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Dihydromyricetin ameliorates lipopolysaccharide–induced hepatic injury in chickens by activating the Nrf2/Keap1 pathway and regulating mitochondrial dynamics

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ABSTRACT

Dihydromyricetin (**DHM**) is a flavonoid found in vine tea that exhibits various pharmacological characteristics, including antibacterial, antiapoptotic, and antioxidant effects. Our previous study revealed that DHM can alleviate chicken hepatic injury, but the underlying mechanism has not been elucidated. To further investigate the protective mechanism of DHM, this study firstly predicted by network pharmacology that the potential regulatory pathways of DHM on hepatic injury. Subsequently, the experimental models were replicated in vivo and in vitro using Hy–Line white broiler chickens and chicken primary hepatocytes treated with DHM and with/without LPS. Network pharmacology results showed that the effect of DHM on hepatic injury might be related to oxidative stress and mitochondrial function. Further experiments showed that DHM significantly reduced LPS–elicited serum ALT and AST activities, promoted antioxidant enzyme activities and scavenged ROS in chicken liver or primary hepatocytes. Molecular docking studies showed that DHM could directly bind to Nrf2 and Keap1. Furthermore, DHM treatment regulated the expression of Nrf2 and Keap1, thereby upregulating the downstream expression of antioxidant factors, including HO–1 and NQO1, in vivo and in vitro. Moreover, DHM modulated the expression of mitochondrial dynamics related factors, including Mfn1/2, Opa1, Drp1, and Fis1, meanwhile, DHM ameliorated mitochondrial structural damage and increased the MMP. Overall, these results suggested that DHM activated the Nrf2/Keap1 pathway and regulated the balance between mitochondrial fusion and fission, ultimately alleviating chicken hepatic injury induced by LPS.

Introduction

In intensive poultry farming, Gram–negative infections can affect the growth rate of poultry or even cause sudden death, resulting in economic losses worldwide (Averós and Estevez, 2018; Goo et al., 2019; Shu et al., 2022). Lipopolysaccharide (**LPS**) is a component and virulence factor of the outer wall of Gram–negative bacteria (Ahmad et al., 2020; Li et al., 2021; Pang et al., 2023; Sun et al., 2020). Poultry in the early growth phase is more susceptible to Gram–negative infections due to the immaturity of the immune system, which may trigger severe hepatic injury (Chen et al., 2023). The pathogenesis of hepatic injury is multifactorial and involves factors, such as apoptosis, inflammatory response and oxidative stress. Excessive reactive oxygen species (**ROS**) can deplete intracellular glutathione and NADPH, which in turn disrupts their redox balance and blocks redox signaling, a condition that triggers oxidative stress in cells (Jin et al., 2006; Wei et al., 2022). In addition, the imbalances caused by oxidative stress include a reduction in the amounts of the enzymes glutathione peroxidase (**GSH–PX**), catalase (**CAT**) and superoxide dismutase (**SOD**), which exacerbate the degree of hepatic injury (Zhao et al., 2021).

The Nrf2/Keap1 pathway is an essential signaling pathway related to oxidative stress. Kelch ECH associated protein 1 (**Keap1**) binds to Nrf2 and promotes ubiquitin–proteasome degradation. The inducer–activated and Nrf2–bound Keap1 is deactivated, resulting in the

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translocation of Nrf2 proteins to the nucleus. This promotes the expression of downstream genes, such as NAD(P)H quinone oxidoreductase 1 (**NQO1**) and heme oxygenase 1 (**HO−1**) (Kansanen et al., 2013; Saha et al., 2020).

The main generator of ROS in a cell is the mitochondria (Kasai et al., 2020; Willems et al., 2015). Mitofusins 1 and 2 (**Mfn1/2**), two−pass proteins located on the outer mitochondrial membrane, orchestrate the fusion of this membrane via heterotypic interactions between neighboring mitochondria (O'Mealey et al., 2017). On the other hand, optic atrophy 1 (**Opa1**), an inner membrane protein, concurrently facilitates matrix connectivity by governing the fusion of inner mitochondrial membranes (Cipolat et al., 2004). Notably, the integrity of the mitochondrial membrane potential is necessary for effective fusion to take place (Legros et al., 2002). Dynamin−related protein 1 (**Drp1**) is a cytoplasmic protein that primarily catalyzes division. It is recruited from the cytoplasm to the expected site of division on the outer mitochondrial membrane, where it binds to the mitochondrial fragmentation factor (**Mff**) on the outer mitochondrial membrane. Fission 1 (**Fis1**) also plays a minor role in division (Otera et al., 2010; Smirnova et al., 2001; Yoon et al., 2003).

In recent years, natural chemicals rich in bioactive components have gained increased attention in animal production because of their benefits of being non-resistant and residue free (Byun et al., 2021; Chen et al., 2017; Gupta and Birdi, 2017; Jingyan et al., 2017; Yao et al., 2016; Zhang et al., 2021; Zhong et al., 2016). Dihydromyricetin, an extract derived from the ampelopsis plant, has multiple pharmacological properties, including hepatoprotective, antioxidant, and mitochondrial protective effects (Guo et al., 2021; Sun et al., 2022; Xie et al., 2015; Zhang et al., 2018). Several studies have shown that DHM has protective effects in mouse models of hepatic injury caused by CCl_4 or acetaminophen (**APAP**). Our preliminary studies revealed that DHM ameliorated LPS−induced hepatic injury by regulating the NLRP3 inflammasome and subsequent pyroptosis. However, the potential mechanism of DHM on chicken liver antioxidant capacity remains unclear. The aim of this study is to investigate the hepatoprotective effects of DHM through the Nrf2/Keap1 pathway and mitochondrial dynamics, and to further explore the underlying mechanism, which provides a theoretical basis for the clinical application of DHM in the treatment of Gram−negative infections.

Materials and methods

Network pharmacology research

The CTD and Swiss Target Prediction databases were searched for DHM−related targets of action. GeneCards was consulted for targets associated with hepatic injury. Using the STRING database, the predicted potential common targets for DHM and hepatic injury were analyzed via PPI. To ensure good realism and accuracy of the data, a confidence level of 0.7 was chosen as the analysis condition. The results of the analysis were then imported into Cytoscape 3.9.1 software to obtain the network diagrams of interactions between common protein targets. GO and KEGG analyses of 13 core target genes were performed via the Metascape database to further investigate the potential therapeutic mechanisms of DHM in hepatic injury. A drug−target−pathway−disease network diagram was constructed via Cytoscape 3.9.1 software.

Chicken embryo hepatocyte culture and treatment

SPF chicken embryos were purchased from the Harbin Institute of Animal Husbandry and Veterinary Medicine of the Chinese Academy of Agricultural Sciences. The liver was extracted under sterile conditions, the gallbladder was removed, the liver was chopped, 0.2% trypsogen solution was added for digestion for 30 min at 37°C, and the mixture was subsequently centrifuged at 1000 rpm for 10 min (Yue et al., 2017). Hepatocytes were cultured in DMEM medium (Gibco, USA) supplemented with 10% FBS (Gibco, USA) and 1% penicillin-streptomycin (Beyotime, China). The experimental procedure involved separating the hepatocytes into five groups, each with three replicates: a control group, a model group (40 μg/mL LPS), and the LPS+DHM group (20 μM, 40 μM and 80 μM DHM).

Cell viability assay

Chicken primary hepatocytes were seeded into 96-well plates at a density of 5×10^5 cells/mL and cultured at 37°C with 5% CO_2 for 7 hours. The medium was then replaced with DMEM containing different concentrations of DHM (purity 98.0%, Shanghai Winherb Medical Technology, China) for 2 hours. LPS (Escherichia coli (O55:B5), Sigma, Germany) was solubilized in HBSS to 10 mg/mL and dilutions in DMEM medium without FBS achieved the required concentration. LPS was then added to the hepatocyte cultures, which were incubated for 12 hours. Subsequently, 20 μL of a 5 mg/mL MTT solution was added, and the cells were incubated for another 4 hours. Finally, 150 μL of DMSO was added, and the OD values were measured at a wavelength of 570 nm.

