Glutamine attenuates Obstructive Cholestasis in Rats via Farnesoid X Receptor-mediated Regulation of Bsep and Mrp2

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Glutamine attenuates Obstructive Cholestasis in Rats via Farnesoid X Receptor-
mediated Regulation of Bsep and Mrp2

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Abstract:

To investigate the protective effect of glutamine (Gln) against obstructive cholestasis is in association with Farnesoid X receptor (FXR) activation. Obstructive cholestasis model was established in male SD rats by giving bile duct ligation (BDL). Serum biomarkers and H&E staining were used to identify the amelioration of the hepatic injury degree of obstructive cholestasis rats after Gln treatment. Immunohistochemistry, RT-PCR, western blot, cultured primary rat hepatocytes with FXR knockdown and dual luciferase reporter assay were performed to elucidate the mechanisms underlying Gln hepatoprotection. We found that Gln treatment protected against obstructive cholestasis induced by BDL through alleviating serum biomarkers ($P < 0.01$, respectively) and reducing hepatocyte injury. An up-regulation of hepatic efflux transporter (Shp, Bsep and Mrp2), an inhibition of hepatic uptake transporter (Ntcp) and bile acid synthesis enzyme Cyp7a1 expression were observed in BDL rats with Gln treatment in vivo. Furthermore, the regulatory effect of Gln on Bsep and Mrp2 expressions was abrogated after FXR knockdown in rat primary cultured hepatocytes. Luciferase assay HepG2 cells also illustrated FXR was a direct target for Gln treatment. In conclusion: The regulation of Bsep and Mrp2 expressions mediated by FXR might an important mechanism for Gln against obstructive cholestasis.

Keywords: Glutamine; Obstructive cholestasis; Bsep; Bile acid; FXR; Mrp2
Introduction

Obstructive cholestasis is characterized by a dramatically increased bile acid levels in liver and serum, which eventually results in impairment of hepatic canalicular bile efflux, acute hepatic toxicity, bile duct proliferation, fibrosis, and even cirrhosis. Various factors can cause cholestasis such as drugs, viral hepatitis, or genetic disorders. Currently, drugs like ursodeoxycholic acid (UDCA) are referred as the primary medicines for the treatment of cholestatic disorders; however, the treatment efficacy is very limited and not satisfied in clinic. Thus, it is an urgency to develop a novel agent to effectively resist the cholestatic disorders.

The pathogenesis of cholestasis may result from the dysfunctions of hepatic transport systems, such as the impairment of bile formation or bile flow in the hepatocyte (Marin et al. 2015; Zollner and Trauner 2006). Bile acids (BAs) are synthesized by hepatocytes from cholesterol in the liver, mainly involved in regulating bile flow in the hepatocytes and cholangiocytes through stimulating some signaling pathways (Kullak-Ublick et al. 2000; Mazuy et al. 2015; Zollner et al. 2006). The intracellular ligand-activated nuclear receptor (NR), farnesoid X receptor (FXR, NR1H4) as well as various transporters and enzymes have been considered to play pivotal roles in regulating BAs homeostasis in the liver, of which FXR is the master regulator of hepatic BAs synthesis and secretion (Ding et al. 2015; Suchy and Ananthanarayanan 2006). BAs are firstly synthesized by the transcription of the rate-limiting enzyme cholesterol 7a-hydroxylase (CYP7a1) in hepatocytes (Trauner et al. 2005); Then in the liver, an orphan nuclear hormone receptor named small heterodimer partner (Shp; NR0B2) is transcriptionally upregulated by BAs- FXR interaction, which
reversely represses Cyp7a1 activity (Zhou and Hylemon 2014); In addition, the activation of FXR can negatively regulate the expression of Na\(^+\)/taurocholate cotransporting polypeptide (Ntcp), resulting in inhibiting the uptake of BAs into the hepatocytes (Stieger 2011); while increase bile acid efflux by two ATP-binding cassette (ABC) transporters bile salt export pump (Bsep) and multidrug resistance-associated protein 2 (MRP2) to induce BAs excretion from the bile canalicular membrane into the canalicular lumen (Stieger and Beuers 2011).

