably mitochondrial atrophy and membrane rupture.

Mitochondrial dysfunction contributes to various cellular pathologies, including oxidative stress [51], apoptosis [52], pyroptosis [53], and autophagy [54]. This dysfunction can compromise the ability of mitochondria to scavenge ROS, leading to their increased production and mitochondrial fragmentation [55]. Under normal conditions, an organism maintains a balance between ROS production and removal by antioxidant enzymes [56]. When ROS production exceeds the antioxidant defense capacity, oxidative stress and cellular damage occur [57]. Excessive ROS can initiate lipid peroxidation, further contributing to liver damage [58]. Biomarkers such as catalase, which detoxifies hydrogen peroxide, and HO-1, involved in heme catabolism, are used to assess oxidative stress [59]. In this study, we posit that mitochondria are a primary intracellular target of boron toxicity. This mitochondrial damage likely serves as a critical initiating event, compromising the electron transport chain and leading to the aberrant generation of reactive oxygen species (ROS). This is substantiated by the significant increase in lipid peroxidation (MDA) and the disrupted redox balance (altered GSSG and T-AOC) we observed. Importantly, the cellular antioxidant defense system appeared to be suppressed rather than adaptively upregulated. The reduced nuclear translocation of Nrf2, the master regulator of antioxidant response, provides a mechanistic explanation for this failure. Consequently, the expression of downstream protective genes such as HO-1 and CAT was dysregulated, creating a permissive environment for oxidative stress to escalate and inflict widespread cellular damage.

Reactive oxygen species (ROS) are vital signaling molecules in ferroptosis. A primary mechanism involves the accumulation of the labile iron pool (LIP) within cells, which promotes lipid peroxidation through free radical generation via the Fenton reaction [60]. Under typical physiological circumstances, extracellular Fe^{3+} enters the cell via TF-bound transport. Fe^{3+} is bound by TF on the cell membrane, forming a TF- Fe^{3+} complex that interacts with the TFR1 on the cell surface, facilitating iron internalization [61]. Ferritin, the main organelle that stores excess iron, is a protein complex composed of FTL and FTH1 subunits [62]. However, excessive ROS can degrade ferritin, releasing stored iron and exacerbating ROS production and cellular damage [63]. Previous studies have shown that aged polystyrene microplastics induce ferroptosis in human hepatocytes by inhibiting FTH1 expression [64]. Additionally, Yu et al. demonstrated that nifedipine disrupts in vivo iron homeostasis by upregulating the expression of DMT1 and TFR1 expression. The precise impact of boron accumulation on iron transport and homeostasis within organisms remains an area of active investigation [65]. Beyond oxidative stress, our data uncover a profound disruption of hepatic iron homeostasis induced by boron, which is pivotal for the induction of ferroptosis. We observed a dose-dependent accumulation of hepatic iron alongside a paradoxical upregulation of the iron import machinery (TF and TFR1). This pattern suggests a failure of the normal feedback inhibition where cellular iron overload should

suppress TFR1 expression. More crucially, we found a dissociative regulation of ferritin subunits: FTL was upregulated while FTH1 was markedly downregulated. Given that FTH1 possesses ferroxidase activity essential for the safe storage of iron as Fe^{3+} , its specific downregulation may severely compromise the liver's capacity to sequester excess iron in a non-reactive form. Therefore, the net effect of these alterations—increased iron influx and potentially defective storage—is the expansion of the labile iron pool (LIP). An elevated LIP catalyzes the Fenton reaction, generating highly toxic hydroxyl radicals that directly drive the lipid peroxidation characteristic of ferroptosis. This distinct iron metabolism signature ($\text{TFR1}\uparrow$, $\text{FTH1}\downarrow$) not only clarifies a key mechanism for boron-induced ferroptosis but also distinguishes it from iron dysregulation patterns reported for other hepatotoxicants.

To further investigate the impact of boron exposure on other ferroptosis pathways, we examined the amino acid and glutathione metabolism pathways involved in this cell death mechanism. System Xc^- (xCT) is a heterodimeric amino acid antiporter composed of the subunits SLC7A11 and SLC3A2, with SLC7A11 being the functional subunit [66]. Located on the plasma membrane, xCT facilitates the exchange of cystine and glutamate in a 1:1 ratio. Cystine is subsequently reduced to cysteine by the enzyme cystine reductase [67]. Cysteine, along with glutamate and glycine, serves as a precursor for the synthesis of the endogenous antioxidant GSH by the enzymes glutamate-cysteine ligase (GCL) and glutathione synthetase (GSS) [68]. GPX4 is the only enzyme capable of both oxidizing GSH to GSSG and reducing toxic lipid peroxides to non-toxic lipid alcohols [69]. This unique enzymatic activity positions GPX4 as a critical player in lipid peroxide elimination and ferroptosis prevention. The most significant mechanistic insight from this study is the targeted suppression of the SLC7A11/GPX4 antioxidant axis by boron. The coordinated downregulation of both SLC7A11 (the functional subunit of system Xc^-) and GPX4 indicates a strategic assault on the two most critical nodes of the cellular defense against ferroptosis. This dual suppression creates a self-reinforcing vicious cycle: (1) reduced SLC7A11 impairs cystine uptake, depleting the precursor for glutathione (GSH) synthesis; (2) GSH deficiency directly diminishes the substrate availability for GPX4; and (3) concomitant reduction in GPX4 protein levels further cripples the enzymatic capacity to neutralize lipid hydroperoxides. Our functional pharmacological experiments provide definitive causal evidence for this pathway's centrality: The aggravation of injury by Erastin (an SLC7A11 inhibitor) synergized with boron, confirming that boron acts on a parallel or overlapping pathway to disable the same defensive axis. Conversely, the robust protection offered by Fer-1 not only confirms the execution of ferroptosis but also validates that lipid peroxidation is the terminal, actionable event in this cascade. Furthermore, the modulation of this axis directly altered oxidative stress parameters, illustrating that in boron toxicity, the SLC7A11/GPX4 axis is not merely a passive victim but a central regulator that integrates and amplifies the oxidative insult from mitochondrial dysfunction and iron overload in hepatocytes. Collectively, these results indicate that boron exposure reduces SLC7A11 and GPX4 expression, disrupts glutathione metabolism via lipid peroxidation, and ultimately leads to ferroptosis and oxidative stress in chicken livers and CEHs.

In conclusion, we propose an integrated model for boron-induced hepatotoxicity where ferroptosis acts as the convergent cell death pathway. In this model, boron simultaneously initiates mitochondrial damage and iron dyshomeostasis ('the fuel and the spark'), and disables the key SLC7A11/GPX4 defense system ('removing the firewall'). This multifaceted attack explains the potent hepatotoxicity observed. While our study establishes the critical role of this axis, several questions remain. For instance, is the downregulation of SLC7A11 and GPX4 a direct transcriptional effect or a consequence of upstream signals such as sustained ER stress or ATF4 activation? Furthermore, future studies should investigate whether nutritional interventions (e.g., N-acetylcysteine supplementation) or pharmacolog-

ical GPX4 activators could mitigate boron toxicity in practical poultry farming. Our work thus not only elucidates a novel toxicological mechanism for boron but also identifies the SLC7A11/GPX4 axis as a potential therapeutic target for countering metalloid-induced liver damage.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ani16050832/s1>, Table S1: Primer sequences of oxidative stress and ferroptosis genes; Figure S1: The survival of CEHs cells exposed to various boron concentrations for a whole day. Figure S2: Cell proliferation activity on CEHs cell of Fer-1 at different doses. Figure S3: Cell proliferation activity on CEHs cell of Erastin at different doses.

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