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Original Article

Dihydroquercetin alleviates mitochondrial dysfunction and inhibits NLRP3-mediated pyroptosis in primary hepatocytes from chronic liver failure patients

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Abstract: Objective: To investigate the protective effects of dihydroquercetin (DHQ) on mitochondrial function in primary hepatocytes from patients with chronic liver failure (CLF) and discuss the underlying molecular mechanism. Methods: Blood samples from 15 CLF patients and 15 healthy subjects were retrospectively analyzed. Primary hepatocytes were isolated using a modified two-step collagenase perfusion method. The optimal concentration of DHQ (20 μmol/L) was determined using the Cell Counting Kit-8 (CCK-8) assay. Cells were divided into a control group, a plasma group, and a DHQ intervention group. The assessed indicators included cell viability (CCK-8), apoptosis rate (flow cytometry), inflammatory factors (IL-1β, IL-6, TNF-α; ELISA), oxidative stress markers (MDA, SOD, GSH-Px), mitochondrial function (JC-1 staining, mtDNA/nDNA ratio) and related protein expression (Western blot). Results: DHQ adjunctive therapy reduced serum circulating mitochondrial DNA levels in patients with CLF, increased serum SOD2 and Humanin expression, inhibited activation of the NLRP3-mediated pyroptotic pathway, improved remission rates of decompensated complications, and showed favorable safety and tolerability in patients with end-stage liver diseases. In vitro, hepatocyte viability was markedly decreased in the CLF group, with increased apoptosis, elevated IL-1β, IL-6, TNF-α, and MDA levels, as well as declined SOD and GSH-Px activities. DHQ reduced apoptosis, up-regulated mitochondrial functional markers (SOD2, Humanin), restored JC-1 red/green fluorescence ratios, inhibited NLR family pyrin domain-containing 3 (NLRP3) inflammasome activation, and suppressed the caspase-1/IL-1β axis. Conclusion: DHQ exerts multidimensional protective effects on hepatocytes, providing a novel strategy and clinical evidence for mitochondria-targeted CLF therapy.

Keywords: Dihydroquercetin, chronic liver failure, mitochondrial dysfunction, mitochondrial quality control, oxidative stress and apoptosis

Introduction

Chronic liver failure (CLF) represents the common end-stage outcome of various chronic liver diseases, characterized by progressive hepatic decompensation, multiple-organ dysfunction, and high mortality. With short-term mortality exceeding 30%, CLF has become a severe public health issue worldwide [1]. Currently, clinical treatment mainly involves etiological control, artificial liver support, and liver transplantation. However, due to factors such as donor shortage and limited treatment time windows, the therapeutic efficacy is significantly restricted. Consequently, developing novel interventions targeting key pathological processes is imperative [2].

Mitochondria are the core organelles regulating energy metabolism and oxidative stress in hepatocytes. Their dysfunction - including reduced ATP synthesis, excessive reactive oxygen species (ROS) production, and dysregulated mitochondrial permeability transition pore (mPTP) opening - has been confirmed as a crucial mechanism driving the progression of CLF. Mitochondrial damage not only directly induces cellular energy crisis but also exacerbates

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hepatocyte death by activating apoptosis and pyroptosis, forming a vicious cycle of "damage - dysfunction - necrosis" [3, 4]. Therefore, targeted protection of mitochondrial function represents a promising therapeutic strategy for CLF.

Natural polyphenolic compounds have become a research hotspot in liver disease intervention due to their antioxidant, anti-inflammatory, and metabolic regulatory properties [5]. Dihydroquercetin (DHQ), a flavonol derivative derived from plants such as larch, has been shown to alleviate liver injury by scavenging ROS and inhibiting the NF-κB inflammatory pathway [6, 7]. However, whether DHQ exerts a direct protective effect on the mitochondria of primary hepatocytes under the specific pathological conditions of CLF, and what are the underlying mechanisms remain unclear. Most existing studies used liver cancer cell lines or acute liver injury models. Primary hepatocytes from CLF patients, due to long-term exposure to an inflammatory microenvironment, accompanied by metabolic reprogramming and mitochondrial epigenetic modifications, exhibit significantly different mitochondrial injury mechanisms from those of normal hepatocytes [8]. Moreover, systematic studies exploring whether DHQ modulates the mitochondrial quality control network, including mitochondrial biogenesis, dynamics, and mitophagic clearance, are lacking.

In this study, primary hepatocytes were isolated from CLF patients to investigate the protective effects of DHQ on mitochondrial function, aiming to provide a theoretical basis for mitochondrial-targeted therapies in CLF. Clinically, if DHQ is confirmed to effectively protect the mitochondrial function of hepatocytes in CLF patients, it may offer a new candidate drug for slowing disease progression, reducing the need for liver transplantation, and improving patient prognosis. Moreover, this research may facilitate the translational application of natural products in the treatment of end-stage liver diseases.

Materials and methods

Cell source

Retained blood samples from 15 CLF patients and 15 matched healthy subjects admitted to our hospital between January and May 2025 were retrospectively analyzed. Primary human hepatocytes were isolated from the discarded non-tumorous liver tissues from 8 out of the 15 CLF patients who underwent orthotopic liver transplantation during the same period. None of these 8 patients had received DHQ treatment before surgery.

Inclusion criteria (CLF patients): patients were required to meet the clinical diagnostic guidelines for CLF [9], with an age between 18 and 65 years, Child-Pugh grade C disease, and a Model for End-Stage Liver Disease (MELD) score ≥20. After confirmation, patients received appropriate treatment, including either conventional treatment or/and combined DHQ treatment. Conversely, those with liver cancer, severe infection, or recent use of mitochondrial-toxic drugs were excluded.

This study was approved by the Ethics Committee of Linyi People's Hospital (No. YX200-639) and conducted in accordance with the Declaration of Helsinki. For the retrospective analysis of residual blood samples, written informed consent was waived by the ethics committee due to the anonymous nature of the samples and absence of additional risk to patients. For the collection of discarded liver tissues for hepatocyte isolation, all liver transplant donors provided written informed consent before surgery.

Clinical treatment

Among the 15 enrolled CLF patients, 7 patients received conventional treatment (labeled as control group) and 8 patients received DHQ treatment (labeled as DHQ group). The control group received standard comprehensive medical therapy according to the Guidelines for the Diagnosis and Treatment of Chronic Liver Failure, including liver-protective treatment, enzyme-lowering therapy, jaundice-relieving treatment, correction of coagulation disorders, prevention and treatment of complications, and control of the underlying cause, administered for 4 weeks. In the DHQ group, oral DHQ was added to standard medical treatment at a dose of 100 mg per administration, three times daily, for 4 weeks.