Glutamine (Gln) as a conditionally essential amino acid is primarily formed and stored in lung and skeletal muscle. The nutritious effect of Gln has been illustrated in various tissues including fibroblasts, lymphocytes, small intestine enterocytes, and macrophages. Though Gln has no antioxidant effect, it can play a critical role against stressful stimuli, such as infection, inflammation, ischemia and even cancer (Miller 1999). In recent years, more scientiffic attention has been attracted to the protective effects of Gln against hepatic diastases. However, whether Gln against obstructive cholestasis is mediated by FXR and its down regulatory genes Mrp2 and Bsep still remains further investigation.

Thus, in this study, rat model of obstructive cholestasis was built to assess the hepatoprotective and choleretic effect of Gln, which may allow a better understanding of the molecular pathway regulated by Gln in cholestasis and provide potential pharmacological evidence targeting the involved regulatory networks.

### Materials and Methods

#### Obstructive cholestasis rat model build

Adult male Sprague-Dawley rats weighing 250-300 g were purchased from the
Laboratory Animal Center of Xi’an Jiaotong University, Xi’an, China [License: SCXK (Shan) 2007-001] and housed at a constant room temperature (24 ± 2°C) and humidity (50%) with an alternating 12h light/dark cycle. Rats were fed with a standard diet of commercial rat chow and tap water *ad libitum*. Obstructive cholestasis animal models were built using a modified method in previous study (Li and Chung 2001). Rats were subjected to laparotomy twice. All procedures were performed under anesthesia with 3% pentobarbital sodium (1.0 ml/kg).

Obstructive cholestasis was induced by double ligation of the common bile duct and Sham group (SH) was produced by separating bile duct locally but not ligated. To minimize tissue handling and dissection the duodenum was extracted with an ophthalmic muscle hook and the common bile duct isolated using very fine forceps. After bile duct ligation (BDL), all SD rats were randomly divided into 4 groups (*n* = 10) and treated as follows: Sham + normal saline (SH + NS) group, the rats in SH group were orally administrated with 0.9% normal saline at the dose of 10 mg/kg per day for 14 consecutive days; BDL + NS group, the rats with BDL were orally administrated with 0.9% normal saline at the dose of 10 mg/kg per day for 14 consecutive days; BDL+ Gln group, rats with BDL were given Gln (Sigma, MO, USA) at the dose of 200 mg/kg orally per day for 14 consecutive days; BDL+UDCA group, rats with BDL were given ursodeoxycholic acid (UDCA, Sigma) at dose of 50 mg/kg orally per day for 14 consecutive days. After final dose of injection, rats were fasted for the following 24 h, the animals were then succumbed and serum and liver tissue samples were obtained immediately. This study was in accordance with the provisions of the Declaration of Helsinki and approved by Animal Research Ethics committee of Xi’an Jiaotong University.
Histological and biochemical assessment

Liver tissues were fixed immediately in 10% neutral buffered formalin and paraffin-embedded blocks were made. Serial sections 5 µm thick were stained with H&E for evaluation of portal inflammation, hepatocellular necrosis and inflammatory cell infiltration. Sections were examined under a light microscope (Eclipse 50i, Nikon, Tokyo, Japan). Liver function markers of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutmyltranspeptidase (γ-GTP), total cholesterol (TC), direct bilirubin (DBIL), total bilirubin (TBIL) and biliary total bile acids (TBA) were analyzed using a commercially available clinical test kit with an automatic biochemical analyzer (AU2700, Olympus, Tokyo, Japan).

Immunohistochemistry and morphometry

The immunohistochemical staining was performed on sections of liver samples. The sections were incubated overnight at 4°C with the anti-Bsep antibody (rabbit polyclonal antibody against Bsep, bs-1954R, Boisynthesis Biotechnology, Beijing, China) at a dilution of 1:200, and the anti-Mrp2 antibody (mouse monoclonal antibody against Mrp2, SC-59611, Santa Cruz, CA, United States) at a dilution of 1:300, respectively. Following several rinses in phosphate buffered solution; the sections were incubated with the biotinylated goat anti-rabbit IgG antibody or biotinylated goat anti-mouse IgG antibody (Boisynthesis Biotechnology, Beijing, China) for 30 min at 37°C. Finally, the sections were colored with DAB at room temperature for 1-15 min, counterstained with hematoxylin for 30 s, dehydrated through gradient ethanol, cleared in xylene and then mounted with permount. Images were obtained.
using a light microscope (Eclipse 50i, Nikon, Tokyo, Japan). Immunoreactivity of Bsep and Mrp2 in rat liver was morphometrically identified by the Image Pro Plus 6.0 image analysis software system (Media Cybernetics, MD, United States).

