Ursolic acid alleviates cholestasis by regulating Nrf2- UGT2B7/BSEP/MRP2 pathway in vivo and in vitro

Xing Wang  
Nanchang University

Wenqiang Xiong  
Nanchang University

Xin Wang  
Nanchang University

Liying Qin  
Nanchang University

Maolian Zhong  
Nanchang University

Yan Liu  
Nanchang University

Yuqing Xiong  
Nanchang University

Xiaoyi Yi  
Jiangxi Cancer Hospital, Jiangxi Cancer Hospital of Nanchang University

Xiaosong Wang  
Jiangxi Cancer Hospital, Jiangxi Cancer Hospital of Nanchang University

Hong Zhang (✉ ZLhospital@163.com)  
Jiangxi Cancer Hospital, Jiangxi Cancer Hospital of Nanchang University

Research Article

Keywords: Ursolic acid, Cholestasis, Nrf2, UGT2B7, BSEP, MRP2

Posted Date: August 14th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3237167/v1

License: © This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Naunyn-Schmiedeberg's Archives of Pharmacology on October 9th, 2023. See the published version at https://doi.org/10.1007/s00210-023-02733-w.
Abstract

Human hepatoblastoma cell line HepG2 has been widely used in the study of liver and liver cancer. α-naphthyl-isothiocyanate (ANIT) is a poison widely used in rodents to simulate human intrahepatic cholestasis. Ursolic acid (UA), a pentacyclic triterpenoid, exhibits various pharmacological actions. However, the role of nuclear factor E2 related factor 2 (Nrf2)-uridine diphosphate glucuronosyltransferase (UGT2B7)/bile salt output pump (BSEP)/multidrug resistance-associated protein 2 (MRP2) pathway in UA against cholestatic liver injury has not been cleared. The purpose of this study is to explore the effect of UA on cholestatic liver injury and its potential mechanism. Compared with the control group, UA could increase the expression of Nrf2, UGT2B7, BSEP, and MRP2 in HepG2 cells by Rt-qPCR and Western Blot. This up-regulation was inhibited after silencing Nrf2. The results of pathological sections and biochemical indexes showed that UA could alleviate cholestatic liver injury induced by ANIT and significantly activate the mRNA and protein of UGT2B7, BSEP, and MRP2 in liver tissue. However, this activation was inhibited in rats silenced with Nrf2. We confirmed that UA can reduce cholestasis. And it's related to Nrf2-UGT2B7/BSEP/MRP2. Therefore, this study expands the understanding of the anti-cholestatic effect of UA and provides a new therapeutic target for cholestasis.

1. Introduction

Impaired bile secretion from hepatocytes or bile duct epithelial cells and obstruction of bile flow formation and excretion leads to blockage of bile flow, which further leads to retention of toxic substances such as bile acid (BA) and eventual hepatocellular damage and cholestatic liver injury (Wagner et al., 2009). The features of intrahepatic cholestasis are impaired bile formation, BA detoxification, and transport (Ding et al., 2008). Ursodeoxycholic acid (UDCA) and obeticholic acid (OCA), which have been used to treat cholestasis, have poor reactivity and safety in clinical applications (John et al., 2021). Therefore, there is an urgent need to develop new drugs to treat cholestasis.

Ursolic acid (UA), a pentacyclic triterpenoid, exists in fruits, leaves, and many herbs. It provides numerous pharmacological benefits, such as anti-inflammation, anti-tumor, anti-diabetes, immunological control, heart protection, liver protection, and so on (Son and Lee, 2020; Shi et al., 2021). Recent studies have shown that UA can improve myocardial ischemia/ reperfusion injury through immune proteasome-PP2A-AMPK (Xu et al., 2023), inhibit colorectal cancer through down-regulating the activity of Wnt/β-catenin signaling axis (Zhao et al., 2023), and ameliorate traumatic brain injury in mice through modulating microRNA-141-mediated PDCD4/PI3K/AKT pathway (Zhang et al., 2023). However, the research on the mechanism of UA in lowering cholestatic liver injury is sparse and deserves further investigation.