Blood sample collection and detection

Fasting morning blood samples (4 mL) were collected from all CLF patients and healthy donors

into clot-promoting tubes. Samples were left at room temperature for 30 minutes and then centrifuged (1505×g, 4°C) for 15 minutes to obtain serum. Liver function indicators, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin (TBIL), were detected using an automatic biochemical analyzer. Inflammatory factors (IL-1β, IL-6, TNF-α) and oxidative stress markers (SOD, MDA, GSH-Px) were quantified by ELISA. In addition, serum levels of mitochondrial-specific superoxide dismutase 2 (SOD2), the mitochondrial-derived peptide Humanin, NLRP3 inflammasome protein, cleaved caspase-1, and N-terminal gasdermin D (NT-GSDMD) were measured. All assays were performed in strict accordance with the kit instructions, and the corresponding absorbance values were read using a microplate reader to calculate sample concentrations. All clinical blood samples were collected at two time points: baseline (within 24 hours before treatment initiation) and week 4 after the last dose of treatment.

Serum qPCR

Real-time quantitative PCR (qPCR) was used to measure the relative copy number of circulating mitochondrial DNA (cmtDNA) in serum. Total DNA was extracted from 200 μL of serum using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. The mitochondrial-specific gene ND1 was used as the target gene, and nuclear gene β-actin served as the internal reference. Primer sequences were as follows: ND1 (F: 5'-CCT CTA CCA AAA TCC TCC C-3'; R: 5'-GAG CGA TGG TGA GAG CTA A-3'); β-actin (F: 5'-TGG AAC GAG GGT CAG ATG T-3'; R: 5'-GCG GCA TTT GAA GGT AGT T-3'). Amplification was performed using SYBR Green PCR Master Mix (Applied Biosystems), and the relative copy number of cmtDNA was calculated using the 2-ΔΔCt method. The use of β-actin as an internal control for serum cmtDNA quantification has been validated in multiple previous studies. In addition, absolute quantification of cmtDNA was additionally performed using a standard curve generated from serial dilutions of a plasmid containing the ND1 gene, and the results were consistent with the relative quantification data (data not shown).

Clinical efficacy and safety

The clinical efficacy endpoint was the remission rate of CLF-related decompensated complications at week 4, including ascites, hepatic encephalopathy, and spontaneous bacterial peritonitis. Criteria for remission or control were applied in accordance with the Guidelines for the Diagnosis and Treatment of Chronic Liver Failure [10]. Safety was evaluated based on the incidence, severity, and outcomes of drug-related adverse events during the intervention period. The severity of adverse events was graded according to the Common Terminology Criteria for Adverse Events (CTCAE, version 5.0). Throughout the study, the time of onset, clinical manifestations, management measures, and outcomes of all adverse events were recorded comprehensively.

Isolation of primary human hepatocytes

Primary human hepatocytes were isolated using a modified ex vivo multi-point injection perfusion method. Approximately 10 g of non-tumorous, discarded liver tissue was collected intraoperatively from the above-mentioned 8 liver transplant recipients with CLF. Tissue blocks were rinsed three times with ice-cold D-Hanks solution to remove residual blood. Each tissue block was placed in sterile Petri dish, and 37°C preheated calcium/magnesium-free perfusion fluid containing 0.5 mM EGTA was injected into multiple sites using a 25-gauge needle at a rate of 5 mL/min for 10 minutes to remove blood cells and chelate divalent cations. Subsequently, the tissue was perfused with 37°C preheated digestion solution containing 0.05% collagenase IV (Sigma-Aldrich) and 2% bovine serum albumin (BSA, Gibco) via multi-point injection at the same rate for 15-20 minutes, until the tissue became soft and translucent. Subsequently, the digested tissue suspension was filtered through a 70 μm cell sieve, and centrifuged (50 g, 5 minutes) to collect hepatocytes. Immediately after isolation, cell viability was assessed using trypan blue staining, and only preparations with ≥90% viability were used for subsequent experiments. Isolated hepatocytes were seeded into 6-well plates at a density of 1×10^6 cells/mL in DMEM/F12 medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco), and cultured at 37°C with 5% CO_2 for 24 hours. Cell viability decreased to approximately 65-70% after 24 hours of culture, which is consistent with the characteristics of primary hepatocytes derived from end-stage CLF patients.

Cell activity detection

CCK-8 assay: Logarithmic-growth-phase cells were inoculated into 96-well plates, and 10 μL of CCK-8 reagent was added per well. Plates were then incubated at 37°C for 2 hours. Absorbance optical density (OD) value was measured using a microplate reader at 450 nm wavelength, and cell survival rate was calculated using the formula: Cell survival rate (%) = (experimental group OD value/normal control group OD value × 100%). Apoptosis assay: Cells were rinsed with PBS twice and then incubated with 5 μL of Annexin V-FITC and 10 μL of PI staining solution at room temperature in the dark for 15 minutes. The proportions of early apoptotic cells (Annexin V^+/PI^-) and late apoptotic cells (Annexin V^+/PI^+) were detected by flow cytometry, and the total apoptosis rate was calculated. Cell viability and apoptosis were detected at 24 hours after DHQ intervention.

DHQ intervention

Different concentrations of DHQ (pre-experiment gradient: 0, 5, 10, 20, 40, 80, 160 μmol/L, prepared in culture medium) were added to the CLF cell culture medium. After 24 hours of treatment, cell viability was detected using the CCK-8 assay to determine a non-cytotoxic and effective intervention concentration of DHQ. Subsequently, the CLF cells were randomly assigned to the following groups: (1) control group: cultured using normal medium; (2) plasma group: cultured using medium containing 10% pooled plasma from healthy donor. This group was designed to simulate the in vivo circulatory environment and exclude the non-specific effects of plasma components, since plasma from CLF patients contains high levels of inflammatory cytokines, damage-associated molecular patterns (DAMPs), and toxic metabolites that may independently induce hepatocyte injury; (3) DHQ group: cultured in medium containing 20 μmol/L DHQ.

Detection of inflammation and stress response

Cell culture supernatants from each group were collected. ELISAs were performed to quantify IL-1β, IL-6, TNF-α, SOD, MDA, and GSH-Px levels. Additionally, the levels of mitochondrial-specific SOD2 (sSOD2) and the mitochondrial-derived peptide Humanin in the supernatants were determined. All inflammatory and oxidative stress markers were measured at 24 hours after intervention.