### Quantitative real time (qRT-PCR) analysis

Total RNA was extracted from 100 mg snap-frozen liver tissue using RNAiso plus reagent (Takara, Dalian, China) according to the manufacturer’s instructions. The quality and quantity of RNA were assessed using 1% agarose gel electrophoresis and spectrophotometric analysis of 260/280 ratios. RNA was reversely transcribed with oligo primer using PrimeScript™ RT reagent Kit (Takara, Dalian, China). The primers of genes were also designed by Takara, and no significant homologous sequence was discovered using National Center for Biotechnology Information, The Basic Local Alignment Search Tool (NCBI BLAST). The housekeeping gene β-actin was used as an internal reference gene to normalize the transcript levels. The primer sequences of FXR, Bsep, Mrp2, Cyp7a1, Ntcp and Shp were listed in Table 1. qRT-PCR was performed on a Step one plus real-time PCR system (ABI, CA, United States) using the SYBR® Premix Ex Taq™ II reagent Kit (Takara, Dalian, China) under the following conditions: 30 s at 95°C followed by 40 cycles of 5 s at 95°C and 30 s at 60°C. The relative expression of the target gene was represented by the $2^{-\Delta\Delta CT}$ value of the tested sample.

### Western blot analysis

The proteins were extracted using Protein Extraction Kit (Promega, WI, United States). Thirty micrograms of proteins were mixed with the sample buffer for sodium dodecyl sulfate
polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresis was performed and the proteins were transferred to the polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with the primary antibodies, including FXR (H-130), Bsep (F-6), Mrp2 (M2IIIC5), Ntcp (M-130), Shp (F-12) and CYP7a1 (H-58) (Santa Cruz, CA, US) at a dilution of 1:200, respectively, while shaking at 4°C overnight. Then the PVDF membranes were incubated with goat anti-rabbit IgG-HRP antibody or goat anti-mouse IgG-HRP antibody (Boisynthesis Biotechnology, Beijing, China) (1:1,000) at room temperature for 2 h and were colored by enhanced electrochemiluminescence reagents (32109, Pierce, IL, United States) after the membrane had been washed repeatedly. Finally, the membranes were imaged and analyzed by the SyngeneG: BOX analytical system and normalized using mouse monoclonal anti-β-actin antibody (sc-47778, Santa Cruz, CA, US).

**Isolation and culture of primary rat hepatocytes**

Hepatocytes from male SD rats were isolated by using a modified two-step collagenase digestion method as described previously (Shen et al. 2012). Hepatocytes were washed with WME medium supplemented with 100 U penicillin/streptomycin (Sigma, USA) and 10% heat-inactivated fetal bovine serum (FBS) (Gibco, USA). Then the hepatocytes were cultured in plates pre-coated with rat tail collagen (Shengyou Biotechnology, Hangzhou, China) and incubated for 12 h at 37°C, then replaced with the fresh DMEM supplemented with 10% FBS, 20 mg/L hepatocyte growth-promoting factor (HGF) (Langdun Biotechnology, Shanghai, China), 0.5 mg/L insulin (Sigma, MO, USA) and 0.1µM Dexamethasone (Sigma, MO, USA) and incubated for another 12 h before treatment. The viability of hepatocytes was measured
by trypan blue exclusion and only cells with viability more than 90% could be used for further study.

**FXR siRNA treatment in cultured primary rat hepatocytes**

Cultured primary rat hepatocytes were seeded onto 24-well plate at a density of $1 \times 10^5$ cells per well. 12 h later, the cells were treated with vehicle (0.1% DMSO) or siRNA. For RNA silencing experiment, siRNA specific targeting at mouse FXR sequence AGG TTC CTT TCT ATG TTT ATA TC were designed as follows: sense: 5’- UAU AAA CAU AGA AAG GAA CCU C3’; Anti-sense: 3’- GUU CCU UUC UAU GUU UA U AUCC5’ (GenePharma, Shanghai) and transiently transfected to cultured primary rat hepatocytes using Lipofectamine 2000 (Invitrogen, US) and incubated for 24 h. After that, 100 µM Gln were added to the medium for another 24 h. the cells were harvested for western blot analysis.