Lactate dehydrogenase (LDH) and antioxidant enzyme release assay

Chicken primary hepatocytes were treated as described above. Collected cells were ultrasonically broken by adding 0.3 mL of saline, and the supernatant was taken to detect the level of LDH release. Collected cell cultures were used to measure hepatocyte CAT, SOD and GSH−PX release levels. The manufacturer's instructions were followed for all procedures (Jiancheng Institute of Bioengineering, China).

Measurement of ROS and the mitochondrial membrane potential

Chicken primary hepatocytes were grown to 80%−90% confluence, the medium was then replaced with DMEM containing different concentrations of DHM, and the cells were cultured for 2 hours before adding LPS, followed by an additional incubation of 12 hours. ROS were detected via DCFH−DA (2, 7−dichlorodihydrofluorescein diacetate), which was used to monitor cellular ROS generation. JC−1 staining was used to assess the mitochondrial membrane potential (MMP). The manufacturer's instructions were followed for all procedures. An inverted fluorescence microscope (Axio Primo Vert A1, Carl Zeiss) was used to image the combined conditions.

Animals and treatment

The Xianfeng Chicken Farm (Harbin, China) supplied Hy−Line white broiler chickens. The 1−day−old chickens were fed for one week in preparation for the studies. The chickens were given regular laboratory feed and unlimited access to fresh drinking water was given to the chickens while they were kept in a house with light (12 h light/dark cycles).

The following procedures were used for conducting the experiments. The chickens were randomly assigned to five groups, each consisting of six replicates: the control group, the model group (60 mg/kg LPS), and the LPS + (0.025%, 0.05%, and 0.1%) DHM group (mass fraction). The DHM and LPS doses were selected on the basis of our earlier experiments. The DHM group of chickens was fed a regular diet supplemented with DHM at different concentrations for 14 days. The model and control groups of chickens were fed a regular diet without DHM supplementation. Liver tissue and blood were collected for further experimental evaluation after 12 h of intraperitoneal saline or LPS injection.

Detection of hepatic injury and antioxidant function specific indicators

A 1% liver tissue homogenate was prepared by mixing approximately 0.1 g of liver with 900 μL of cold saline and homogenized using

Table 1
Primer sequences used in this study

Gene Name	Primer Sequence	Product Length
β−actin	Forward: CTCTGACTGACCGCGTTACT Reverse: TACCAACCATCACACCCTGAT	172 bp
Mfn1	Forward: CATCGTTGTTGGCGGAGTGATTTG Reverse: AAACTGCTGCTTGAAGGCTCTCTC	142 bp
Mfn2	Forward: AAATCCCAGTCTGCCTCCTTTGC Reverse: CCACGCCACCAACCACGATG	128 bp
Opa1	Forward: TCCAGCAGCACAGACAATGAACTC Reverse: AAGTCTCCCACGCCACCTCTAC	116 bp
Drp1	Forward: TGCGGAGTCTGTTCATTGCCAAC Reverse: CCAACCCCAGCTTCCTGTTTCG	143 bp
Fis1	Forward: GGCAACACGCAGGGACTGAG Reverse: GGGCACGAGAGATGGCAAACC	145 bp
Nrf2	Forward: GGGACGGTGACACAGGAACAAC Reverse: GCTCTCCACAGCGGGAAATCAG	97 bp
Keap1	Forward: GCATCACAGCAGCGTGGAGAG Reverse: GGCGTACAGCAGTCGGTTCAG	118 bp
HO−1	Forward: GCTGGGAAGGAGAGTGAGAGGAC Reverse: GCGACTGTGGTGGCGATGAAG	107 bp
NQO1	Forward: CCCGAGTGCTTTGTCTACGAGATG Reverse: ATCAGGTCAGCCGCTTCAATCTTC	107 bp

Ultra Turrax (Shanghai Jing Xin, China). Commercially available kits were used to determine the serum ALT, AST and liver CAT, SOD and GSH−PX levels. The manufacturer's instructions were followed for all procedures.

Transmission electron microscopy (TEM) analysis

Following sacrifice, the livers were harvested, diced to 1 mm^3. The samples were examined via TEM (JEOL, Japan) after fixation in osmium tetroxide, dehydration in a graded series of ethanol, embedding in epoxy resin, sectioning with an ultramicrotome (Leica, Germany) and staining with uranyl acetate.

Quantitative real−time PCR (qRT-PCR) analysis

Total RNA was extracted from liver tissue and chicken primary hepatocytes using TRIzol Reagent (Takara, China). After quantification via a NanoDrop instrument (Thermo Scientific, USA), a PrimeScript^{TM} RT Reagent Kit (Takara, China) with gDNA Eraser was used for reverse transcription into cDNA. A TB Green® Premix Ex TaqTM II (Tli RNaseH Plus) Kit (Takara, China) and a LightCycler® 96 Instrument (Roche, Switzerland) were used for qRT-PCR. All of the primers used are listed in Table 1. The following system components were used: 6 μL of nuclease−free water, 1 μL of forward primer (20 mM), 1 μL of reverse primer (20 mM) and 2 μL of cDNA. Gene expression levels were calculated via the $2^{-\Delta\Delta Ct}$ method.

Western blotting analysis

To extract protein, liver tissue and primary hepatocytes were lysed with RIPA buffer containing PMSF (1 mM). Before protein was transferred to PVDF membranes, SDS−PAGE was used to separate the proteins. The following primary antibodies were added to the membranes and incubated overnight at 4°C: anti−Nrf2 (1:1000, No. WL02135, Wanleibio, China), anti−Keap1 (1:1000, No. WL03285, Wanleibio, China), anti−HO−1 (1:500, No. WL02400, Wanleibio, China), anti−NQO1 (1:500, No. WL04860, Wanleibio, China), and anti−Drp1(1:500, No. WL03028, Wanleibio, China), anti−Mnf1 (1:1000, No. bs-17693R, Bioss, China), anti−Mnf2 (1:1000, No. bs-2988R, Bioss, China), anti−Opa1 (1:1000, No. bsm-54144R, Bioss, China), anti−Fis1 (1:1000, No. bsm-60551M, Bioss, China), and β-actin (1:1000, No. bs-0061R, Bioss, China). After that, the goat anti-rabbit IgG (1:5000, No. bs-80295G-HRP, Bioss, China) or the goat anti-mouse IgG (1:5000, No. bs-0296G-HRP, Bioss, China) were added to the membranes and incubated for 2 h at 37°C. FluorChem RFR1045 (ProteinSimple, Silicon Valley, USA) was used for imaging and ImageJ was used for gray value analysis of the bands.

Molecular docking

The structure of DHM was obtained from the PubChem database and transformed via ChemBio 3D Ultra 20.0 software into a three−dimensional (3D) structural formula. The PDB formats for the Nrf2 and Keap1 proteins were obtained from the UniProt website. The Discovery Studio 2019 Client was used to perform DHM and protein preprocessing and molecular docking, whereas AutoDock Vina was used for binding affinity calculations. The docking results were visualized via PyMOL (Anaconda3).

Statistical analysis

Data were processed using GraphPad Prism (version 8.0, GraphPad Software Inc.). All data in the experiment were analyzed by one-way analysis of variance (ANOVA) or unpaired t-test. And expressed as mean ± standard deviation. $p < 0.05$ or $p < 0.01$ was considered significant difference.

