Bicyclol Alleviates Signs of BDL-Induced Cholestasis by Regulating Bile Acids and Autophagy-Mediated HMGB1/p62/Nrf2 Pathway

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Cholestasis is a liver disease characterized by the accumulation of toxic bile salts, bilirubin, and cholesterol, resulting in hepatocellular damage. Recent findings have revealed several key steps of cholestasis liver injury including the toxicity of bile acids and accumulation of proinflammatory mediator. In this study, we investigated the protective effect of bicyclol in cholestasis caused by bile duct ligation (BDL), as well as relevant mechanisms. Bicyclol attenuated liver damage in BDL mice by increasing the levels of hydrophilic bile acid such as α-MCA and β-MCA, regulating bile acid-related pathways and improving histopathological indexes. High-mobility group box 1 (HMGB1) is an extracellular damage-associated molecular pattern molecule which can be used as biomarkers of cells and host defense. Bicyclol treatment decreased extracellular release of HMGB1. In addition, HMGB1 is also involved in regulating autophagy in response to oxidative stress. Bicyclol promoted the lipidation of LC3 (microtubule-associated protein 1 light chain 3)-II to activate autophagy. The nuclear factor, E2-related factor 2 (Nrf2) and its antioxidant downstream genes were also activated. Our results indicate that bicyclol is a promising therapeutic strategy for cholestasis by regulating the bile acids and autophagy-mediated HMGB1/p62/Nrf2 pathway.

Keywords: liver injury, cholestasis, BDL, high mobility group box 1, autophagy, Nrf2, bicyclol

INTRODUCTION

Cholestasis is a pathological condition caused by obstruction or cessation of bile flow resulting in intrahepatic cholestasis and accompanied by cell injury, inflammatory infiltration, cell apoptosis, liver fibrosis, and even cirrhosis (Yokoda and Carey, 2019). Currently, the main drug treatment for chronic cholestatic liver injury is ursodeoxycholic acid (UDCA). However, some patients do not respond to this treatment (Wagner and Fickert, 2020). Hence, novel treatment approaches, which could be based on a comprehensive understanding of the mechanisms of the different stages of disease progression, are urgently needed.
A previous study found that primary bile acids conjugated with glycine or taurine were significantly increased in serum of patients with cholestasis liver injury (Woolbright et al., 2015). Bile acids (BAs), such as taurocholic acid (TCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GDCA), and glycocydoxycholic acid (GDCa), can be used as biomarkers of liver injury or as indicators of drug-induced liver injury (DILI) (Yang et al., 2019). The hydrophobicity of bile acids was affected by the number and orientation of the hydrophilic region, where lithocholic acid (LCA) was the most hydrophobic while cholic acid (CA) was less hydrophobic (Ashby et al., 2018). On the other hand, the conjugated groups also affected the hydrophobicity of bile acids, where unconjugated was the most hydrophobic and glycine conjugated was more hydrophobic than taurine or sulphate conjugated (Ashby et al., 2018). The toxicity of bile acids is mostly dependent on its hydrophobicity. According to previous studies, TCA, the major endogenous BAs in rodents, significantly increases the mRNA level of inflammatory cytokines monocyte chemotactic protein 1 (Ccl2) and macrophage inflammatory protein 2a (cxcl2) (Cai et al., 2017). The toxicity of BAs during obstructive cholestatic liver injury likely occurs because of biliary BAs leakage. Previous studies proved that biliary BAs at 0.5x dose resulted in significant hepatocyte necrosis (Woolbright et al., 2015).