**Dual luciferase reporter assay**

FXR expression plasmid was constructed by cloning the FXR gene sequence into pCI-neo mammalian expression vector (Promega). Bsep luciferase reporter vector was constructed by cloning the upstream DNA fragment of promoter region into the luciferase vector pGL4.14 [Lcu2/Hygro] (Promega) (Sang et al. 2008). For vitro transfection study, HepG2 cells were seeded in 24-well plates at a density of $1 \times 10^5$ cells/well in DMEM media supplemented with 10% FBS and cultured for 24h. Then, the cells were cotransfected with FXR expression plasmid, Bsep promoter luciferase reporter vector and the null-Renilla luciferase plasmid as an internal control. After transfection for 24 h, cells were treated with vehicle (0.1% DMSO), 100 µM CDCA (Sigma, MO, USA) or 10, 40, 70, 100 µM Gln for 24
h. the luciferase activities were measured with a Dual-luciferase Reporter assay System (Promega) according to manufacturer’s instructions. The firefly luminescence was normalized to the Renilla luminescence signal, and the ratio of treatment over control served as fold activation.

**Statistical analysis**

All results were expressed as mean ± standard deviation (SD). Statistical analyses between two groups were performed by a Student’s *t*-test and multiple comparisons were performed by a one-way ANOVA. A *P* < 0.05 was considered statistically significant.

**Results**

**Morphological observation and liver function test of obstructive cholestasis rat**

After BDL, rat skin stained yellow and lethargy; the obstructive rats became depressed, inactive and anorectic gradually and passed dark yellow urine. During and after the biliary drainage procedures, hemorrhage and dehydration were the main death reasons. Finally, 40 rats were enrolled into this study and treated as follows: the BDL rats were randomly injected with Gln (BDL + Gln group), UDCA (BDL + UDCA group), or normal saline (BDL + NS group) for 14 days. When compared to SH + NS group, the urine and skin color of BDL + NS rats still stained yellow and showed serious depression with poor appetite, lighter weight, and reduced activity. While in BDL + Gln and BDL + UDCA groups, the skin and urine of these rats gradually returned to normal and their appetite and activity recovered remarkably.

**Levels of Serum Hepatic Biomarkers in Rats**

To study the liver biomarker changes in different treated rats, ALT, AST, ALP, γ-GTP, TC,
DBIL, TBIL and TBA were analyzed. As shown in figure 1, the biochemical indicator levels of ALT, AST, ALP and γ-GTP in BDL + NS group were significantly increased when compared to SH +NS control group (P < 0.01, respectively). Whereas, after treatment with Gln or UDCA for 14 days in BDL rats, their levels gradually restored and showed a declined tendency (P < 0.01, respectively). Furthermore, consistent with serum biochemistry findings, BDL-induced elevation of bile salts including TC, DBIL, TBA and TBIL were also reduced by Gln and UDCA (P < 0.05, respectively). Notably, there was no significant difference between Gln and UDCA treatments. Taken together, these results directly illustrated that Gln possessed the function in attenuating BDL-induced obstructive cholestasis.

Gln attenuated liver damage in obstructive cholestasis rat

To further investigate the histological changes of obstructive cholestasis in rat tissues, the liver sections stained with H&E were determined by histopathological assessment. Inflammatory cell infiltration, hepatocyte necrosis, bile duct proliferation, and liver fibrosis were graded by (-, negative; +, slight; ++, moderate; ++++, severe), and shown in Table 2 and Figure 2. Histopathological study of sham operated rats revealed a normal liver lobular architecture and no pathological changes in livers. Whereas, the liver of obstructive rats (BDL + NS) showed severe bile duct proliferation and areas of confluent hepatocyte necrosis, fibrous expansion of the portal tracts with severe fibrosis and even pseudolobule formed (Fig. 2 and Table 2). Our results also found that after treatment with Gln and UDCA, the histopathologic parameters in BDL + Gln and BDL + UDCA groups were markedly decreased, and the liver displayed normal morphological features of hepatocytes and preserved lobular
architecture with only mild bile duct proliferation and slight inflammatory cell infiltration in comparison to SH + NS rats. Thus, consistent with the results of serum biomarkers detection, the histological examination demonstrated that Gln supplementation alleviated BDL-induced liver damage.