Results

Network pharmacology prediction of DHM on hepatic injury

A total of 126 DHM−related genes were collected from the CTD and Swiss Target Prediction databases, and 11,345 hepatic injury related targets were collected from GeneCards (Fig. 1A). Using Cytoscape 3.9.1 software, 103 common targets for DHM and liver injury were identified, and a PPI network consisting of 97 nodes was constructed (Fig. 1B). Fifteen targets with more adjacent nodes were selected as core genes (degree values ≥90): Akt1, IL6, Bcl2, IL1b, Casp3, Egfr, Mmp9, Hif1a, Pparg, Esr1, Mapk3, Src, and Egf. These core genes were used to construct a core target PPI network (Fig. 1C). These core targets play important roles in oxidative stress, the inflammatory response and mitochondrial function. Therefore, we speculate that DHM could alleviate hepatic injury in chickens through these effects. The GO molecular function enrichment (Fig. 1D) analysis revealed 497 biological processes, 9 cellular components and 30 molecular functions. The top 9 moderate values for each category are selected, and a bubble chart is plotted. These pathways are related mainly to the response to reactive oxygen species, the regulation of muscle cell proliferation, the response to oxidative stress, the cellular response to chemical stress, etc. The KEGG analysis (Fig. 1E) results revealed 94 signaling pathways, with the top 15 signaling paths in terms of selectivity value, and are presented in the form of a bubble diagram. These pathways are related mainly to HIF−1, estrogen, AGE−RAGE in diabetic complications, TNF signaling pathways, etc. A network diagram of the relationships between 13 key targets and the top 15 signaling pathways was created via Cytoscape 3.9.1 software to reveal the role of DHM in hepatic injury (Fig. 1F). On the basis of these findings, we speculate that the mitigating effect of DHM on hepatic injury may be related to oxidative stress, the inflammatory response and mitochondrial function. Further in vitro and in vivo experiments are needed to validate this speculation.

DHM improved chicken primary hepatocytes injury and oxidative stress

The extracorporeal experimental procedure is shown in Fig. 2A. Chicken primary hepatocytes were basically adherent to the bottom wall after 48 h of cultivation, with irregular morphology (Fig. 2B). To determine whether DHM protects hepatocytes from injury, the model was replicated after hepatocytes were exposed to 40 μg/mL LPS for 12 h. Compared with the model group, DHM significantly increased hepatocyte viability and reduced LDH levels ($p < 0.01$) (Fig. 2C—**D**). The

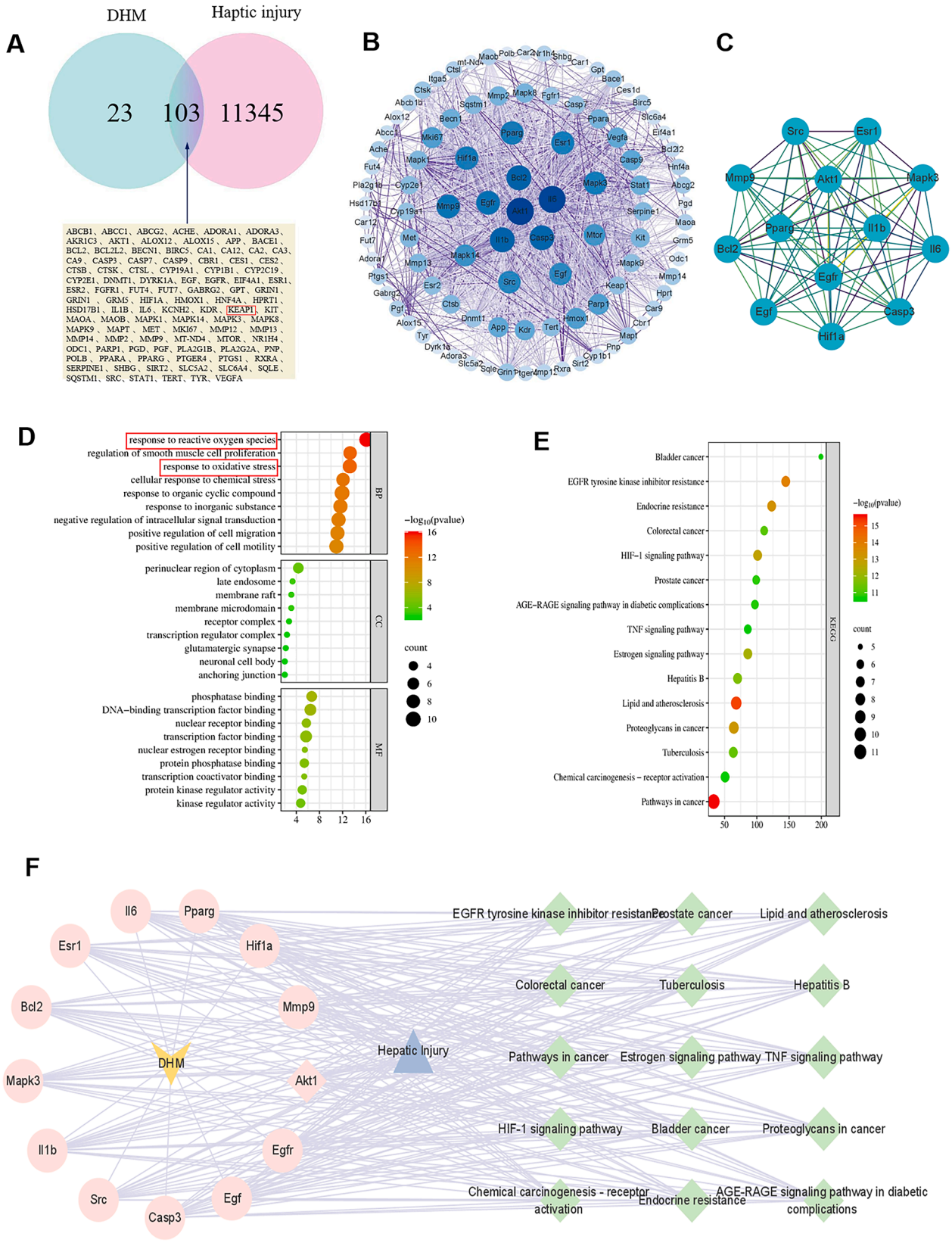

Fig. 1. Effects of DHM on hepatic injury based on network pharmacology. (A) Wayne plots of DHM and hepatic injury targets. (B) Constructing PPI networks with collective targets. (C) Construction of a PPI network with core targets. (D) GO enrichment analysis of core target genes. (E) KEGG pathway enrichment analysis of DHM for the treatment of hepatic injury. (F) The Network of relationships between DHM, the target, and the top 15 KEGG pathways.