Cells qPCR

Total DNA, containing both nuclear DNA and mtDNA, was extracted from cells in each group using a DNeasy Blood & Tissue Kit (Qiagen). With the nuclear gene β-actin serving as the internal reference and the mtDNA-specific gene ND1 (NADH dehydrogenase 1) as the target gene, the mtDNA/nDNA ratio was detected by SYBR Green qPCR. Primer sequences for ND1 and β-actin were the same as those used for serum qPCR: ND1 (F: 5'-CCT CTA CCA AAA TCC TCC C-3'; R: 5'-GAG CGA TGG TGA GAG CTA A-3'); β-actin (F: 5'-TGG AAC GAG GGT CAG ATG T-3'; R: 5'-GCG GCA TTT GAA GGT AGT T-3'). mtDNA/nDNA ratio was detected at 48 hours after intervention.

Western blot

Cells were lysed using RIPA buffer (Beyotime Biotechnology) supplemented with a protease inhibitor cocktail (Roche). Protein concentration was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific). Proteins were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore). After blocking, membranes were incubated overnight at 4°C with the following primary antibodies (diluted at 1:1000, Cell Signaling Technology): Bax, Bcl-2, cl-caspase-3, p62, LC3-II, Beclin-1, NLRP3, GSDMD, NT-GSDMD, cl-caspase-1, IL-1β, and GAPDH. Membranes were then incubated with HRP-labeled secondary antibodies (diluted at 1:2000, Cell Signaling Technology) at room temperature for 1 hour. Protein bands were developed using the enhanced chemiluminescence (ECL) substrate (Thermo Fisher Scientific). Finally, the bands' gray values were analyzed using ImageLab software, and the relative expression of target proteins was analyzed at 48 hours after intervention.

Mitochondrial membrane potential

Cells were inoculated onto coverslips in 24-well plates. After the treatment, the culture medium was discarded, and cells on the plates were washed with PBS. Subsequently, cells were incubated with 5 μg/mL JC-1 working solution for 20 minutes, followed by nuclear re-staining with DAPI. Fluorescence was observed under a fluorescence microscope: red fluorescence

Table 1. Clinical data of the cell donors

Groups	Age	BMI	Sex Male/female	Family history of CLF Yes/no
CLF (n=15)	55.33±9.60	23.62±1.65	10/5	2/13
Healthy controls (n=15)	53.13±8.33	23.82±2.56	8/7	4/11
t or χ^2	0.671	0.254	0.556	Fisher's exact
P	0.508	0.801	0.456	0.651

Note: Abbreviations: BMI, body mass index; CLF, chronic liver failure; n, number of participants; P, probability value.

(JC-1 aggregates) indicated normal mitochondrial membrane potential, whereas green fluorescence (JC-1 monomers) suggested depolarization. The red/green fluorescence ratio was quantified using ImageJ software. Mitochondrial membrane potential was assessed at 48 hours after intervention.

Statistical analysis

Data were analyzed using SPSS 32.0 software (IBM). Categorical variables were compared using Fisher's exact test due to the small sample size and low expected frequencies in some cells. Continuous variables were compared using the independent-samples t-test between two groups, and one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test among multiple groups. Differences were considered statistically significant at $P<0.05$.

Results

Baseline characteristics of cell donors

CLF patients and healthy donors were well-matched in terms of baseline characteristics, including age, sex, family history, and body mass index (BMI), with no statistically significant intergroup differences observed ($P>0.05$, **Table 1**). This baseline equivalence minimizes potential confounding effects of these factors on subsequent cellular-level comparisons.

Differences in liver function, inflammatory response, and oxidative stress

Compared with healthy controls, serum levels of AST, ALT, and TBIL were significantly elevated in CLF patients ($P<0.05$, **Figure 1A**), indicating obvious hepatic dysfunction. In addition, CLF patients exhibited increased levels of IL-1β, IL-6, TNF-α, and MDA, as well as reduced levels of SOD and GSH-Px ($P<0.05$, **Figure 1B**, **1C**). These findings are in line with the characteristic manifestations of CLF, namely, intensified inflammatory and oxidative stress responses [11].

Protective effects of DHQ on mitochondrial function and inhibition of the pyroptotic pathway in patients with CLF

After DHQ intervention, serum circulating mtDNA levels were significantly lower than those in the conventional treatment group ($P<0.05$, **Figure 2A**). Concurrently, DHQ increased serum levels of the mitochondrial-specific antioxidant enzyme SOD2 and the mitochondrial protective peptide Humanin in patients with CLF ($P<0.05$, **Figure 2B**, **2C**). Evaluation of the pyroptotic pathway further showed that DHQ reduced serum levels of NLRP3, cleaved caspase-1, and the pyroptosis executor protein NT-GSDMD in CLF patients ($P<0.05$), suggesting that DHQ may attenuate NLRP3 inflammasome activation by preserving mitochondrial function, while concurrently suppressing the caspase-1/IL-1β pyroptotic signaling axis (**Figure 2D-F**). Notably, serum NLRP3 concentration in the control group showed a slight increase after 4 weeks of conventional treatment, which may be attributed to the progressive nature of CLF. Conventional treatment alone appears insufficient to fully halt the inflammatory cascade in end-stage liver disease, leading to persistent NLRP3 inflammasome activation. In contrast, DHQ adjunctive therapy significantly reversed this trend, demonstrating its potent anti-inflammatory effect.

Evaluation of the effects of DHQ on clinical complications and medication safety in patients with CLF

When added to standard medical treatment, DHQ significantly improved the remission rates of ascites, hepatic encephalopathy, and spontaneous bacterial peritonitis in CLF patients ($P<0.05$). As for safety, only 2 patients in the DHQ group experienced mild and transient