Uridine diphosphate glucuronosyltransferase (UGT) 2B7 is a glycoprotein mainly expressed in the human liver, kidney, and lung (Yang et al., 2017). It is crucial for the glucuronidation of endogenous compounds such as steroids and bile acids and the clearance of exogenous compounds such as drugs (Sastre et al., 2015). Bile salt output pump (BSEP), a transmembrane protein, is mainly expressed in hepatocyte tubes. BSEP is an important rate-limiting factor in the clearance of bile salts from hepatocytes to bile. The
enterohepatic circulation of bile salts and bile flow may be affected by impaired BSEP transport, which may result in cholestatic liver damage (Cheng et al., 2016). Multidrug resistance-associated protein 2 (Mrp2), a tubular efflux transporter highly expressed in hepatocytes, is essential for the biliary excretion pathway of glutathione, glutathione conjugates, and glucuronic acid (Dietrich et al., 2001; Hayashi et al., 2012; Kong et al., 2012). Nuclear factor erythroid 2-related factor Nrf2 (nuclear factor erythroid 2-related factor2, Nrf2) is one of the members of the basic leucine chain transcription factor family (Alekhya Sita et al., 2019). It is believed that the Nrf2 signaling pathway will develop into a possible target for the treatment of liver illness since it plays an essential role in cellular anti-oxidative stress, bile acid synthesis, and enterohepatic circulation (Ma et al., 2020; Fawzy et al., 2023; Liu et al., 2023; Song et al., 2023; Xiang et al., 2023).

Therefore, this study intends to explore the potential mechanism of UA in alleviating cholestatic liver injury based on Nrf2-UGT2B7/BSEP/MRP2 pathway by using HepG2 cells and ANIT-induced rats of cholestasis, to provide the theoretical and scientific basis for targeted treatment of cholestatic liver injury and the development and clinical application of UA.

2. Materials and methods

2.1 Chemicals and Reagents

UA (purity ≥ 93%), α-naphthalene-isothiocyanate (ANIT, purity 98%), and chloral hydrate (purity > 99%) were purchased from Shanghai Macklin Biochemical Technology Co., Ltd (Shanghai, China). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphokinase (ALP), and γ-glutamyl transferase (γ-GT) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Total bilirubin (T-BIL), direct bilirubin (D-BIL), and total bile acid (TBA) assay kits were provided by Changchun Huili Biotechnology Co., Ltd (Changchun, China). Hematoxylin staining solution, tissue fixative (4% paraformaldehyde), and phosphate buffer saline (PBS) were from Servicebio Biotechnology Co., Ltd (Wuhan, China). Antibodies for GAPDH, UGT2B7, and MRP2 were purchased from Proteintech Group (USA). Antibodies for BSEP and Nrf2 were provided by Abcam (UK). Antibodies for horseradish peroxidase-labeled goat anti-rabbit IgG and goat anti-mouse IgG were from Proteintech Group (Wuhan, China)

2.2 Cell Culture and Treatment

HepG2 cells were acquired from the Typical Culture Repository of the Chinese Academy of Sciences (Shanghai, China). They were cultured in IMDM medium (HyClone, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Biological Industries, Israel) in an incubator at 37°C, 5% CO₂ for 24h. Cells in the logarithmic growth phase were incubated for 48h with UA (8, 16, 32 µM), which were dissolved in DMSO. Sulforaphane (SF) was employed as a positive control, and DMSO as a negative control.

2.3 Construction of HepG2 cell line silencing Nrf2
Plasmids loaded with shRNA-Blank and shRNA-Nrf2 fragments were extracted according to E.Z.N.A. Endo-free plasmid mini kit II kit (OMEGA Company, USA). The concentration and purity of the plasmid were determined by the nucleic acid protein detector, and the OD260/OD280 was between 1.8 and 2.0. HepG2 cells, at the density of 3×10^5 cells/mL, were plated in the 6-well plate and cultured. When the cells fused to 70–90%, transfected according to the instructions of Lipofectamine 3000. Western blot combined with qRT-PCR was used for verification.

2.4 Plasmid

Shanghai Novobio Scientific was commissioned to construct a pEGFP-Nrf2 expression plasmid by synthesizing the human NFE2L2 (Nrf2) gene sequence into the overexpression vector pEGFP-N1. Promoter regions of three genes UGT2B7/BSEP/MRP2 (2000 bp before and 200bp after transcription start) were synthesized into luciferase vector pGL4.10 to construct reporter genes pGL4.10-UGT2b7, pGL4.10-BSEP, and pGL4.10-MRP2. And sequencing verification. The activity of firefly luciferase constructed by pGL4.10 was normalized using the sea kidney luciferase produced by the pRL-TK vector as an internal control.