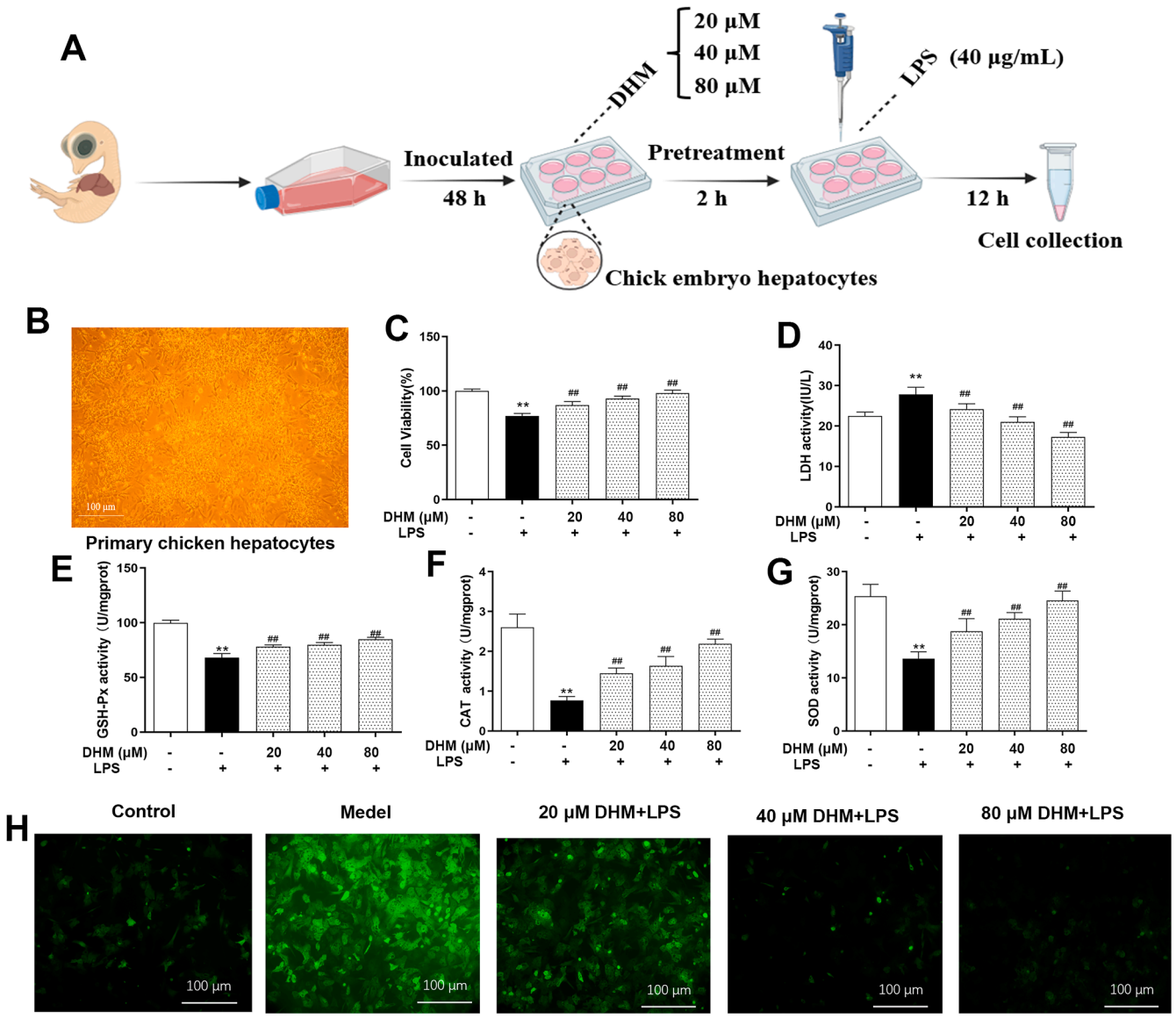

Fig. 2. DHM attenuates LPS–induced chicken primary hepatocytes injury and oxidative stress. (A) Schematic diagram of the animal experiment process. (B) Morphology of chicken primary hepatocytes. (C) Cell viability by MTT. (D) LDH activity in cell cultures. (E–G) Antioxidant enzyme GSH–PX, CAT and SOD activity in hepatocyte. (H) ROS fluorescence. Magnification: 400 ×; bar: 100 μm. The significance for comparisons between the control and model groups is noted as $^{**}p < 0.01$. The significance for comparisons between the model and DHM+LPS groups is noted as $^{\#\#}p < 0.01$.

antioxidant enzyme activities results revealed that LPS significantly decreased the levels of GSH–PX, CAT and SOD; in contrast, DHM significantly increased the levels ($p < 0.01$) (Fig. 2E—**G**). In addition, the ROS fluorescence results revealed that DHM reduced the accumulation of intracellular ROS (Fig. 2H).

DHM activated the Nrf2/Keap1 pathway in primary hepatocytes

The Nrf2/Keap1 pathway is a key signaling pathway associated with oxidative stress. To clarify whether DHM directly binds to Nrf2/Keap1, we performed molecular docking. The results revealed that the binding affinities of DHM for Nrf2 and Keap1 were -6.5 kcal/mol and -9.0 kcal/mol, respectively. Visualization showed that DHM formed hydrogen bonds, π–alkyl hydrophobic interactions, π–π stacking interactions, and hydrophobic interactions in the crystal structure of the Nrf2 complex (Fig. 3A); and formed hydrogen bonds, Pi–Alkyl hydrophobic interactions, and Pi–Sulfur interactions in the crystal structure of the Keap1 complex (Fig. 3B). These results suggested that the DGR binding pocket of Keap1 was tightly bound to DHM through these interaction forces, which effectively prevented the binding of Keap1 and Nrf2. In primary hepatocytes, the impact of DHM on the Nrf2/Keap1 pathway was further investigated. The results (Fig. 3C–**K**) displayed that LPS significantly downregulated the mRNA and protein expression of Nrf2, HO–1 and NQO1, whereas Keap1 was upregulated. In contrast, DHM markedly increased the mRNA and protein expression of Nrf2, HO–1 and NQO1 and reduced Keap1 expression ($p < 0.01$). Moreover, the trend of change was more pronounced in the 80 μM DHM group, so this dose was chosen for the subsequent experiments. These results indicated that DHM effectively aggregated the Nrf2 protein and increased the expression levels of the downstream antioxidant factors HO–1 and NQO1.

DHM mitigated mitochondrial dynamic imbalance in primary hepatocytes

Mitochondria are major sources of ROS within the cell and are especially vulnerable to oxidative stress. To investigate whether DHM mitigated LPS-induced hepatocyte mitochondrial dynamic imbalance, the mRNA and protein expression levels of key division proteins (Drp1