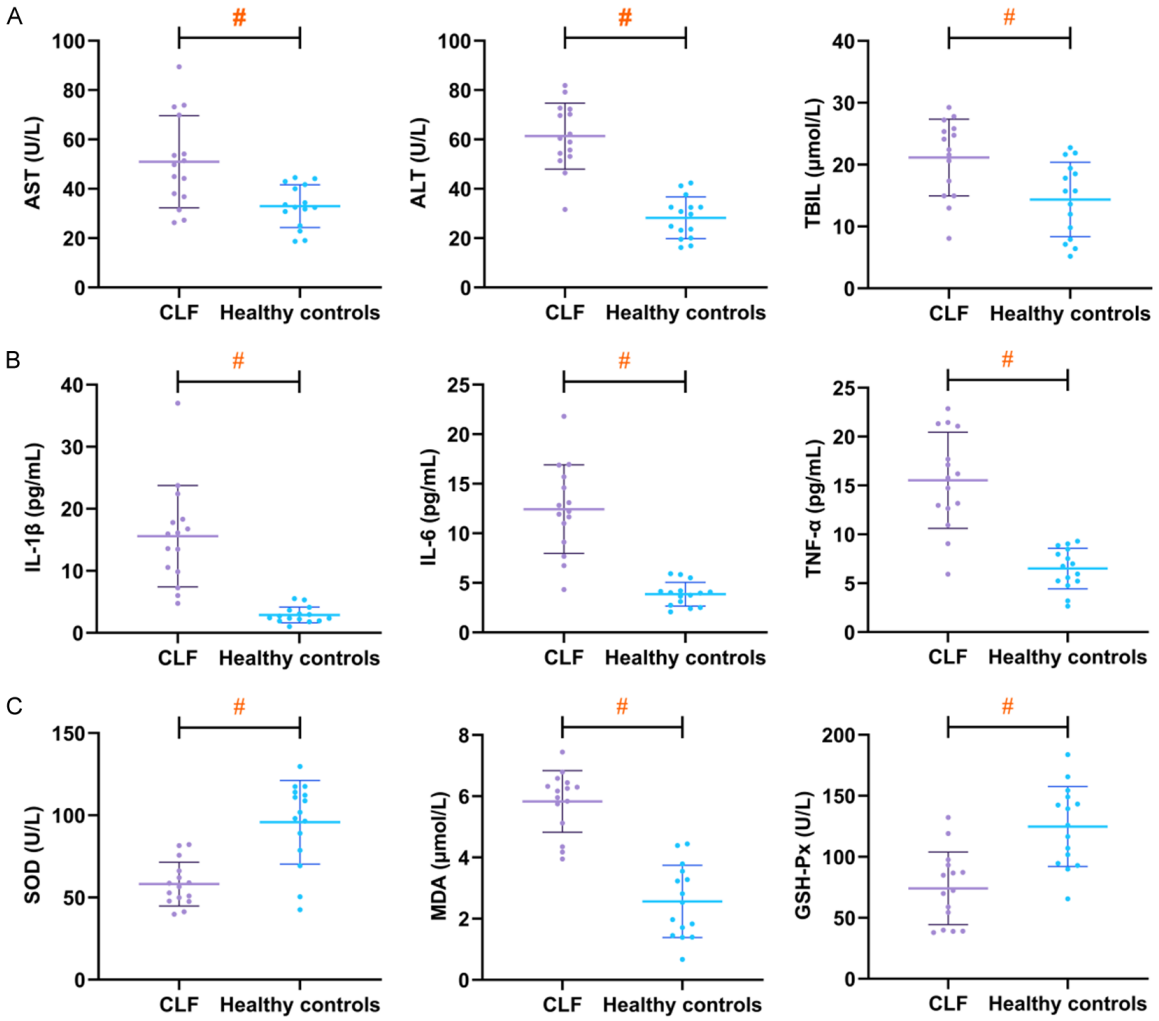

Figure 1. Comparison of liver function, inflammatory factors, and oxidative stress markers in cell donors. A. Comparison of liver function indicators. B. Comparison of inflammatory factors. C. Comparison of oxidative stress markers. Multiple comparisons were performed using independent samples t-test, #P<0.05. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CLF, chronic liver failure; GSH-Px, glutathione peroxidase; IL-1β, interleukin-1β; IL-6, interleukin-6; MDA, malondialdehyde; SOD, superoxide dismutase; TBIL, total bilirubin; TNF-α, tumor necrosis factor-α; P, probability value.

adverse events, corresponding to an overall incidence of 25.00%. No serious adverse events were observed, and no cases of drug-related liver or kidney injury or treatment discontinuation due to adverse reactions occurred, confirming that DHQ is well tolerated and safe in Child-Pugh class C patients with end-stage liver disease (**Table 2**).

Comparison of hepatocyte viability between the groups

CCK-8 assay showed that the proliferative capacity of primary human hepatocytes from CLF patients was significantly impaired compared with that from healthy controls, with cell viability decreasing to approximately 65% at 24 hours after culture (P<0.05, **Figure 3A**). Flow cytometry analysis further revealed that the total apoptosis rate in CLF hepatocytes was significantly higher than that of healthy controls at 24 hours after culture (P<0.05, **Figure 3B**). These results confirm that the isolated primary CLF hepatocytes retained the disease-specific injury phenotype, providing a reliable cellular model for subsequent intervention experiments.

Effect of DHQ on hepatocyte activity

All subsequent in vitro experiments were performed using primary CLF hepatocytes, with

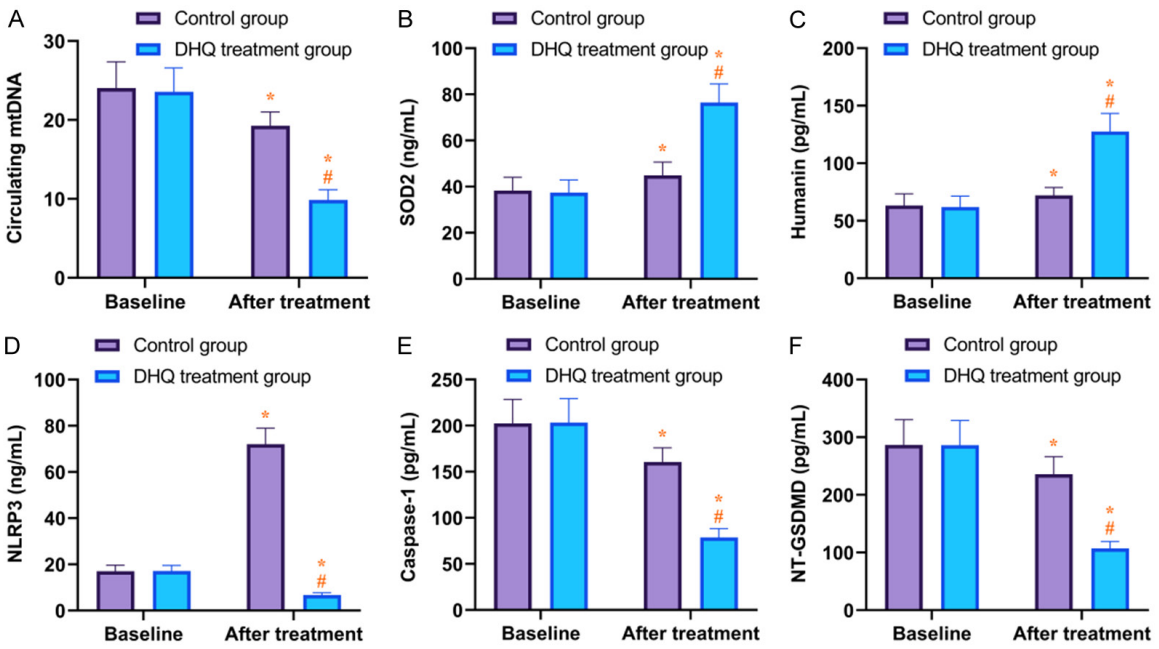

Figure 2. Protective effects of DHQ on mitochondrial function and inhibition of pyroptotic pathway in CLF patients. A. Relative copy number of serum circulating mtDNA. B. Serum SOD2 concentration. C. Serum Humanin level. D. Serum NLRP3 concentration. E. Serum cleaved-caspase-1 level. F. Serum NT-GSDMD concentration. Comparisons between groups at each time point were performed using independent samples t-test; comparisons within groups between baseline and post-treatment were performed using paired samples t-test, #P<0.05 *vs.* control group; *P<0.05 *vs.* baseline. Abbreviations: CLF, chronic liver failure; DHQ, dihydroquercetin; mtDNA, mitochondrial DNA; NLRP3, NLR family pyrin domain-containing 3; NT-GSDMD, N-terminal gasdermin D; SOD2, superoxide dismutase 2; P, probability value; vs., versus.