High-mobility group box-1 (HMGB1) protein, a member of the high-mobility group 1 (HMG-1) family, modulates cell death in acute liver injury by NF-kB signal pathway activated by advanced glycan end products (RAGE) and toll-like receptor 4 (TLR4) (Eguchi et al., 2014). HMGB1 is involved in many liver diseases. Previous studies have found that the level of liver HMGB1 protein was correlated with alcoholic steatohepatitis (ASH) and primary biliary cirrhosis (PBC) in patients with chronic Hepatitis C virus (HCV), as well as in chronic carbon tetrachloride (CCL4) treated mice (Ge et al., 2018). HMGB1 levels also increased in BDL induced cholestatic mice (Woolbright et al., 2013; Dondorf et al., 2017), while carnosic acid provided protection against BDL induced fibrosis by inhibiting HMGB1 expression (Zhang et al., 2017a). HMGB1 is a proinflammatory cytokine released by injured cells and the innate immune system, which alerts other cells that infection or sterile injury has occurred (Andersson and Tracey, 2011). Neutrophils and Cxcl2, Ccl2 mRNA levels were significantly decreased in mice with hepatocyte-specific Hmgb1 knockout (Huebener et al., 2019).

Autophagy deficiency leads to the rise of HMGB1 levels and promotes ductular reaction and tumorigenesis via its receptor RAGE (Kambhu et al., 2018). HMGB1 regulates autophagy under the circumstances of oxidative stress (Kang et al., 2011). The oxidation HMGB1 binding to Beclin1 causes the dissociation of Bcl-2 from Beclin1, leading to the induction of autophagy (Tang et al., 2010). So, autophagy and HMGB1 are closely connected. Autophagy includes five processes: initiation, elongation, closure, maturation, and degradation. LC3B and p62 are the most commonly used markers of autophagy. The carboxyl terminus of the LC3 protein is specifically cleaved to form LC3-I with the exposed carboxyl-terminal glycine, which is combined with phosphatidylethanolamine to form LC3-II (Kuma et al., 2017). LC3-II targets the inner and outer membranes of autophagosomes to participate in the elongation step of autophagy (Kuma et al., 2017; Qian et al., 2017). P62 as a receptor for ubiquitinated proteins and organelles is selectively transported into the autophagosomes, after which it is degraded by autophagy (Ichimura and Komatsu, 2010).

The high levels of p62 associated with the suppression of autophagy competitively interacts with Kelch-like ECH-associated protein-1 (Keap1), the nuclear factor Nrf2-binding site, resulting in activation of Nrf2 and its target genes (Komatsu et al., 2010; Jiang et al., 2015). The activation of Nrf2 protects from various liver diseases through different molecular mechanisms (Xu et al., 2018). After activation, Nrf2 is translocated to the nucleus to activate heme oxygenase-1 (HO-1), NADPH quinone oxidoreductase-1 (NQO1), glutamate cysteine ligase (GCLC), and other genes to resist damage caused by oxidative stress (Galicia-Moreno et al., 2020). Nrf2-knockout mice are more susceptible to hepatic injury after BDL, as vitamin A activates Nrf2 to protect liver function in BDL rats, thus proving that Nrf2 activation is beneficial in cholestatic liver injury (Weerachayaphorn et al., 2012; Shin et al., 2013; Wang et al., 2014).

Bicyclol (4,4′-dimethoxy-5,6,5′,6′-bis (methyleneoxy)-2-hydroxymethyl-2′-methoxy carbol benzyphenyl) is a synthetic drug widely used to treat HCV (Li et al., 2018; Huang et al., 2019). Bicyclol has been proven to induce autophagy and inhibit cell proliferation through PI3K/AKT and MEK/ERK pathways (Wang et al., 2016b). Previous studies in our laboratory have shown that bicyclol can activate Nrf2 to act against CCL4 induced hepatotoxicity (Zhao et al., 2020). What’s more, Zhen, Yong-Zhan et al. have found that BDL-induced liver fibrosis can be significantly attenuated by bicyclol through reversing fibrogenic gene expression (Zhen et al., 2015). Nonetheless, there are still few studies on the mechanism of the protective effect of bicyclol in BDL-induced liver damage.

In this study, we aimed to explore the protective effect of bicyclol in BDL induced liver injury. Our research was mainly based on two aspects: the synthesis, excretion, and composition of bile acid, and the pathway associated with autophagy-mediated by HMGB1.