2.5 Transient transfection and dual luciferase reporter assay

HepG2 cells were inoculated in the 24-well plate at a concentration of 3×10^5 cells/mL with 500 µL. When the cells converged to 70–90%, transient transfection was performed according to the instructions of Lipofectamine 3000. In brief, Lipofectamine 3000 and plasmid were diluted with Opti-MEM medium and incubated at room temperature. Then the transfection complex was added, mixed thoroughly, and cultured after the plasmid had been incubated with Lipofectamine 3000 for an additional 15 minutes. The Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was used to quantify the activities of the firefly and renilla luciferases, and SpectraMax M5 multifunctional microplate reader (Molecular Devices, USA) was employed to measure luminescence. The firefly luciferase activity was normalized compared to renilla luciferase activity, and the ratio of normalized activity in the test samples to that in the negative control was determined as the relative luciferase activity.

2.6 Animals and Treatment

SPF male SD rats (180-220g) were purchased from Hunan Slaike Jingda Laboratory Animal Co., Ltd. (Changsha, Hunan, China, No. SYXK < Xiang > 2016-0002). All rats were kept in good experimental conditions (temperature: 23 ± 1°C, humidity: 55–60%, 12-hour light, and 12-hour dark cycle). All animal experiments were approved by the Ethics Committee of Nanchang University (registration number: NCUSYDWFL-202016, date: 11-05-2020).

The rats were randomly divided into 8 groups (n = 6): control group, ANIT group (60mg/kg), ANIT combined with UA (10mg/kg, 20mg/kg, and 40mg/kg) group, control group silencing Nrf2, ANIT group silencing Nrf2 and ANIT combined with UA (40mg/kg) group silencing Nrf2. The rat silencing Nrf2 was established by tail vein injection of lentivirus. From day 1 to day 14, mice in the control group and ANIT group were given 1% Tween80, while other groups were given UA. On day 12, the control group was given...
olive oil solution, ANIT group, and ANIT combined with the UA group were given ANIT (60 mg/kg, dissolved in olive oil). After successful modeling, 10% chloral hydrate was injected intraperitoneally. All rats were sacrificed on day 14, and venous blood and liver were collected.

2.7 Sample Collection and Pretreatment

Centrifuge the blood samples (4°C, 3500rpm, 15min), transfer the supernatant (clear and transparent), and store them at -80°C for later use. The liver tissue was washed with PBS, the part was fixed in 4% paraformaldehyde at room temperature for 24 h, and the rest was stored at -80°C.

2.8 Biochemical Index and histological analysis

The activities of ALT, AST, ALP, υ-GT, and the levels of DBIL, TBIL, and TBA in serum were operated according to the instructions of the kits. Liver tissues were fixed for 24h, embedded in paraffin, and cut into 4µm sections. After staining with hematoxylin-eosin (HE), the sections were observed with a light microscope (Nikon Eclipse E100, Nikon, Japan).

2.9 Construction of the rat model with silencing Nrf2

The lentiviruses (1*108TU) loaded with shRNA-Blank and shRNA-Nrf2 were injected into the rats through the tail vein respectively. This operation was carried out on the sterile operating bench.

2.10 Western Blot

Firstly, preparation of liver tissue homogenate with RIPA lysate buffer (APPLYGEN, Beijing, China), the content of protein in the supernatant was determined according to the instructions of the BCA assay kit (APPLYGEN, Beijing, China). Then, 30µg of protein extract was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10%, w/v) and transferred to polyvinylidene fluoride membrane (PVDF, Millipore, USA). After sealing the bands with 5% skim milk for 2h, incubated with primary antibody overnight at 4°C. Next, the membranes were washed with TBST (n = 3, 10min) and incubated with the corresponding horseradish peroxidase coupled secondary antibody at room temperature for 1h. Finally, visualization was achieved with an ECL kit (Fdbio Science, Hangzhou, China).