cells cultured in normal medium serving as the control group. To determine the optimal non-cytotoxic concentration of DHQ, primary CLF hepatocytes were treated with a gradient of DHQ concentrations for 24 hours. Results showed that DHQ at concentrations ≤20 μmol/L had no significant cytotoxicity, whereas concentrations ≥40 μmol/L caused a dose-dependent decrease in cell viability. Among the non-cytotoxic concentrations, 20 μmol/L DHQ exhibited the most significant protective effect against hepatocyte injury and was therefore selected for subsequent experiments (**Figure 4A**). Cell viability assays revealed no statistical differences between the control and plasma groups (P>0.05, **Figure 4B**); however, the cell growth ability of the DHQ group was enhanced, and the apoptosis rate was decreased (P<0.05, **Figure 4C**). Consistently, apoptosis-related protein analysis showed no significant differences in Bax, Bcl-2, or cl-caspase-3 between the control and plasma groups (P>0.05), whereas in the DHQ group, Bax and cl-caspase-3 levels decreased, and Bcl-2 levels increased (P<0.05, **Figure 4D**).

Impact of DHQ on the biological behavior of hepatocytes

Subsequently, cell biological behaviors in each group were examined. No significant differences were noted between the control and plasma groups (P>0.05). In the DHQ group, however, the levels of IL-1β, IL-6, TNF-α, and MDA were all decreased, while SOD and GSH-Px were increased (P<0.05, **Figure 5A**, **5B**). Similarly, analysis of autophagy and pyroptosis-related proteins indicated that DHQ increased the expression of p62, LC3-II, Beclin-1, and the LC3-II/I ratio compared to the control and plasma groups (P<0.05). The simultaneous increase in Beclin-1 (autophagy initiation marker), LC3-II/I ratio (autophagosome accumulation marker), and p62 (autophagic substrate) suggests that DHQ may promote autophagosome formation while partially inhibiting autophagolysosomal degradation, leading to autophagosome and p62 accumulation. Alternatively, the induction of autophagy initiation may exceed the degradation rate. Autophagic flux assays using chloroquine (CQ) will be performed in future studies to clarify this mechanism. Con-

Table 2. Clinical efficacy and medication safety of DHQ in patients with CLF

Groups	Remission of CLF decompensation			Adverse drug reactions			
	Ascitic fluid	Hepatic encephalopathy	Spontaneous peritonitis	Gastrointestinal complaints (nausea, bloating)	Dizziness	Other	Total
Control group (n=7)	1 (14.29)	2 (28.57)	2 (28.57)	0 (0.00)	1 (14.29)	0 (0.00)	1 (14.29)
DHQ treatment group (n=8)	6 (75.00)	7 (87.50)	8 (100.0)	1 (12.50)	0 (0.00)	1 (12.50)	2 (25.00)
P	0.041	0.041	0.007				>0.999

Note: All *P* values were calculated using Fisher's exact test. Abbreviations: CLF, chronic liver failure; DHQ, dihydroquercetin; n, number of participants; P, probability value.

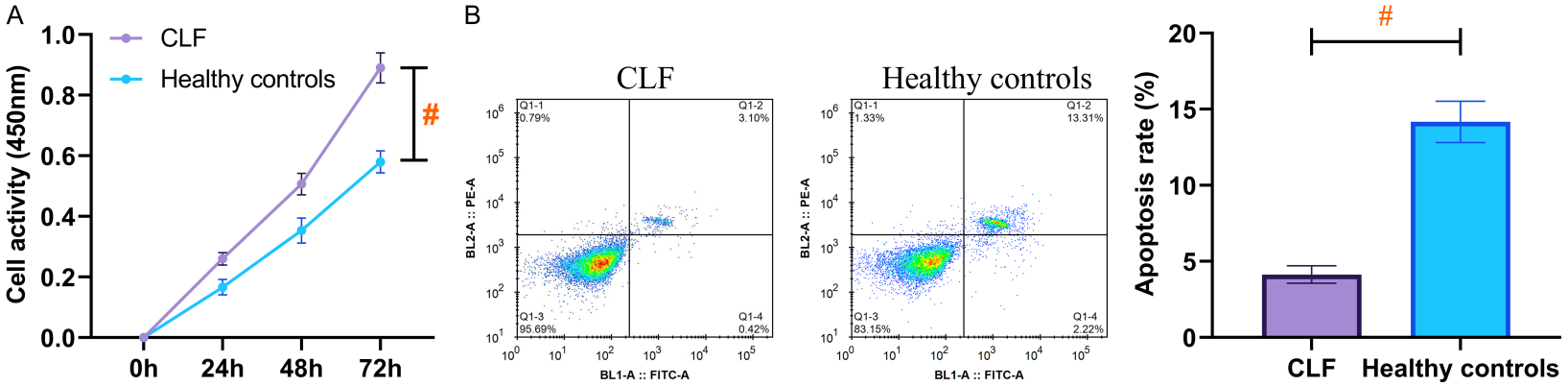

Figure 3. Comparison of hepatocyte viability between the two groups. A. Cell growth curve. B. Cell apoptosis rate. Multiple comparisons were performed using independent samples t-test, #P<0.05. Abbreviations: CLF, chronic liver failure; P, probability value.