**MATERIALS AND METHODS**

**Animals**

Eleven-weeks old male (22–23 g) c57BL/6 mice were purchased from Beijing HFK Bioscience CO., LTD. All mice were housed in the SPF animal room with a constant temperature (22°C), and a 12/12 h light/dark cycles and were given free access to water and food. Before the experiment, mice were allowed to adapt to the environment for 1 week. All animal studies (including the mice euthanasia procedure) were done in compliance with the regulations and guidelines of the Animal Ethical and Welfare Committee (AEWC) at the Institute of Radiation Medicine.
Chinese Academy of Medical Sciences and conducted according to the AAALAC and the IACUC guidelines.

The mice were randomly divided into three groups: Sham group (n = 8), BDL group (n = 6), and BDL + Bicyclol group (n = 6); the different numbers in different groups are due to the injury following BDL surgery. BDL was employed to induce cholestatic liver injury as described in a previous study (Fickert et al., 2002). Briefly, after anesthesia with isoflurane, the bile duct was separated from the portal vein and hepatic artery, then ligated using a 6–0 suture. The sham group underwent similar laparotomy without BDL. BDL + Bicyclol group was orally administered with bicyclol at a dose of 100 mg/kg (Hu and Liu, 2006; Zhen et al., 2015) body weight daily for 14 days, while sham and BDL group were treated with volume-matched 0.5% carboxymethylcellulose sodium (CMC-Na). Bicyclol was a kind gift from the Beijing Union Pharmaceutical Company (Beijing, China) with a purity of over 99%. Animals were sacrificed on days 14 after the operation, after which the blood and liver were collected and stored at −80°C for further research.

**Serum Biochemical Analysis**
The serum levels of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGT), serum bile acid (TBA), and total cholesterol (TC) were measured by the automated chemistry analyzer (AU5800, Beckman Coulter, United States) of the clinical laboratory (Tianjin medical university general hospital).

**Histology**
Liver tissue was fixed in 4% paraformaldehyde at room temperature and blocked with paraffin wax, after which they were sectioned into 4 μm thick slices and stained with hematoxylin and eosin (H&E) according to the standard H&E protocol. The inflammatory cell infiltration and necrosis were assessed by two experienced pathologists according to Ishak scoring criteria, including piece-meal necrosis, fusion necrosis, lytic necrosis, and portal inflammation (Ishak et al., 1995). Images were taken by Leica microscope (Leica DM5000B; Germany) at 200X magnification.

**Measurement of Gallbladder Bile Acids Composition**
Gallbladder bile was collected, and its composition was measured with liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Yang et al., 2017). Briefly, standard samples were added into 50 μl bile samples, the supernatant was extracted after oscillation and centrifuged at 12,000 rpm for 10 min and then further concentrated on the concentrator. The concentrate was reconstituted in 100 μl of methyl/water (50/50) and analyzed by the LC-MS/MS system. The chromatographic separation was performed on the ACQUITY UPLC HSS T3 column (2.1×100 mm, 1.8 um). Data acquisition systems mainly included Ultra Performance Liquid Chromatography (Shim-pack UFLC SHIMADZU CBM30A) and Tandem mass spectrometry (Applied Biosystems 6500 QTRAP).

**Immunofluorescence**
The slides with liver samples were dewaxed with xylene and various ethanol concentrations and blocked with 5% bovine serum albumin (BSA) for 30 min. After that, the slides were incubated with primary antibodies against p62/SQSTM1 (no. NBP1-48320SS; Novus) at 4°C overnight. After being washed with PBS, the sections were incubated with secondary antibodies at 37°C for 1 h. The nuclei were stained with 4′-6′-diamino-2-phenylindole dihydrochloride (DAPI), and then sealed with resins. The sections were observed under a fluorescence microscope (Leica DM500B; Germany).