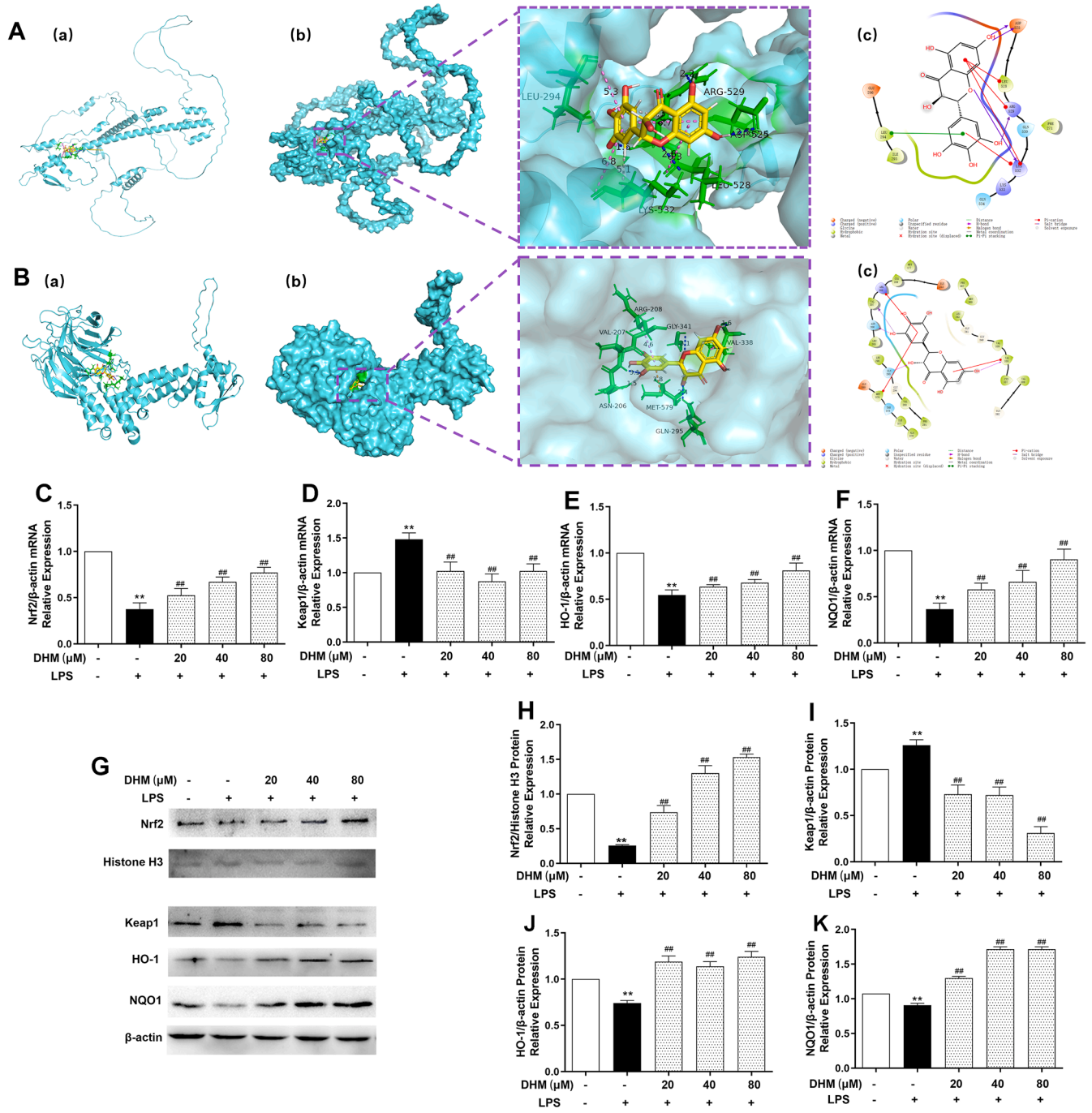

Fig. 3. Changes in the Nrf2/Keap1 pathway in LPS–induced chicken primary hepatocyte injury. (A) DHM and Nrf2 molecular docking results. (B) DHM and Keap1 molecular docking results, (a) band diagram, (b) 3D diagram, (c) the hydrogen–bond interaction. (C) Nrf2, (D) Keap1, (E) HO−1 and (F) NQO1 mRNA expression. (G) Protein bands of Nrf2, Keap1, HO−1 and NQO1. (H) Nrf2 (nuclear), (I) Keap1, (J) HO−1 and (K) NQO1 protein levels. The significance for comparisons between the control and model groups is noted as $^{**}p < 0.01$. The significance for comparisons between the model and DHM+LPS groups is noted as $^{\#\#}p < 0.01$.

and Fis1) and key fusion proteins (Opa1, Mfn1 and Mfn2) were measured. The results (Fig. 4A—**K**) indicated that the mRNA and protein expression of Drp1 and Fis1 were markedly reduced in DHM+LPS group. However, the expression of Opa1, Mfn1 and Mfn2 were significantly increased, compared with LPS group ($p < 0.01$). Additionally, the MMP is an essential indicator of mitochondrial functional status. We measured the MMP of hepatocytes, and found that the MMP decreased in the LPS group compared with the control group, whereas DHM inhibited the LPS-induced the decrease (Fig. 4L). These results indicated that DHM effectively attenuated mitochondrial function damage.

DHM alleviated LPS–induced chicken hepatic injury and oxidative stress

The animal experimental procedure is depicted in Fig. 5A. The chicken hepatic injury model was replicated by 60 mg/kg LPS on the basis of previous experiments (Shi et al., 2022). The results showed that LPS markedly increased the activities of serum ALT and AST, whereas DHM treatment significantly decreased the activities ($p < 0.05$ or $p < 0.01$) (Fig. 5B-C). To further investigate the impact of DHM on hepatic oxidative stress, the levels of antioxidant enzymes were examined in chicken livers. The results showed that LPS significantly reduced the levels of liver GSH−PX, CAT and SOD, which were markedly increased

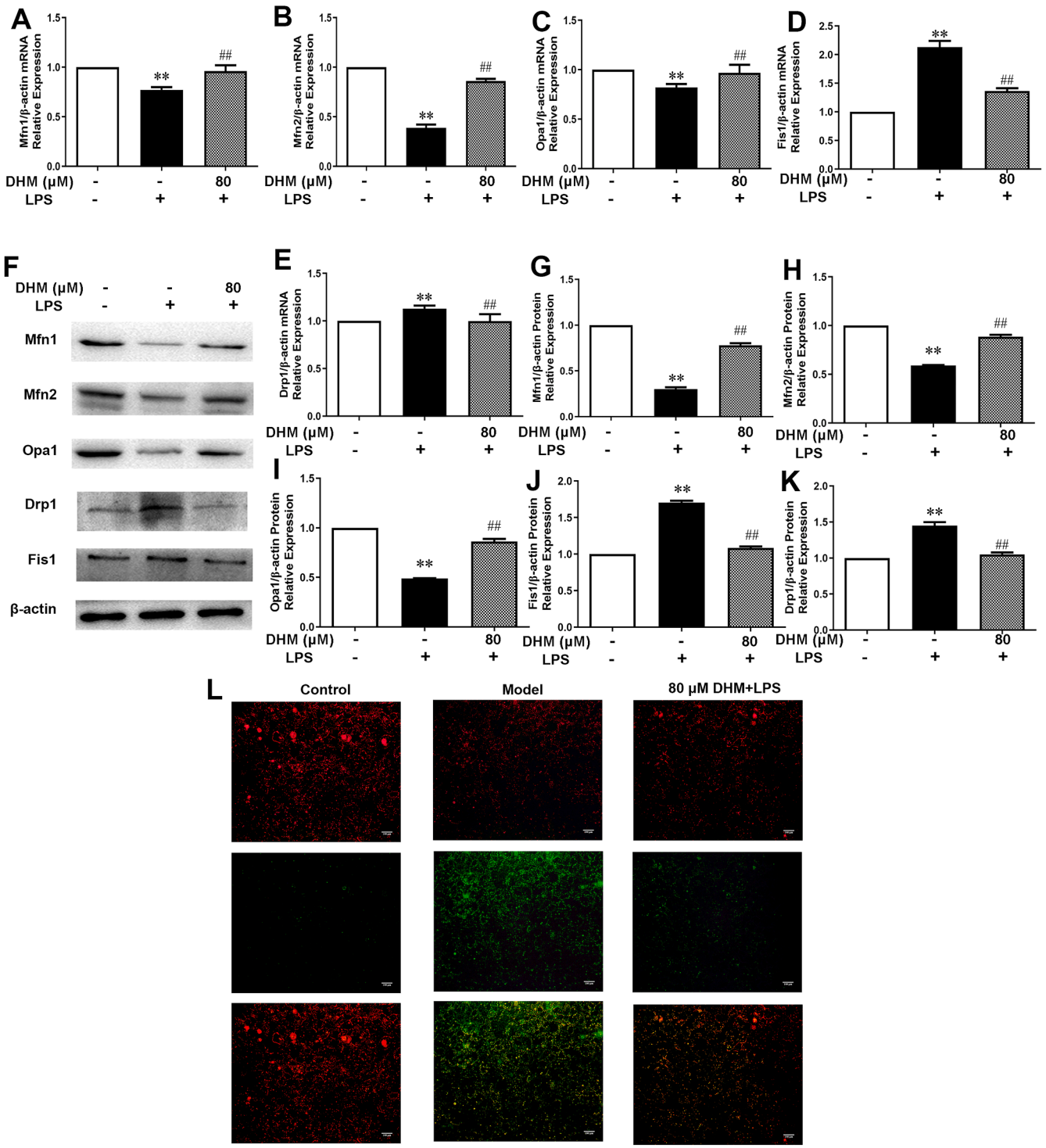

Fig. 4. Changes in the levels of mitochondrial fusion division related factors in LPS–induced chicken primary hepatocyte injury. (A) Mfn1, (B) Mfn2, (C) Opa1, (D) Fis1 and (E) Drp1 mRNA expression. (F) Protein bands of Opa1, Mfn1, Mfn2, Fis1 and Drp1. (G) Mfn1, (H) Mfn2, (I) Opa1, (J) Fis1 and (K) Drp1 protein levels. (L) The MMP of chicken hepatocytes. Magnification: 100 ×; bar: 100 μm. The significance for comparisons between the control and model groups is noted as $^{**}p < 0.01$. The significance for comparisons between the model and DHM+LPS groups is noted as $^{\#\#}p < 0.01$.

by DHM ($p < 0.01$) (Fig. 5D–**F**). These results indicated that DHM effectively ameliorates LPS–induced chicken hepatic injury and oxidative stress.