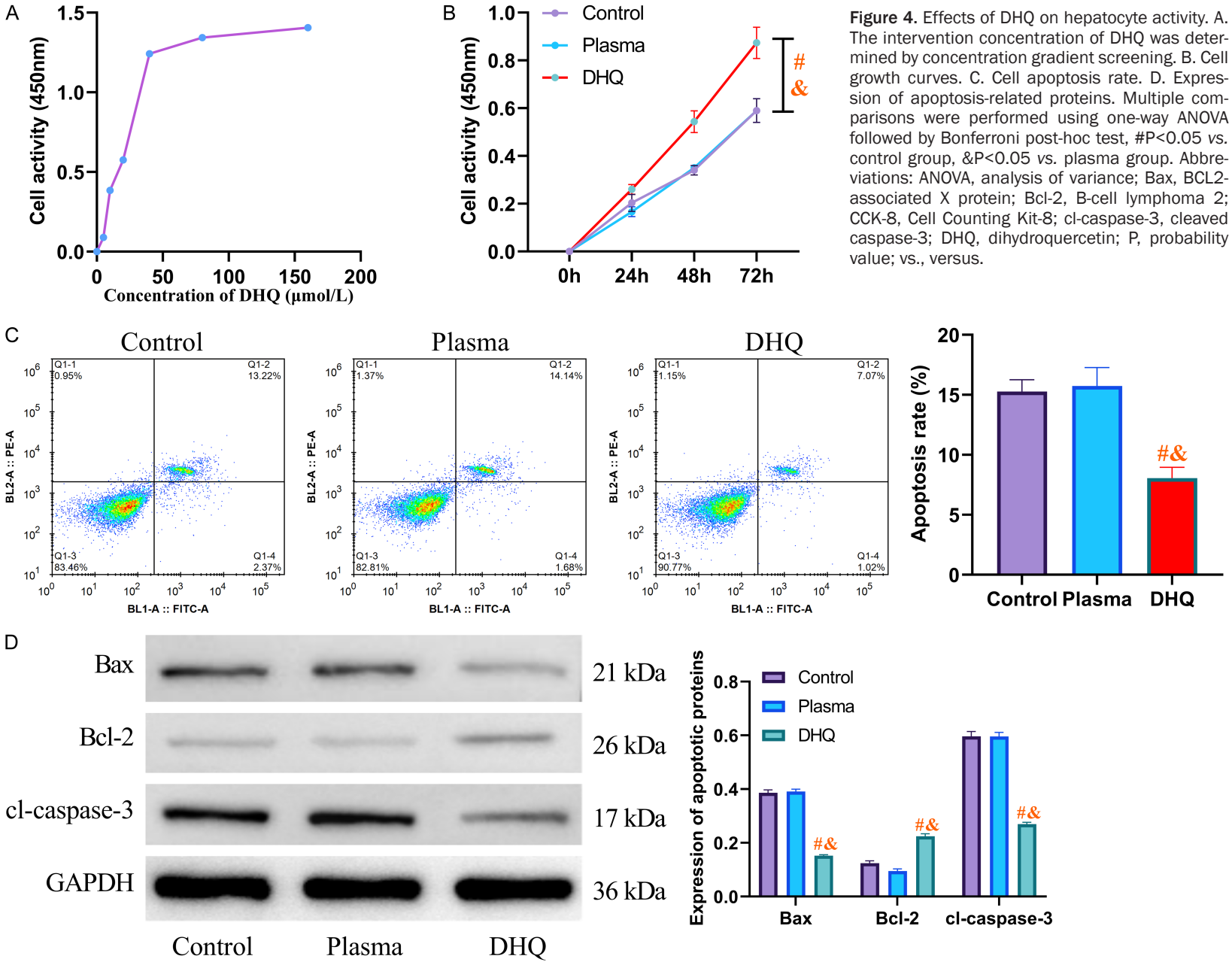

Figure 4. Effects of DHQ on hepatocyte activity. A. The intervention concentration of DHQ was determined by concentration gradient screening. B. Cell growth curves. C. Cell apoptosis rate. D. Expression of apoptosis-related proteins. Multiple comparisons were performed using one-way ANOVA followed by Bonferroni post-hoc test, #P<0.05 *vs.* control group, &P<0.05 *vs.* plasma group. Abbreviations: ANOVA, analysis of variance; Bax, BCL2-associated X protein; Bcl-2, B-cell lymphoma 2; CCK-8, Cell Counting Kit-8; cl-caspase-3, cleaved caspase-3; DHQ, dihydroquercetin; P, probability value; vs., versus.