2.11 Quantitative real-time polymerase chain reaction

Total RNA in liver tissue was extracted according to the instructions of the TransZol Up Plus RNA Kit (TransGen Bio., Beijing, China). OD260/OD280 ranged from 1.8 to 2.1, and OD260/OD230 ranged from 2.0 to 2.4. Total RNA was reverse transcribed into cDNA with EasyScript® One-Step gDNA Removal and cDNA Synthesis Super Mix (TransGen Bio., Beijing, China). QRT-PCR was performed using the SYBR® Premix Ex Taq™ (TaKaRa Bio., Kyoto, Japan) in a Thermal Cycler Dice Real Time System. The primer sequence is shown in Table 1. The parameters of the amplification reaction were set as follows: pre-denaturation (95°C, 20s), denaturation (95°C, 10s), annealing (55°C, 15s) for 40 cycles, extension (95°C, 10s), and dissolution for 81 cycles(55°C, 30s).
Table 1
Primer sequences for quantitative real-time PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer(5'-3')</th>
<th>Reverse Primer(5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrf2</td>
<td>ATCCAGACAGACACCAGTGGATC</td>
<td>GGCAGTGAAGACTGAACCTTTCA</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>GTTCAGATTTGCTCAACGC</td>
<td>CTGTCAGAGGCTTCAGAG</td>
</tr>
<tr>
<td>BSEP</td>
<td>TGGAAAGGAATGGTGATGGG</td>
<td>CAGAAGGCCAGTGCAATAACACA</td>
</tr>
<tr>
<td>MRP2</td>
<td>CAGTCACGGCTTCTTCTTG</td>
<td>AGGTTCGGCTGGGACTTCT</td>
</tr>
<tr>
<td>GAPDH</td>
<td>ATGGAGAAGGCTGGGGCTCACCCT</td>
<td>AGCCCTTCCACGATGCCAAAGTTGT</td>
</tr>
</tbody>
</table>

2.12 Statistical analysis

SPSS19.0, GraphPad Prism 5.0, and Image J were used for statistical analysis, and the data were expressed in the form of Mean ± SD. T-test was used for comparison between two groups, and One-way ANOVA was used for comparison between multiple groups. P < 0.05 was considered a significant difference.

3. Result

3.1 UA increased the expression of Nrf2, UGT2B7, BSEP and MRP2 in HepG2 cells

To investigate the changes in the expression of nuclear receptor Nrf2, metabolic enzyme UGT2B7, cholate transporter BSEP, and MRP2 in hepatocytes after UA administration, Western blot, and qRT-PCR were employed to analyze. Compared with the control group, mRNA and protein expressions of Nrf2, UGT2B7, BSEP, and MRP2 in HepG2 cells treated with SF and UA for 48h were effectively improved, with significant differences (P < 0.05). In addition, the activation of UA was dose-dependent (Fig. 1).

3.2 Effects of UA on UGT2B7, BSEP, and MRP2 reporter genes in HepG2 cells transiently co-transfected with Nrf2 expression plasmid.

To further verify whether Nrf2 was involved in the regulation of UGT2B7/BSEP/MRP2, the effect of UA on reporter gene activity in HepG2 cells via transient co-transfection of luciferase reporter gene plasmid was evaluated. Table 2 showed the results of luciferase activity of different reporter genes. Compared with the null group, the luciferase activities of the UGT2B7 and BSEP reporter genes in SF and UA groups were enhanced but not remarkable after transplanted pEGFP-Nrf2, pRL-TK, and the correspondent reporter genes. Nevertheless, the fluorescence enzyme activities with the MRP2 reporter gene were significantly increased, which were 225% and 107%, respectively.
### Table 2
The relative luciferase activity of UGT2B7, BSEP, and MRP2 reporter