**Cell Culture and Treatment**
Alpha mouse liver 12 cell line AML12 were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured in Dulbecco’s Modified Eagle Media: Nutrient Mixture F-12 (DMEM—F12) (#119435; Gibco) supplemented with 10% fetal bovine serum (Gibco), 1% Insulin—Transferrin—Selenium (ITS) (#41400045; Gibco), 40 ng/ml dexamethasone. Cells were grown in a humidified atmosphere with 5% CO2 at 37°C. Once the cells reached confluency near 80%, they were digested with 0.125% trypsin-EDTA and seeded into 6-well plates. For analysis of protein and mRNA levels, cells were stimulated for 24 h with 100 μM TCA and/or 500 μM bicyclol. Taurocholic acid (TCA) was purchased from Sigma-Aldrich.

**Western Blot**
Liver tissues and cells were homogenized in RIPA buffer (Solarbio, China) that contained PMSF (Solarbio, China) and phosphatase inhibitors on ice. The homogenates were centrifuged, and supernatants were extracted, after which 1/3 volume of loading buffer was added and stored at −80°C for further study. The protein was quantified by a BCA protein assay kit (Solarbio, China). Equal amounts of proteins were separated by SDS-PAGE gels and transferred to nitrocellulose membranes. After blocking with 5% non-fat milk, membranes were incubated with primary antibodies overnight at 4°C. The primary anti-CYP7A1 (#sc-518007, Santa Cruz), anti-BSEP (#sc-74500, Santa Cruz), anti-FXR (#ab235094, Abcam), anti- SQSTM1/p62 (#39749, Cell Signaling Technology), anti-LC3A/B (D11) (#3868, Cell Signaling Technology), anti-Beclin-1 (#3495, Cell Signaling Technology), anti-HMGB1 (#6893, Cell Signaling Technology), anti-RAGE (ab216329, Abcam, Cambridge, MA, United States), anti-TLR4 (#A17436, ABclonal Technology), anti-NRF2 (#12721, Cell Signaling Technology), anti-Keap1 (#A17061, ABclonal Technology) and anti-β-actin (#3700, Cell Signaling Technology) were used. The secondary horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG antibodies (Zhongshan Golden Bridge Biotechnology, Beijing, China) were incubated at room temperature for 1 h. After fully washing, the ECL Reagent (Solarbio, China) and Image Lab (Bio-Rad, America) were used to analyze the bands.
Real-Time RT-PCR
Total RNA was extracted from liver tissues, and AML12 cells using Trizol reagent and the concentration was determined by an enzyme-labeled instrument. The RNA was reverse-transcribed into cDNA using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, United States). The reaction mix was performed as follows: 50°C (2 min); 95°C (5 min); followed by 50 cycles of 95°C (15 s) and 60°C (30 s).

TABLE 1 | Specific qPCR primers used in this study.

| Gene name | Forward primer (5'-3')                      | Reverse primer (5'-3')                      |
|-----------|---------------------------------------------|---------------------------------------------|
| Gapdh     | GGAGAAAACCTGGAAGTATG                        | TGGGAGTTTGCTGTTGGAAGTC                     |
| Cyp7a1    | AACAACTCGCAGTACTAGATAG                     | GTGTAAGTGAAATTTCCTTGGAAGT                   |
| Fox        | GGCCCTCGGTGACACCACCA                       | TGTACAGGGCGTTTGTGA                           |
| Bsep      | CTGCCAAGGATGTAATGCA                        | GGATGGAACCTGAGAAGAAGG                        |
| Tnf-α      | GGGCGCTGCTCAGGCTGCTGCT                    | GGGTCTGACGAGAAGAAGAAGAAGA                   |
| Il-1β      | GCCATGACTGAAGAAGAAGAAGA                    | AGGATGAGGAAGAAGAAGAAGAAGA                   |
| Nqo1      | CTTTGTCTGGAGAAGAAGAAGA                    | TGCTAGAATGACTGCGAAAGAAGA                    |
| Hmox1     | GATAAGGCCAACAGCAGAAGA                     | CAGTGAAGGCGCATGAAAGAAGA                     |