**Bile efflux transporter Bsep and Mrp2 distributions in obstructive cholestasis**

To elucidate the mechanism underlying the protective effect of Gln on obstructive cholestasis, the canalicular efflux transporter Bsep and Mrp2 were determined by immunohistochemical staining. The integrated optical density (IOD) was taken with the Image-Pro Plus 6.0 software to qualify the stained density. As shown in Figure 3A, the expression levels of Bsep and Mrp2 were mainly localized in the basolateral membrane of hepatic cells. The expressions of Mrp2 and Bsep in the BDL + NS group were significantly decreased in comparison to SH + NS group ($P < 0.01$, respectively) (Fig. 3). After treatment with Gln and UDCA for 14 days in BDL group, the expressions of Bsep and Mrp2 were significantly re-enhanced to the compatible level of SH group ($P < 0.01$, respectively).

**Alteration of bile acids regulatory molecules of obstructive cholestasis involved in Gln treatment**

To further verify the hepatoprotective effect of Gln on obstructive cholestasis was related to the restoration of bile flow system. Western blot and RT-PCR were used to investigate the alterations of hepatic key molecules including FXR, Bsep, Mrp2, Cypa1, Ntcp and Shp in livers of rats with different treatments. The mRNA and protein levels of FXR, Bsep, Mrp2 and SHP were remarkably decreased, while Ntcp and Cyp7a1 expressions were significantly
increased in BDL + NS group than in SH + NS group ($P < 0.01$, respectively); In contrast, after treatment with Gln and UCDA for 14 days, the expressions of FXR, Bsep, Mrp2, Shp, Ntcp and Cyp7a1 were restored to the normal levels when compared to the SH + NS group ($P > 0.05$, respectively) (Fig. 4). However, no significant difference was found between Gln and UCDA treatments. Taken together, these data strengthened the hypothesis that Gln plays a crucial role in protecting liver against obstructive cholestasis through bile acid synthesis and bile efflux transporter pathway.

The effect of Gln is abrogated by FXR silencing in rat primary cultured hepatocytes

Since genes including Bsep, Mrp2, Ntcp, Shp, and Cyp7a1 involved in regulating hepatic bile acid transport are downstream target genes of FXR. We hypothesized that Gln may activate FXR to regulate expression level of genes in bile acid homeostasis. To verify this hypothesis, siRNA was used to knock down the expression of FXR in cultured primary rat hepatocytes. Western blot confirmed that the FXR expression was hardly detected after specific siRNA targeting FXR transfection. In addition, the expressions of Mrp2 and Bsep induced by Gln were remarkably abrogated by FXR silence ($P < 0.01$, respectively) (Fig. 5A and B). Therefore, it was further demonstrated the involvement of FXR activation in the hepatoprotective effect of Gln against BDL-induced liver injury.

Gln increased Bsep expression by serving as a partial FXR agonist

Since significant increase expression of Bsep was detected in BDL rats with Gln treatment, we then investigated whether this effect was directly caused by the main upstream regulator FXR. Dual luciferase reporter assay was firstly performed in HepG2 cells.
cotransfected with FXR expression plasmid and FXR target gene Bsep promoter reporter vector. As demonstrated in Figure 5C, CDCA (100 µM), a characterized FXR agonist, significantly increased the luciferase activity of FXR reporter gene compared to vehicle group ($P < 0.01$), while Gln (at doses of 10, 40, 70 and 100 µM) also significantly increased the luciferase activity of FXR at a dose-dependent manner ($P < 0.05$, $P < 0.05$, $P < 0.01$, $P < 0.01$, respectively), which directly demonstrated the positive regulatory role of Gln on FXR activation.