DHM activated the Nrf2/Keap1 signaling pathway in chicken

To further confirm whether DHM activating the Nrf2/Keap1 pathway in LPS–induced chicken hepatic injury, the mRNA and protein expression levels of Nrf2, Keap1, and their downstream antioxidant factors NQO1 and HO–1 were measured. The results (Fig. 6A–**I**) revealed that LPS significantly decreased the mRNA and protein expression levels of Nrf2, HO–1 and NQO1 and increased the expression of Keap1 in the liver ($p < 0.05$ or $p < 0.01$). DHM treatment markedly attenuated these changes, in particular, 0.1% DHM significantly

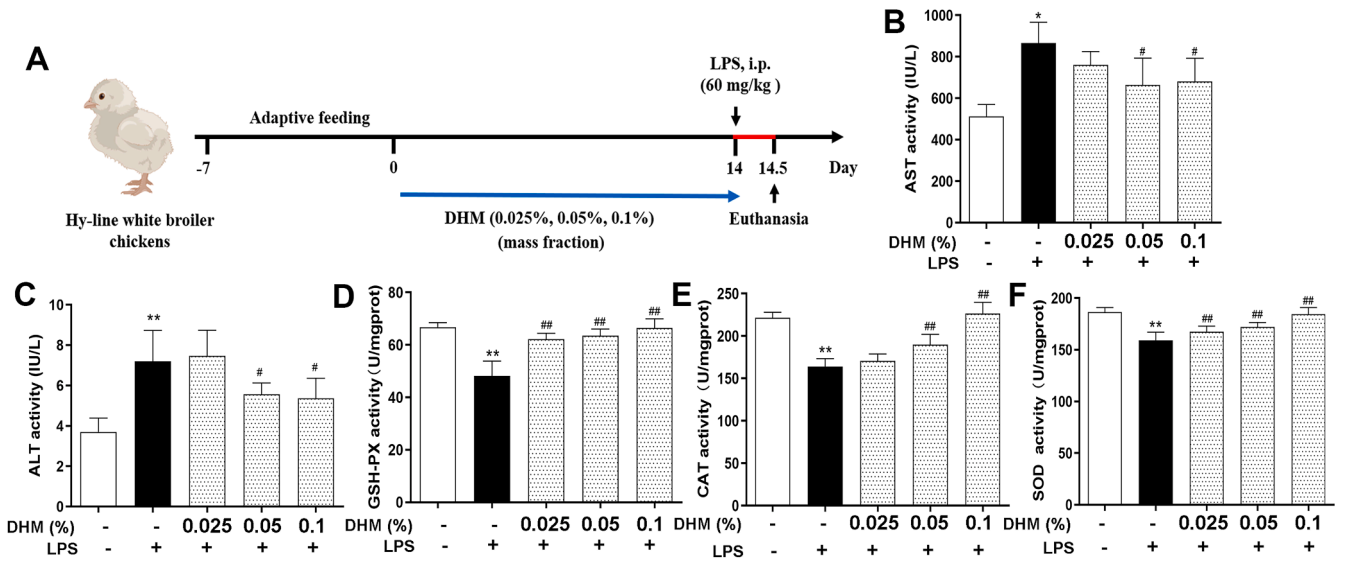

Fig. 5. Effects of DHM on LPS–induced chicken hepatic injury and oxidative stress. (A) Schematic diagram of the animal experimentation process. (B) AST and (C) ALT activities in the serum. (D) GSH–PX, (E) CAT, and (F) SOD activities in the liver. The significance for comparisons between the control and model groups is noted as $^{*}p < 0.05$ and $^{**}p < 0.01$. The significance for comparisons between the model and DHM+LPS groups is noted as $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$.

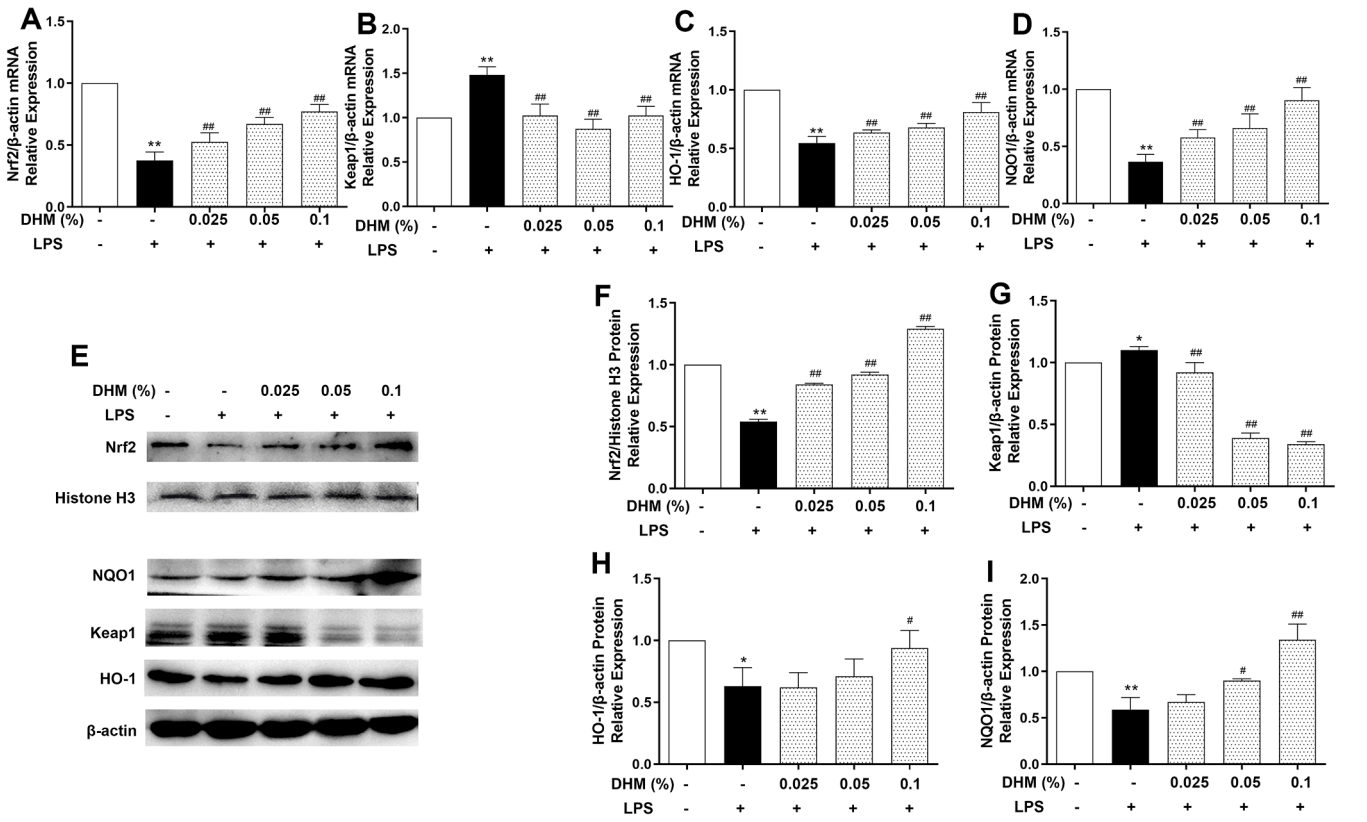

Fig. 6. Changes in the Nrf2/Keap1 pathway in LPS–induced chicken hepatic injury. (A) Nrf2, (B) Keap1, (C) HO−1 and (D) NQO1 mRNA expression. (E) Protein bands of Nrf2, Keap1, HO−1 and NQO1. (F) Nrf2 (nuclear), (G) Keap1, (H) HO−1 and (I) NQO1 protein levels. The significance for comparisons between the control and model groups is noted as $^{*}p < 0.05$ and $^{**}p < 0.01$. The significance for comparisons between the model and DHM+LPS groups is noted as $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$.

increased the expression of Nrf2, HO−1 and NQO1, and markedly suppressed Keap1. ($p < 0.05$ or $p < 0.01$). Hence, 0.1% DHM was chosen for subsequent experiments. These results revealed that DHM treatment activated the Nrf2/Keap1 pathway.