FIGURE 1 | Bicyclol markedly improved the liver enzyme, histology, and liver gross appearance in BDL mice. (A–F) Serum biochemistry including AST, ALT, ALP, GGT, TBA, and TC. (G) H&E stain liver histology (Scale bar, 100 um). (H) Gross liver appearance (Scale bar, 10 mm). AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, γ-glutamyl transpeptidase; TBA, total bile acid; TC, total cholesterol. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs. sham; #p < 0.05, ##p < 0.01, ###p < 0.001, ####p < 0.0001 vs. BDL.
Relative expression of target genes was calculated by the $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH. The primer sequences are shown in Table 1.

**Statistical Analysis**

Data are expressed as mean ± SEM and analyzed with GraphPad Prism 8.0.1 (GraphPad Software, United States). One-way ANOVA followed by Tukey’s multiple comparisons test was used to calculate the statistical significance. A $p$-value of $<0.05$ was considered statistically significant.

**RESULTS**

**Protective Effects of Bicyclol on Liver Injury in Bile Duct Ligation Mice**

To explore the effects of bicyclol on BDL induced mice liver injury, we tested liver enzymes including AST, ALT, ALP, GGT, TBA, and TC, and liver pathology. The serum AST, ALT, ALP, GGT, TBA, and TC levels increased in BDL compared with the sham group (Figures 1A–F). Bicyclol significantly decreased the AST, ALT, ALP, and GGT levels. However, the TBA and TC levels were comparable between
**FIGURE 3** | Effects of bicyclol on genes related to bile metabolism. (A–C) The mRNA levels of bile metabolism-related genes (Fxr, Cyp7a1, Bsep) in BDL and bicyclol treatment mice. The mRNA levels (D–F) and protein levels (G) of bile metabolism-related genes (Fxr, Cyp7a1, Bsep) in AML12 cells after TCA and bicyclol exposure. Cr represents control group without special treatment, TCA means cells were stimulated with 100 μM TCA for 24 h, TCA + Bicyclol means cells were pretreated with 500 μM bicyclol for 2 h, then stimulated with 100 μM TCA for 24 h. Data are expressed as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs. sham or Cr; #p < 0.05, ##p < 0.01 vs. BDL or TCA.
BDL and BDL + Bicyclol groups. As indicated by H&E staining, BDL-induced inflammatory cell infiltration and hepatic necrosis in mice liver, the effects of which were then ameliorated by bicyclol (Figure 1G). The beneficial effects were also proved by liver appearance, where livers from BDL mice had many yellow-white granules on the surface. In contrast, yellow-white granules of the liver were significantly improved in the bicyclol treatment group (Figure 1H). These results revealed that bicyclol could relieve BDL induced liver injury.

**Bicyclol Treatment Changed Bile Acid Composition**

In those mice with bile duct ligation, the gallbladder volume significantly increased, and the bile was stagnant (Figure 2A); thus, we collected the bile in the gallbladder and analyzed the composition. In total, we tested 25 different kinds of bile acids, finding four BAs CA, GCDCA, α-muricholic acid (α-MCA), β-muricholic acid (β-MCA) levels that differed between BDL and bicyclol groups (Figure 2). A heatmap was plotted to show the levels of four bile acids (Figure 2B). CA, GCDCA, α-MCA, β-MCA in three samples of the bicyclol group were higher than that in the BDL group. In addition, violin plot analysis showed that the levels of bile acids, including CA, GCDCA, α-MCA, and β-MCA were higher in the bicyclol group than in the BDL group (Figure 2C). Previous studies have found that cholate and β-muricholate were the main components of mouse bile acid pool (Kerr et al., 2002), with the following order from hydrophobic to hydrophilic: LCA > deoxycholic acid (DCA) > chenodeoxycholic acid (CDCA) > CA > UDCA > MCA (de Aguiar Vallim et al., 2013). Our results showed that bicyclol could increase hydrophilic bile acid, mainly the α-MCA, β-MCA levels.