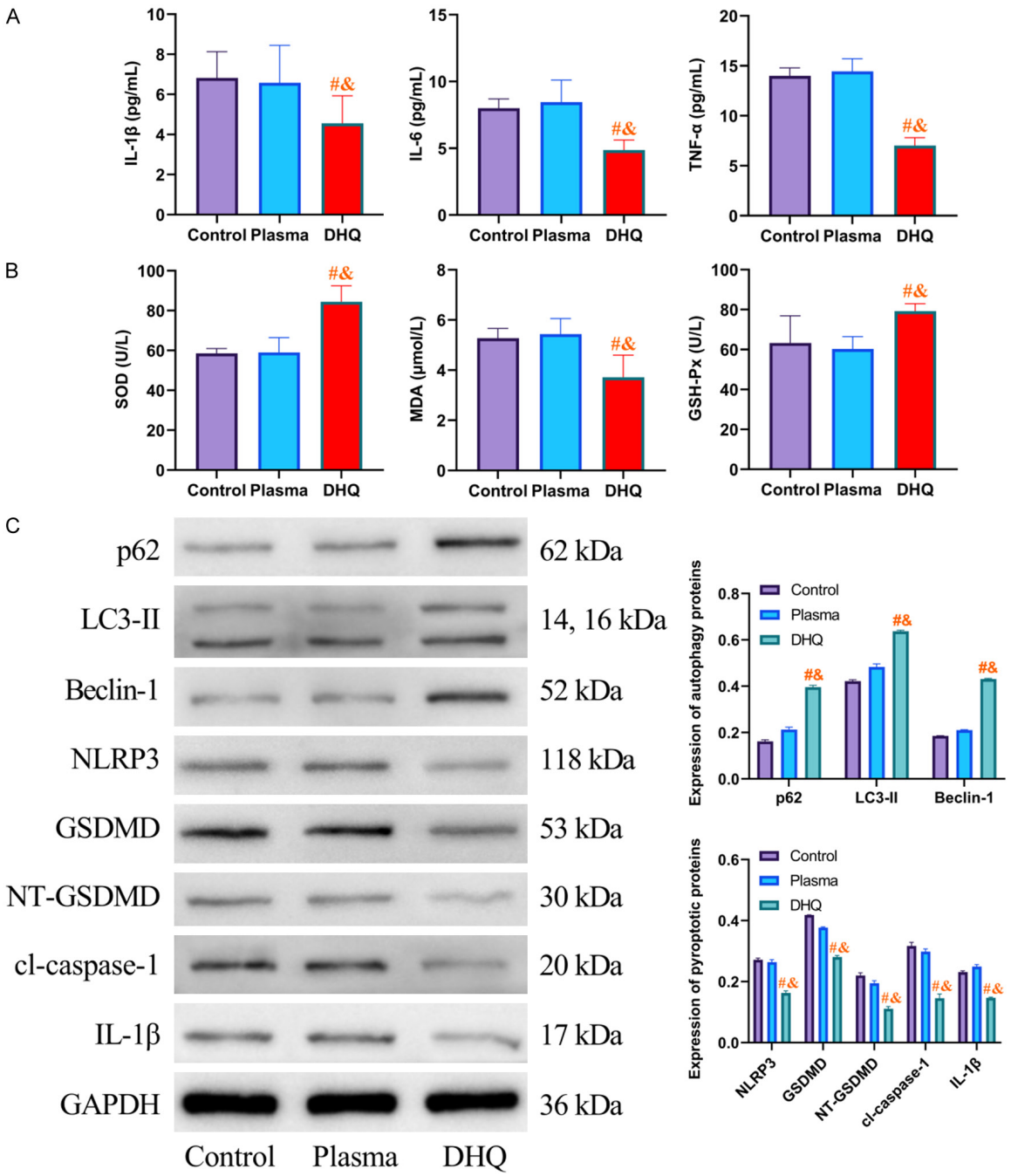

Figure 5. Effects of DHQ on inflammatory factors, oxidative stress response, autophagy and pyroptosis in hepatocytes. A. Comparison of inflammatory factors. B. Comparison of oxidative stress responses. C. Comparison of autophagy- and pyroptosis-related proteins. Multiple comparisons were performed using one-way ANOVA followed by Bonferroni post-hoc test, #P<0.05 *vs.* control group, &P<0.05 *vs.* plasma group. Abbreviations: ANOVA, analysis of variance; CLF, chronic liver failure; DHQ, dihydroquercetin; GSDMD, gasdermin D; GSH-Px, glutathione peroxidase; IL-1β, interleukin-1β; IL-6, interleukin-6; LC3, microtubule-associated protein 1 light chain 3; MDA, malondialdehyde; NLRP3, NLR family pyrin domain-containing 3; NT-GSDMD, N-terminal gasdermin D; p62/SQSTM1, sequestosome 1; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α; P, probability value; vs., versus.

comitantly, DHQ reduced protein levels of NLRP3, GSDMD, NT-GSDMD, cl-caspase-1, and IL-1β (P<0.05, **Figure 5C**), indicating suppression of the pyroptotic pathway.

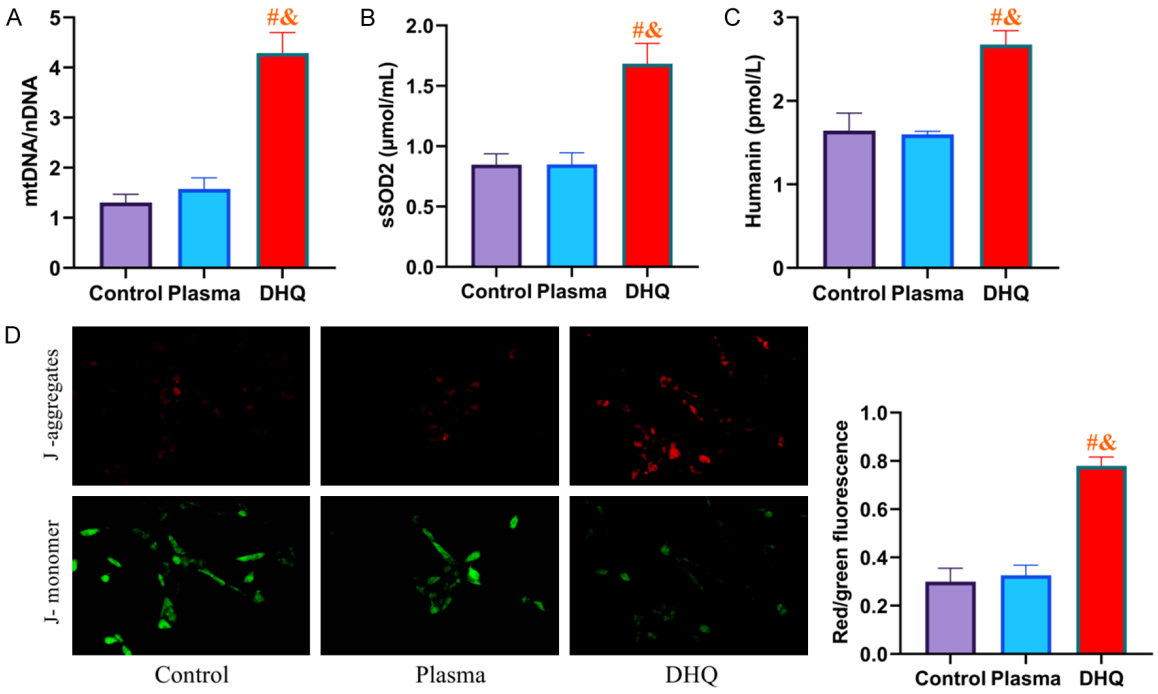

Figure 6. Effects of DHQ on mitochondrial function in hepatocytes. A. Comparison of mtDNA/nDNA levels. B. Comparison of sSOD2 levels. C. Comparison of Humanin levels. D. Comparison of mitochondrial membrane potential. Multiple comparisons were performed using one-way ANOVA followed by Bonferroni post-hoc test, #P<0.05 *vs.* control group, &P<0.05 *vs.* plasma group. Abbreviations: ANOVA, analysis of variance; DHQ, dihydroquercetin; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; sSOD2, supernatant superoxide dismutase 2; P, probability value; vs., versus.

Influence of DHQ on mitochondrial integrity and function in hepatocytes

Mitochondrial function was further assessed in each group. The DHQ group showed significantly higher levels of mtDNA, sSOD2, and Humanin than the control and plasma groups (P<0.05, **Figure 6A-C**). Furthermore, JC-1 fluorescence staining revealed an elevated proportion of red fluorescence in the DHQ group (P<0.05, **Figure 6D**), suggesting restoration of mitochondrial membrane potential and reversal of mitochondrial damage.

Discussion

This study is the first to systematically investigate the protective effect of DHQ on the mitochondrial function of primary hepatocytes from CLF patients. Our results showed that primary hepatocytes from CLF patients exhibited significant mitochondrial dysfunction, manifested as decreased cell viability and enhanced apoptosis. DHQ intervention alleviated all these injuries, restored the JC-1 red/green fluorescence ratio, and upregulated mitochondrial function-related markers (sSOD2, Humanin). The underlying mechanism involves two pathways: DHQ may restore the balance of mitochondrial dynamics by regulating the mitochondrial quality-control network; DHQ also attenuates hepatocyte pyroptosis by inhibiting activation of the NLRP3 inflammasome and suppressing the Caspase-1/IL-1β signaling axis. These findings collectively highlight the potential therapeutic value of DHQ in the treatment of end-stage liver diseases.