DHM regulated mitochondrial dynamic imbalance in chicken

To further validate the effect of DHM on mitochondrial dynamic imbalance in the in vivo model, we examined the protein and mRNA expression of factors related to mitochondrial dynamics in LPS–induced chicken hepatic injury. The results revealed that compared with the LPS

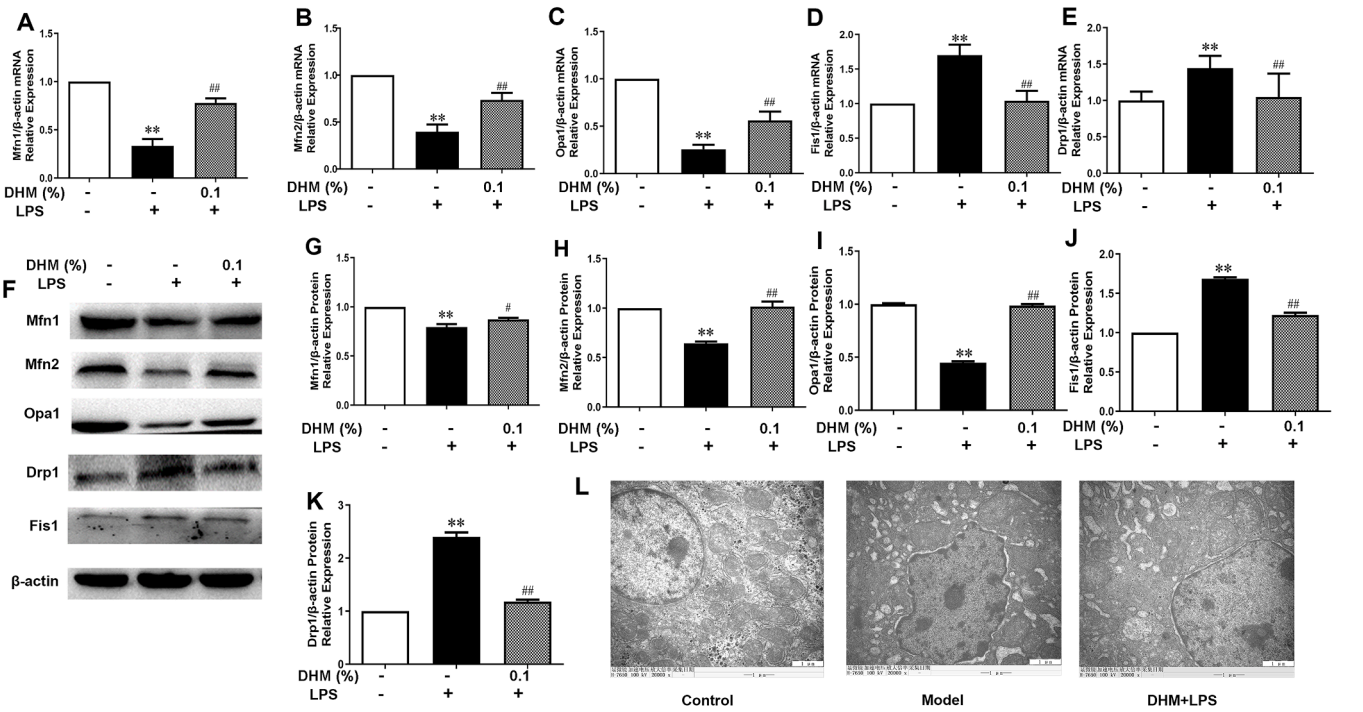

Fig. 7. Changes in the expression of mitochondrial fusion division related factors in LPS–induced hepatic injury. (A) Mfn1, (B) Mfn2, (C) Opa1, (D) Fis1 and (E) Drp1 mRNA expression. (F) Protein bands of Opa1, Mfn1, Mfn2, Fis1 and Drp1. (G) Mfn1, (H) Mfn2, (I) Opa1, (J) Fis1 and (K) Drp1 protein levels. (L) Morphology of hepatocyte mitochondria by ultramicropathology in chickens. Magnification: 20,000 ×; scale bar: 1 μm. The significance for comparisons between the control and model groups is noted as $^{**}p < 0.01$. The significance for comparisons between the model and DHM+LPS groups is noted as $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$.

group, the mRNA and protein expression of Fis1 and Drp1 were significantly decreased in the DHM+LPS group, whereas Opa1, Mfn1, and Mfn2 were significantly increased ($p < 0.05$ or $p < 0.01$) (Fig. 7A—**K**). Furthermore, the results of ultramicroscopic pathology revealed that the subcellular morphology of the liver was normal in the control group, whilst the nuclear membrane of the cell nucleus was intact, the mitochondrial content was abundant, the bilayer membrane structure was intact, and the mitochondrial crista structure was clear. The model group presented irregular nuclear shrinkage, enlarged nuclear membrane gaps, numerous vacuoles in the endoplasmic reticulum and mitochondria, mitochondrial swelling, and unclear mitochondrial cristae. In contrast, the morphology of the nucleus and mitochondria in the DHM + LPS group tended to be normal (Fig. 7L). These results indicated that DHM can effectively regulate mitochondrial dynamic homeostasis and attenuate mitochondrial structural damage.

Discussion

In the poultry industry, Gram–negative bacterial diseases cause increased mortality and severe economic losses. In addition, Gram–negative bacterial infections are always accompanied by the release of LPS from the cell wall, which enters the liver via the portal vein (Kim et al., 2013; Napetschnig and Wu, 2013). Considering that the liver is a central organ in metabolism, it is necessary to develop new drugs for the treatment of hepatic injury. DHM has been extensively studied for its various biological functions and pharmacological activities, including anti–inflammatory, hepatoprotective and antioxidant effects.

Network pharmacology is an invaluable tool for analysing drug-disease interactions and mechanisms (Ge et al., 2023). A collective of 103 targets for DHM and liver injury were obtained from an online database. The 15 core targets screened were closely related to oxidative stress and mitochondrial function (Liou et al., 2016; Mori et al., 2019; Shi et al., 2021; Yang et al., 2023). GO analysis showed that ROS and oxidative stress are important biological processes. These studies suggest that the mitigating effect of DHM on hepatic injury could be related to oxidative stress, inflammatory response and mitochondrial function. Previous studies have suggested that the mitigating effect of DHM on hepatic injury could be related to the inflammatory response (Chang et al., 2020; Shi, Wang, Zhang, Ishfaq, Li, Zhang, Si, Li, Li and Liu, 2022). Thus, the aim of this study was to determine the hepatoprotective effects of DHM on oxidative stress and mitochondrial function.