<table>
<thead>
<tr>
<th>Component</th>
<th>Group</th>
<th>SF</th>
<th>UA</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGL4.10 + pRL-TK + pEGFP-N1</td>
<td>Control</td>
<td>1.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>pGL4.10 + pRL-TK + pEGFP-Nrf2</td>
<td></td>
<td>1.06 ± 0.16</td>
<td>1.00 ± 0.14</td>
</tr>
<tr>
<td>pGL4.10-UGT2B7 + pRL-TK + pEGFP-N1</td>
<td></td>
<td>1.04 ± 0.10</td>
<td>1.15 ± 0.12</td>
</tr>
<tr>
<td>pGL4.10-UGT2B7 + pRL-TK + pEGFP-Nrf2</td>
<td></td>
<td>1.22 ± 0.09</td>
<td>1.24 ± 0.10</td>
</tr>
<tr>
<td>pGL4.10-BSEP + pRL-TK + pEGFP-N1</td>
<td></td>
<td>1.06 ± 0.14</td>
<td>1.05 ± 0.20</td>
</tr>
<tr>
<td>pGL4.10-BSEP + pRL-TK + pEGFP-Nrf2</td>
<td></td>
<td>1.18 ± 0.13</td>
<td>1.18 ± 0.13</td>
</tr>
<tr>
<td>pGL4.10-MRP2 + pRL-TK + pEGFP-N1</td>
<td></td>
<td>0.987 ± 0.14</td>
<td>1.06 ± 0.19</td>
</tr>
<tr>
<td>pGL4.10-MRP2 + pRL-TK + pEGFP-Nrf2</td>
<td></td>
<td>1.18 ± 0.10</td>
<td>3.25 ± 0.23***</td>
</tr>
</tbody>
</table>

(Mean ± SD, n = 3. * P < 0.05, ** P < 0.01, ***P < 0.001)

### 3.3 Upregulation of UA on UGT2B7, BSEP and MRP2 in HepG2 with Nrf2 silencing was weakened

To determine whether UA regulated the expression of downstream UGT2B7, BSEP, and MRP2 via activating Nrf2, we silenced Nrf2 with shRNA for further investigation. The results of Western blot and qRT-PCR revealed that Nrf2 expression in HepG2 was significantly decreased after transfection with shRNA-Nrf2 in comparison to the group transfected with shRNA-Blank, which suggested the HepG2 cell line with Nrf2 silenced was successfully developed (Fig. 2). Compared with the null group, the activation of SF and UA on the mRNA and protein expression of UGT2B7, BSEP, and MRP2 were inhibited after the silencing of Nrf2 (Fig. 2). Moreover, the changes in MRP2 expression were statistically significant (P < 0.05).

### 3.4 UA attenuates ANIT-induced cholestatic liver injury in rats

To clarify the effect of UA on cholestatic liver injury, we initially analyzed changes in liver biomarkers in serum. As shown in Fig. 3, after treatment with ANIT for 48 h, ALT, AST, ALP, γ-GT, T-BIL, D-BIL, and TBA were significantly higher than those of normal controls (P < 0.001). These variations were remarkably reversed after the administration of UA. (P < 0.001, Fig. 3A-G). The liver sections of rats were stained with HE for histopathological observation (Fig. 3H-L). We noticed considerably localized necrosis and apparent inflammatory cell infiltration after ANIT administration. With UA concentration increasing, the
lesion area was gradually alleviated, and the inflammatory response was progressively improved. Besides, this change was most pronounced at 40mg/kg. Therefore, it was chosen for the following experiments.

3.5 UA increases the expression of Nrf2, UGT2B7, BSEP and MRP2 in rats with ANIT-induced

To explore whether the variations of bile flow rate in ANIT-treated and UA-pretreated rats were associated with bile acid metabolizing enzymes and transport proteins, RT-PCR and Western blot were applied to identify the changes of Nrf2, UGT2B7, BSEP, and MRP2 expression levels in rat liver. The mRNA and protein levels of Nrf2, UGT2B7, BSEP, and MRP2 were significantly lowered in the ANIT group compared with the normal control group (Fig. 4). After pretreatment of ANIT-induced rats with UA, the expression of the four target genes was found to be increased by western blot and RT-PCR (Fig. 4). The differences were all statistically significant (P < 0.05).

3.6 Effect of UA on the expression of Nrf2, UGT2B7, BSEP, and MRP2 in rats with Nrf2-silenced ANIT-induced cholestatic liver injury

To examine whether the induction of metabolic enzymes UGT2B7 and transporters BSEP and MRP2 by UA was mediated by NRF2 activation, we employed the tail vein injection of lentivirus to develop the rat model of silenced Nrf2 for validation. Compared with the corresponding shRNA-blank, the mRNA and protein levels of Nrf2 were significantly suppressed in the control, ANIT and ANIT combined UA groups containing shRNA-Nrf2 (Fig. 5). This indicated that the rat model with Nrf2 silencing was effectively established. In cholestatic rats with Nrf2 silenced, the activation of UA on UGT2B7, BSEP, and MRP2 was markedly inhibited in comparison to the null group (Fig. 5, P < 0.05). Taken together, these data revealed that UA may regulate the expression of metabolic enzyme UGT2B7 and transporters BSEP and MRP2 via the mediation of Nrf2.