Clinically, CLF patients exhibited significant liver function impairment, as reflected by elevated AST, ALT, and TBIL levels compared to healthy controls. This is consistent with previously reported pathological features of CLF, which include severe hepatocellular injury and impaired bile excretion [12]. In terms of inflammatory response, higher serum levels of IL-1β, IL-6, and TNF-α in the CLF group suggest that a chronic inflammatory microenvironment is a core contributor to CLF progression [13]. Oxidative stress assessment revealed increased

MDA levels as well as declined SOD and GSH-Px activities in the CLF group, confirming the vicious circle of mitochondrial ROS overproduction and antioxidant system imbalances [14].

Circulating mtDNA (cmtDNA) is a key marker of mitochondrial injury, and previous studies have shown that its levels are positively associated with the degree of hepatocyte necrosis and short-term mortality in CLF patients [15]. In the present study, DHQ significantly reduced serum cmtDNA levels in CLF patients, providing direct in vivo evidence that DHQ can alleviate structural mitochondrial damage in hepatocytes. As for markers of mitochondrial function, earlier studies have shown that loss of mitochondrial-specific SOD2 aggravates liver injury, whereas Humanin can inhibit hepatocyte death by stabilizing mitochondrial membrane potential [16]. DHQ increases serum SOD2 and Humanin levels in CLF patients, thus offering direct clinical biomarker evidence for its mitochondrial-protective effects in vivo. Regarding the pyroptotic pathway, previous work has already established that NLRP3 inflammasome-mediated pyroptosis is a major mechanisms underlying hepatocyte death in CLF. Our clinical findings further indicate that DHQ inhibits the NLRP3/caspase-1/NT-GSDMD pyroptotic axis, thereby filling a gap in clinical research on natural products targeting the pyroptotic pathway in CLF. Notably, this study also evaluated the effect of DHQ adjunctive therapy on decompensated complications and medication safety in patients with CLF. DHQ treatment improved the remission rates of ascites, hepatic encephalopathy, and spontaneous bacterial peritonitis. These benefits are unlikely to reflect symptomatic control alone; rather, they appear to represent downstream clinical consequences of the mitochondrial-protective, anti-inflammatory, and antioxidant actions of DHQ, providing a new candidate for adjunctive treatment in CLF. Previous clinical safety data for DHQ have mainly been derived from healthy individuals or patients with metabolic disorders. In contrast, the present study is the first to demonstrate its safety and tolerability in Child-Pugh class C patients with end-stage CLF. Only mild and transient adverse reactions were observed, with no serious adverse events reported. This is consistent with the favorable safety profile reported previously for DHQ [17] and provides important clinical support for its use in patients with end-stage liver disease.

At the cellular level, primary hepatocytes in CLF patients exhibited a notably increased apoptosis rate than those from healthy controls, supporting the activation of apoptosis mediated by the mitochondrial pathway. Under the intervention of DHQ, the damage to primary hepatocytes in CLF was effectively reversed. Based on the results of this study, the protective effects of DHQ on CLF hepatocytes are primarily mediated through two core interconnected pathways: mitochondrial protection and inhibition of NLRP3-mediated pyroptosis. First, DHQ significantly ameliorated mitochondrial dysfunction in CLF hepatocytes, as evidenced by the restored mitochondrial membrane potential, increased mtDNA copy number, and upregulated expression of mitochondrial protective factors SOD2 and Humanin. SOD2 is the primary mitochondrial antioxidant enzyme responsible for scavenging superoxide anions [18], where Humanin, a mitochondria-derived peptide stabilizes mitochondrial membrane potential and inhibits cell death [19]. The upregulation of these two factors directly reduces mitochondrial ROS production and prevents mitochondrial permeability transition pore (mPTP) opening, thereby reducing mtDNA leakage into the cytoplasm [20]. Second, DHQ significantly inhibited the activation of the NLRP3/Caspase-1/GSDMD pyroptotic axis, which is consistent with the reduced circulating mtDNA levels in both serum and cell supernatant [21]. Taken together, these findings suggest that DHQ exerts its hepatoprotective effect by preserving mitochondrial integrity, reducing mtDNA leakage, and subsequently inhibiting NLRP3 inflammasome activation and pyroptosis. The observed changes in autophagy-related proteins may represent a secondary response to mitochondrial protection, and the precise mechanisms require further investigation.

Certainly, this study has several limitations. First, the clinical sample size was relatively small, which may limit the statistical power and generalizability of the clinical findings. As a preliminary exploratory investigation, further multicenter, large-sample randomized controlled trials are needed to determine whether DHQ can improve long-term outcomes in CLF patients, including 90-day all-cause mortality and the

need for liver transplantation. In addition, pharmacokinetic data should be incorporated to optimize individualized dosing in this patient population. Second, the in vitro primary hepatocyte model has inherent limitations. Although it preserves the patient-specific mitochondrial dysfunction and pathological characteristics of CLF, it cannot simulate the complex in vivo microenvironment of CLF, including portal hypertension, gut microbiota dysbiosis, immune cell infiltration, and multi-organ crosstalk. These factors play crucial roles in CLF progression and may influence the therapeutic efficacy of DHQ. In contrast, CLF animal models can recapitulate the systemic pathological changes of the disease but lack human-specific genetic and metabolic features. Therefore, combining primary human hepatocyte models with animal models will provide more comprehensive and reliable evidence for the clinical translation of DHQ. Third, this study did not investigate whether DHQ regulates key regulators such as mitochondrial DNA repair enzymes (e.g., POLG) or mitochondrial autophagy receptors (e.g., OPTN), which is also a crucial aspect that requires focused attention in subsequent research. Subsequent studies should also combine blood drug concentration monitoring with pharmacokinetic analyses to further validate the therapeutic dosing of DHQ. Meanwhile, the synergistic effect between DHQ and existing treatment modalities (e.g., artificial liver support systems) should be explored. Finally, establishing an animal model of CLF and conducting long-term follow-up to systematically evaluate the impact of DHQ on liver fibrosis reversal and survival rate will be crucial for promoting its clinical translation.

Conclusion

DHQ enhances mitochondrial function in primary hepatocytes from CLF patients and suppresses inflammation and apoptosis by regulating the mitochondrial quality control network. Its protective mechanism involves multi-dimensional synergistic effects, including antioxidant stress, restoration of mitochondrial dynamics, and promotion of mitochondrial biogenesis. Future studies should focus on optimizing intervention strategies in preclinical research and exploring potential synergistic effect with existing treatment methods.

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Disclosure of conflict of interest

None.

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