**Bicyclol Prevented Liver Injury by Preventing Hepatic Bile Acid Synthesis and Promoting Bile Acid Excretion**

Cyp7a1 is the key enzyme in the classic bile acid synthesis pathway (Chiang and Ferrell, 2019). Fxr is a nuclear receptor that suppresses Cyp7a1 expression (Xu et al., 2016). In our study, liver Cyp7a1 mRNA expression level was elevated by BDL and significantly reduced post bicyclol treatment (Figure 3A). BDL significantly reduced Fxr expression at the mRNA level, which was then reversed by bicyclol (Figure 3B). Bsep expression at the mRNA level was also decreased in the BDL group and increased in BDL + Bicyclol group (Figure 3C). TCA resulted in the most abundant BAs in blood, which was significantly increased in cirrhosis and hepatocellular carcinoma (HCC) patients, and induced inflammatory gene expression in liver cells. Consequently, we treated TCA cells to mimic the cell damage caused by bile (Chen et al., 2011; Wang et al., 2016a). TCA caused a down-regulation of Fxr [F (2,7) = 7.736], which was reversed by bicyclol. Fxr further caused a decrease of Cyp7a1 and an increase of Bsep (Figures 3D–F). At the same time, bicyclol significantly up-regulated the protein expression of BSEP and inhibited the protein level of CYP7A1 although there was no significant difference, even if the FXR protein expression was not changed by bicyclol (Figure 3G). These results suggested that bicyclol could activate Fxr to suppress Cyp7a1 expression, which led to a reduction of BA synthesis and acceleration of BA excretion.

**Bicyclol Ameliorates Bile Duct Ligation-Induced Liver Injury by Down-Regulation of High-Mobility Group Box-1**

Inflammatory factors, including tumor necrosis factor-α (TNF-α), interleukin-1 β (IL-1β), interleukin-6 (IL-6), transforming growth factor-β (TGF-β), are involved in drug-induced liver injury, cholestasis, alcoholic and non-alcoholic fatty liver diseases, and other chronic liver disease processes (Szabo and Csak, 2012). Severe bile duct damage often triggers inflammation. To further verify the liver damage caused by BDL and the effect of bicyclol, we tested the mRNA levels of TNF-α and IL-1β. The results showed that bicyclol recovered the BDL-induced IL-1β, TNF-α mRNA higher expression (Figures 4A,B). HMGB1, as a member of damage-associated molecular patterns (DAMPs) and its receptor RAGE, promote neutrophil infiltration in necrotic tissue, thus aggravating necrosis (Huebener et al., 2015). So, we also measured the protein expression changes of HMGB1 in the liver and AML12 cells. The HMGB1 expression levels were upregulated in the BDL or TCA groups, and this upregulation was offset by bicyclol (Figures 4C,E). Similarly, BDL and TCA also caused the up-regulation of RAGE protein and was also reversed by bicyclol (Figures 4D,F). TLR4 can be activated by HMGB1, the expression of TLR4 was upregulated at TCA and TCA + Bicyclol groups compare to Cr group (Figure 4G).

**Bicyclol Stimulated Autophagy in Mice and Alpha Mouse Liver 12 Cells**

To analyze the effect of bicyclol on autophagy, two important markers of autophagy were detected. The conversion from LC3-I (soluble form) to LC3-II (lipated form) and p62 protein are autophagy activation indicators. Western blot showed that the expression of LC3-II levels was increased, and p62 was decreased in the BDL group. Also, bicyclol treatment further upregulated the LC3-II, but the p62 levels were also increased by bicyclol (Figure 5A). In vitro, bicyclol enhanced the TCA induced accumulation of LC3-II and p62 (Figure 5C). We also observed the expression of p62 in the liver by immunofluorescence (Figure 5B). The results showed that the nucleus of the cholestasis site was broken and dissolved, there was no normal structure, and the fluorescence of p62 was significantly reduced. Bicyclol significantly improved cell necrosis with p62 fluorescence increased dramatically (Figure 5B). These results were consistent with the western blot findings. Besides these, the expression levels of pro-autophagy protein Beclin-1 were upregulated by TCA, even though there was no significant difference (Figure 5D).