DHM increased the viability of primary hepatocytes and reduced LDH release in the culture medium, thus effectively reducing primary hepatocyte injury. In addition, important indicators of liver function are serum ALT and AST activities (Ding et al., 2018). Our study confirmed that DHM could decrease the ALT and AST activities in the serum. GSH–PX, SOD and CAT are recognized as key antioxidant entities crucial for oxidative stress. In this study, DHM increased the CAT, SOD and GSH–PX contents in the liver. DHM also has the same promoting effect on primary hepatocytes, indicating that DHM could improve the antioxidant ability of the liver, thus inhibiting LPS–induced oxidative damage. Mitochondria are considered to be a major source of ROS. Studies have reported that DHM could significantly reduce the ROS content in H_2O_2–induced HUVECs, thereby alleviating oxidative stress (Hou et al., 2015). Our experimental data further confirmed that DHM effectively reduced the ROS content in LPS–induced primary hepatocyte injury.

The Nrf2/Keap1 pathway plays a crucial role in preventing oxidative stress. Nrf2 dissociates from Keap1 and enters the cell nucleus when cells are exposed to oxidative stress. Flavonoids effectively ameliorate hepatic injury by regulating the Nrf2/Keap1 pathway (Shi et al., 2018; Wang et al., 2021b; Wang et al., 2017). Recent studies have shown that dietary addition of DHM can improve the antioxidant capacity of fattening pigs by increasing CAT and GSH–PX activities and activating the ERK/Nrf2/HO–1 signaling pathway. Zhongyang Guo reported that the flavonoid compound DHM improved lipid metabolism and liver antioxidant capacity by affecting Nrf2 signaling in male Kunming mice or fattening pigs (Guo et al., 2022). In this study, molecular docking predicted that DHM could bind directly to Nrf2 and interact with amino acid residues of Keap1, occupying the binding pocket of Keap1 and Nrf2 by binding more tightly, thereby activating the Nrf2 pathway. The main

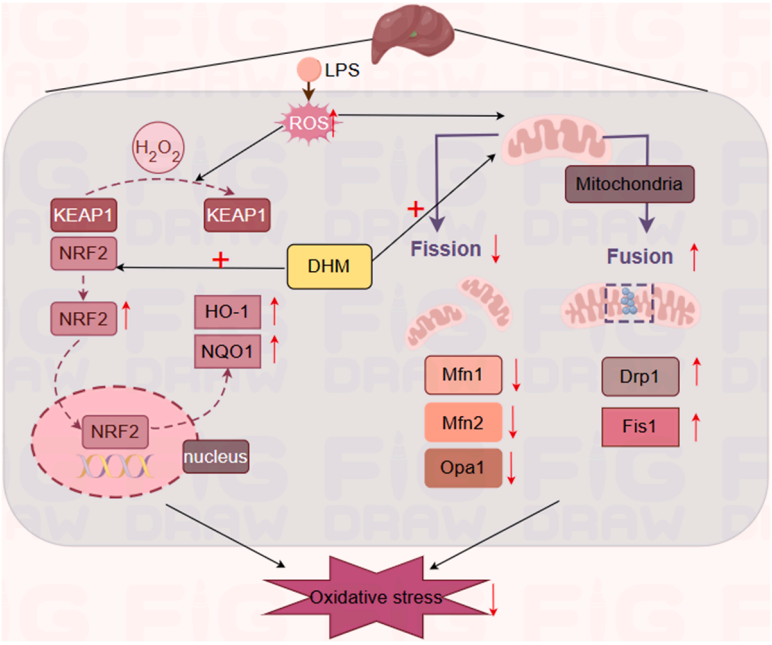

Fig. 8. Mechanism of DHM regulates LPS–induced hepatic oxidative stress in chickens.

structural domains of Keap1 comprise NTR, BTB, IVR, DGR, and CTR. The DGB domain of a six–β–bladed propeller structure recognizes and binds substrate proteins, which can interact with Nrf2. Some studies have found that the region including VAL293–LYS583 is the DGR domain of the chicken Keap1 protein. In this study, DHM upregulated Nrf2, NQO1, and HO–1 protein and mRNA expression and decreased Keap1 protein and mRNA expression in LPS–challenged hepatocyte injury. Moreover, DHM also activated Nrf2 signaling in the LPS–induced chicken hepatic injury. As mentioned above, DHM improved antioxidant stress in chickens through the activation of Nrf2/Keap1.

Oxidative stress can provoke mitochondrial dysfunction and cause ultrastructural damage. In our study, the electron microscopy results showed that LPS resulted in subcellular structural damage in the liver, with disorganized nuclei and mitochondria and fragmented mitochondrial cristae. In contrast, DHM normalized the morphology of the nucleus and mitochondria. When mitochondrial function is impaired, a series of negative effects are triggered, including overproduction of ROS, injury to the MMP, and increased apoptosis (Galley, 2010; Pardo et al., 2019; Xu et al., 2021). To further explore the detailed mechanisms by which DHM regulates mitochondrial function, we analyzed the expression of key proteins involved in mitochondrial fusion and fission. Mfn1/2, located on the mitochondrial outer membrane, and Opa1, situated on the mitochondrial inner membrane, facilitate mitochondrial fusion. Conversely, mitochondrial fission is predominantly controlled by Drp1 and its adaptor Fis1. Hui–Li Wang reported that DHM treatment reduced mitochondrial fragmentation in heat stress–induced apoptosis (Wang et al., 2021a). Our experiments clearly demonstrated that DHM effectively increased the protein and mRNA levels of Mfn1, Mfn2, and Opa1, while decreasing Drp1 and Fis1 in LPS–induced hepatic injury in vivo and in vitro. Furthermore, DHM regulated LPS–induced mitochondrial damage. These results suggested that DHM promotes the expression of mitochondrial fusion proteins and inhibits the expression of mitochondrial division proteins.

Conclusion

In summary, DHM ameliorated LPS–induced hepatic injury in chickens. DHM effectively increased antioxidant enzyme activities, mitigated ROS accumulation and inhibited hepatic oxidative stress via the upregulation of Nrf2/Keap1. Furthermore, DHM attenuated mitochondrial damage, improved mitochondrial function by increasing the MMP, and regulated the balance between mitochondrial fusion and division. The mechanism is shown in Fig. 8. These findings indicated that DHM could be a good natural pharmaceutical ingredient for the prevention of hepatic injury. This study provides a basis for the application of DHM in the poultry industry and new ideas for the development of new drugs.

CRediT authorship contribution statement

Liang Yuan: Writing – original draft, Conceptualization, Investigation, Methodology, Data curation. **Xinru Jiang:** Formal analysis, Investigation, Methodology. **Yani Ren:** Methodology, Data curation. **Bingke Ma:** Validation, Data curation. **Zhenghua Ji:** Methodology, Data curation. **Shibo Wang:** Methodology, Data curation. **Beili Hao:** Conceptualization, Investigation. **Changwen Li:** Supervision. **Rui Li:** Supervision. **Fangping Liu:** Writing – review & editing, Conceptualization, Methodology, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics statement

Experiments were approved by the Institutional Animal Care and Use Committee (NEAUEC20220326) of the Northeast Agricultural University (Harbin, China), and we conducted in accordance with the principles and specific guidelines.

Abbreviations used

CAT, catalase; DHM, dihydromyricetin; Drp1, dynamin-related protein 1; Fis1, fission 1; GSH-PX, glutathione peroxidase; HO-1, hema oxygenase 1; Keap1, Kelch-like ECH-associated protein 1; LPS, lipopolysaccharide; MDA, malondialdehyde; Mfn1, mitofusins 1; Mfn2, mitofusins 2; NQO1, NAD(P)H quinone oxidoreductase 1; Nrf2, nuclear

factor erythroid 2-related factor 2; Opa1, optic atrophy 1; ROS, reactive oxygen species; SOD, superoxide dismutase.

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Data availability

No data was used for the research described in the article.

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