4. Discussion

Cholestasis is a pathological condition caused by obstruction of bile flow. The accumulation of toxic bile acids can trigger inflammatory reactions, leading to liver fibrosis, cirrhosis, and ultimately liver failure (Ou et al., 2016). Promoting bile acid efflux and detoxification are the main strategies for treating cholestasis (Kong et al., 2018). The drugs currently approved for cholestatic liver injury are limited and the treatment effect is not satisfactory (Leung et al., 2020; Wagner and Fickert, 2020).

HepG2 cells, which were established from chronic hepatitis C-associated liver tumors, have the same characteristics and characteristics as adult hepatocytes (Li et al., 2009). Elevated serum transaminases and bile acids, inflammatory cell infiltration, and hepatobiliary cell necrosis, followed by bile duct
obstruction and hyperplasia are the main features of ANIT-induced cholestasis (Desmet et al., 1968; Hertzog et al., 1975; Amin et al., 2006). ALP, T-BIL, and TBA are biomarkers of cholestasis. ALT as well as AST are biomarkers related to liver injury (Ferreira et al., 2003). We found that compared with the control group, their levels were significantly increased in rats treated with ANIT for 48h, and the differences were statistically significant. Moreover, focal necrosis, hemorrhage, and infiltration of inflammatory cells were observed in the pathological sections. The above data confirmed that ANIT successfully induced intrahepatic cholestasis in rats.

Studies have found that the expression of bile acid transporters, such as BSEP, was up-regulated, the adaptive response mediated by Nrf2 was enhanced, and the cell damage was alleviated after treatment of HepG2 cells induced by rifampicin with TUDCA (Zhang et al., 2017). Wang et al. found BSEP/NTCP expression was activated through the epigenetic activation of Nrf2 in the prevention of rifampicin-induced liver injury (Yang et al., 2020). Our findings in the current study were also compatible: the expression of BSEP was reduced when Nrf2 was downregulated. Bi et al. found that Nrf2 directly regulated the expression of MRP2 in primary mouse hepatocytes (Chen et al., 2015). In our study, it was confirmed that the regulation of UA on MRP2 was mediated by Nrf2. Glucuronidation was another important mode of bile acid metabolism. Gian et al. discovered that UGT2B7 was one of the most relevant uridine diphosphate glucuronosyltransferases in the metabolism of toxic bile acids via glucuronidation (Kastrinou Lampou et al., 2023). We found for the first time that UA could increase the expression of UGT2B7 protein and mRNA in HepG2 cells and ANIT-induced rats. And this activation was inhibited after the down-regulation of Nrf2 expression. We speculated that Nrf2 may be the upstream factor regulating UGT2B7.

This study still had some limitations. The results indicated that in HepG2 cells silencing Nrf2, the inhibitory effect of UA on the up-regulation of UGT2B7 and BSEP protein and mRNA was not significant. This was inconsistent with the results in ANIT-induced rats. In addition to the species difference, it may be because HepG2 cells are hepatoma cells, which are different from the cholestatic liver injury studied in this study.

In summary, we found that UA alleviated intrahepatic cholestasis by reducing hepatocyte injury, improving inflammatory cell infiltration, and promoting bile acid efflux, and Nrf2-UGT2B7/BSEP/MRP2 pathway may be one of the mechanisms of UA against cholestatic liver injury.

**Declarations**

**Ethical Approval**

The experimental procedures were implemented in accordance with the Guide for the Care and Use of Laboratory Animals. All animal experiments were approved by the Ethics Committee of Nanchang University. (registration number: NCUSYDWFL-202016, date: 11-05- 2020).

**Competing interests**
All authors declare that they do not have any conflicts of interest regarding the publication of this paper.