**Bicyclol Regulated Nuclear Factor, E2-Related Factor 2 Mediated Antioxidant Response**

Cholestasis is related to oxidative stress. Under basal conditions, Nrf2 binds to Keap1 in the cytoplasm; under oxidative stress,
FIGURE 4 | Effect of bicyclol on inflammatory cytokines, HMGB1, and its receptor RAGE and TLR4. The mRNA level of Tnf-α (A) and IL-1β (B) in mouse liver tissue. (C,D) Immunoblots for HMGB1 and RAGE in mouse liver. (E,F) Immunoblots for HMGB1 and RAGE in AML12 cells. (G) Immunoblots for TLR4 in AML12 cells. *p < 0.05, **p < 0.01, ***p < 0.001 vs. sham or Cr; #p < 0.05, ##p < 0.01 vs. BDL or TCA. Protein levels were normalized to levels of β-actin.
FIGURE 5 | Effect of bicyclol on autophagy. (A) P62 and LC3-II protein levels in mice were detected by western blot. (B) Paraffin sections of liver tissue were stained with p62 (red) and counterstained with DAPI to visualize nuclei (blue) by immunofluorescence. Scale bar, 30 μm. (C) Immunoblots for p62 and LC3-II in AML12 cells. (D) Immunoblots for Beclin-1 in AML12 cells. *p < 0.05 vs. sham or Cr; **p < 0.05, ***p < 0.01, ****p < 0.001 vs. BDL or TCA. Protein levels were normalized to levels of β-actin.
Figure 6 | Effects of bicyclol on Nrf2 and its target genes. (A–C) The mRNA levels of Nrf2, Nqo1, Hmox1 in BDL and bicyclol treatment mice. (D–F) The mRNA levels of Nrf2, Nqo1, Hmox1; The protein levels of Nrf2 (G) and Keap1 (H) in AML12 cells after TCA and bicyclol exposure. Dates are expressed as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs. sham or Cr; #p < 0.05, ##p < 0.01 vs. BDL or TCA.
Nrf2 dissociates from the Nrf2/Keap1 complex and translocate to the nucleus, thereby initiating the transcription of anti-oxidative stress genes. As shown in Figure 6A, Nrf2 mRNA level was significantly increased in BDL + Bicyclol group compared with the Sham and BDL groups. In addition, the downstream target genes, including NADPH: quinone oxidoreductase 1 (Nqo1) and heme oxygenase-1 (Hmox1), were also determined. The results showed that Nqo1 and Hmox1 were upregulated in BDL and BDL + Bicyclol groups compared with the Sham group (Figures 6B,C). The level of Hmox1 was further upregulated by bicyclol. The Nrf2 and Nqo1 mRNA levels were increased in bicyclol-treated AML12 cells; however, there were no significant differences in TCA-treated AML12 cells (Figures 6D,E). In addition, the protein level of Nrf2 also increased by bicyclol (Figure 6G). The expression level of Keap1 was significant increased after TCA exposure, while pretreatment with bicyclol reversed the upregulation (Figure 6H).

**DISCUSSION**

Cholestasis is a liver disease characterized by the accumulation of toxic bile salts, bilirubin, and cholesterol, resulting in hepatocellular injury, which eventually develop into fibrosis and cirrhosis. UDCA, as an exogenous hydrophilic bile acid that replaces endogenous hydrophilic bile acids and obeticholic acid as FXR agonists, have been used to treat PBC (Gulamhusein and Hirsch, 2003). Cyp7a1 overexpressed mice have increased bile acid pool and a higher level of hepatic cholesterol (Li et al., 2011). Nuclear receptors are also involved in BAs balance. Fxr regulates most of the bile acid formation processes, including transport and detoxification, and limits the overload of bile acids in liver cells (Wagner et al., 2011). Fxr participates in BAs balance through a variety of ways, including inhibiting liver Cyp7a1 transcription, inducing intestinal Fgf-15 to activate liver FGF receptor 4 (FGFR-4), repressing Na/taurocholate cotransporter (NTCP), activating the bile salt export pump Bsep and similar (Wagner et al., 2011). The Fxr agonist obeticholic acid (OCA) inhibits the expression of Cyp7a1 by activating liver Fxr and Shp expression and changes the bile acid pool size and composition to inhibit intestinal cholesterol absorption (Xu et al., 2016). Our results showed that treatment with bicyclol in BDL mice up-regulated Cyp7a1 transcription while down-regulating Cyp7a1 and up-regulating the expression of Bsep. Many previous studies have proven that gut microbes are involved in the synthesis and excretion of bile acids and the conversion of components, such as VSL#3 probiotics (including eight different probiotic strains) (Degirolamo et al., 2014) and probiotic Lactobacillus rhamnosus GG (LGG) (Liu et al., 2020), while bile acids can also affect bacterial structure in vitro and in vivo (Tian et al., 2020). Besides affecting the growth of intestinal bacteria, bile also participates in anti-adhesion and neutralizing endotoxins (Wiest et al., 2014). The lack of intestinal bile leads to bacterial translocation and intestinal inflammation, which promotes the progression and development of colorectal cancer (Jia et al., 2018). Therefore, intestinal bacteria and pathological changes after BDL should also be investigated due to the lack of bile in the intestinal.

HMGB1 can have both favorable and unfavorable consequences. On the one hand, hepatocyte-specific HMGB1 knockout mice suffer from increased mitochondrial damage and cell death during liver ischemia/reperfusion (Huang et al., 2014). On the other hand, hepatocyte HMGB1 is involved in the
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The mRNA level of Nrf2 was not significantly up-regulated in the BDL group, while the levels of Nqo1 and Hmox1 were substantially up-regulated. Bicyclol not only up-regulated the mRNA level of Nqo1 and Hmox1, but it also up-regulated the level of Nrf2. In vitro cell experiments, TCA only up-regulated the level of Nrf2 mRNA without significant difference, while bicyclol up-regulated the mRNA and protein levels of Nrf2 and Nqo1. So, bicyclol also improved liver damage caused by BDL by activating Nrf2 and its downstream targets Nqo1 and Hmox1, which is consistent with previous studies (Zhang et al., 2014; Zhao et al., 2020).

CONCLUSION

These results indicate that bicyclol can improve liver damage caused by BDL in obstructive cholestasis. The bicyclol treatment promotes hydrophilization of bile acids, inhibits bile acid synthesis through Fxr/Cyp7a1, and promotes the excretion of bile acids through the Fxr/Bsep pathway. In addition, the fact that bicyclol treatment exerts its therapeutic effect through p62-Nrf2 anti-inflammatory and antioxidant pathways further the research on cholestasis liver injury.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of Radiation Medicine Chinese Academy of Medical Sciences (IRM-DWLL-2018104, November 20, 2018).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Review Board (or Ethics Committee) of Radiation Medicine Chinese Academy of Medical Sciences.

AUTHOR CONTRIBUTIONS

JWZ, JZ, and LZ designed the research; MR, TY, XX, PJ, and LZ performed the experiments; MR, LC, SS, XL, and SZ analyzed data; MR, JWZ, and LC wrote the paper; JWZ, JZ, and LZ read and revised the manuscript.

FUNDING

This research was funded by National Key R&D Program of China (No. 2019YFC0119500), National Natural Science Foundation of China (No. 81960107) and Natural Science Foundation of Tianjin (NO18JCQNJC80700).
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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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