**Author Contributions**

The authors declare that all data were generated in-house and that no paper mill was used. Participated in research design: Hong Zhang, Yuqing Xiong. Conducted experiments: Xing Wang, Xin Wang, Liying Qin, Wenqiang Xiong, Maolian Zhong, Yan Liu, Xiaoyi Yi. Xiaosong Wang. Performed data analysis: Xiaoyi Yi, Xiaosong Wang, and Hong Zhang. Wrote or contributed to the writing of the manuscript: Xing Wang, Xin Wang, Liying Qin, and Hong Zhang.

**Funding**

This work was supported by National Nature Science Foundation of China (No. 81660622).

**Availability of data and materials**

Data are available from the corresponding author upon reasonable request.

**References**


Figures

**Figure 1**

Effects of UA on Nrf2, UGT2B7, BSEP, and MRP2 expression.

HepG2 cells were treated with SF (10 μM) and UA (8, 16, 32 μM) for 48 h. DMSO (0.1%) was used as the negative control. The experiments were compared to the negative control group. (A) Relative mRNA, (B) protein levels were quantified by qRT-PCR and western blot analyses, respectively. The experiments were compared to the negative control group. UGT2B7, BSEP, and MRP2 were normalized to GAPDH, whereas Nrf2 was normalized to Lamin B1. All values were expressed as the mean ± SD. of three independent experiments. (* p < 0.05, ** p < 0.01, *** p < 0.001).
Figure 2

Effects of UA on Nrf2, UGT2B7, BSEP and MRP2 in Nrf2-silenced HepG2 cells.

HepG2 cells were transfected with shRNA-Black or shRNA-Nrf2 recombinant plasmid. The (A) mRNA and (B) protein levels in these cells were determined by qRT-PCR and western blot analyses, respectively. Then, the HepG2 cells stably transfected with shRNA-Black or shRNA-Nrf2 were treated with SF or UA (32 μM) for 48 h. The expression levels were determined by qRT-PCR and western blot analyses, respectively. DMSO (0.1%) was used as the negative control. UGT2B7, BSEP, and MRP2 were normalized to GAPDH, whereas Nrf2 was normalized to Lamin B1. The values were expressed as the mean ± SD. of three independent experiments (# P < 0.05 and * P < 0.05 compared with shRNA-Blank-control and shRNA-Blank of the corresponding group, respectively).
Figure 3

Protective effect of UA on ANIT-induced cholestatic liver injury in rats.

Biochemical indexes in serum: (A) ALT, (B) AST, (C) ALP, (D) γ-GT, (E) TBIL, (F) DBIL, and (G) TBA; Images of HE stained liver sections: (H) control, (I) ANIT, (J) UA (10 μM), (K) UA (20 μM), (L) UA (40 μM), (200 × magnification). Data are presented as mean ± SD (n = 6). *P<0.05, ***P<0.001, compared with control group; #P<0.05, ##P<0.01, ###P<0.001, compared with ANIT group.
Figure 4

Effect of UA on Nrf2, UGT2B7, BSEP, MRP2 mRNA in rats with ANIT-induced cholestatic liver injury.

(A) The mRNA expression of Nrf2, UGT2B7, BSEP, and MRP2 were measured by RT-qPCR analysis. (B) The protein expression of Nrf2, UGT2B7, BSEP, and MRP2 were measured using western blot. Data are presented as mean ± SD (n = 6). *p < 0.05 and #p < 0.05 compared with the vehicle and ANIT group, respectively. **p < 0.01 and ##p < 0.01 compared with the vehicle and ANIT group, respectively.

Figure 5

Effects of UA on Nrf2, UGT2B7, BSEP, and MRP2 expression in rats treated with ANIT after silencing Nrf2.

The (A) mRNA and (B) protein levels in liver tissue were determined by qRT-PCR and western blot analyses, respectively. Olive oil was used as the negative control. UGT2B7, BSEP, MRP2, and Nrf2 were normalized to GAPDH. Data are presented as mean ± SD (n = 4) (*P < 0.05, **P < 0.01, and ***P < 0.001 compared with control group; #P < 0.05, ##P < 0.01 and ###P < 0.001 compared with ANIT group; &P < 0.05, &&P < 0.01 and &&&P < 0.001 compared with shRNA-Blank group, respectively.)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.