**Discussion**

Bile acids (BAs) as the major composition of bile, plays a pivotal role in lipometabolism (Geier et al. 2007; Morgan et al. 2016). The impairment of bile secretion and flow could lead to cholestasis syndrome such as lesions, primary sclerosing cholangitis, epatocellular and cholangiocellular secretory defects (Matsuzaka et al. 2015). Furthermore, Tarantino et al. have reported that acute drug-induced liver injury (DILI) in patients suffering from non-alcoholic fatty liver disease (NAFLD) could present as a cholestatic form (Tarantino et al. 2007), because it’s well known that NAFLD patients with biochemical cholestasis have a histological picture of bile damage as Sorrentino et al. suggested (Sorrentino et al. 2005). Previous researches have demonstrated that if the activation of FXR is blocked, liver cholestasis could be aggravated by increasing the endogenous synthesis of BAs while decreasing bile acid efflux (Li and Guo 2015; Trauner et al. 1998). Current studies also reported that knockout of the expression of Bsep could induce the occurrence of cholestasis in rats (Funk et al. 2001). Moreover, the dysregulated expressions of Bsep and Mrp2 contributed
to the onset of related cholestatic diseases such as intrahepatic cholestasis of pregnancy (ICP) in adults and Dubin-Johnson syndrome (Cui et al. 2009; Kajihara et al. 1998; Wendum 2010). Donner et al. also declared that zonal downregulation of Bsep in obstructive cholestasis was associated with portal inflammation and is mediated by TNF-alpha and IL-1beta (Donner et al. 2007).

Aldemir et al. has reported that Gln could reduce the incidence of bacterial translocation and preserve intestinal mucosal integrity in common bile duct ligated rats (Aldemir et al. 2003). Xu et al. demonstrated that preconditioning with Gln significantly improved hepatic structure and function by inhibiting reactive oxygen species, inflammation, and hepatocyte apoptosis after ischemia/reperfusion injury in rats with obstructive jaundice (Xu et al. 2014). Alecrim et al. indicated that Gln supplementation had no positive effect on colonic scar strength in rats with extrahepatic biliary obstruction (Alecrim et al. 2015). Thus, it is urgent to deeply reveal the hepatoprotective mechanism underlying Gln on obstructive cholestasis. In the present study, Gln apparently mitigated serum transaminases in OJ-induced intrahepatic cholestasis rats such as ALT, AST, ALP, γ-GTP and bile acids TC, TBIL, DBIL, TBA levels, which were commonly used as crucial criteria for the diagnosis of hepatobiliary disorders (Knight 2005; Lalisang 2012; Scheig 1996). Furthermore, H&E experiment revealed that obstructive cholestasis rats with Gln treatment remarkably alleviated severe necrotic and degenerative changes of hepatobiliary cells, the bile duct proliferation, and the inflammatory cell infiltration, indicating the hepatoprotective effects of Gln involved in obstructive cholestasis.
Obstructive cholestasis with Gln treatment remarkably increased Bsep and Mrp2 distributions and expressions, resulting in the restoration in bile flow and biliary bile acid output (Chen et al. 2016; Kast et al. 2002). In addition, hepatic uptake of bile acids takes place at the basolateral membrane of hepatocytes and is mediated through Ntcp activation, which mediates \( \text{Na}^+ \)-dependent uptake of all physiological bile acid in their conjugated form (Stieger 2011). Besides transporters, bile acid synthetic enzymes like Cyp7a1 play important roles in bile acid homeostasis (Fu et al. 2014). In this study, Ntcp and Cype7a1 were remarkably reduced in obstructive cholestasis with Gln treatment to prevent synthesizing excessive bile acids entering hepatocytes. It has been demonstrated that Bsep and Shp are direct target genes of FXR and could be induced by FXR in livers; Furthermore, Shp has been proved to be the upstream gene of Ntcp and Cyp7a1, which could be induced by FXR to suppress the expressions of Ntcp and Cyp7a1 (Kwong et al. 2015; Li and Guo 2015). Whereas, treating with Gln in BDL rats significantly reduced FXR downstream target genes Cyp7a1 expression via FXR-Shp axis, lead to suppressing bile acid synthesis. Taken together, Gln as the key regulatory factor for BAs may become the effective therapeutic agent against obstructive cholestasis.

FXR plays an important role in regulating transporters and enzymes of BAs. Considering the significant effect of Gln on gene expression of Bsep and Mrp, which are the PXR target genes, \textit{in vitro} experiments was performed in cultured primary rat hepatocytes, FXR siRNA was applied to silence FXR expression and the result demonstrated that Mrp2 and Bsep expression regulated by Gln was directly abrogated in hepatocytes. Thus, we proposed that
Gln mediated activation of FXR pathway against BDL-induced cholestasis. Moreover, previous study has demonstrated that Bsep was predominantly regulated by FXR (Song et al. 2013). In this study, we speculated that Bsep upregulation in BDL rats with Gln treatment might be due to FXR agonism. The dual luciferase reporter assay confirmed that Gln could stimulate FXR activation dose-dependently in HepG2 cells, which was similar to the effect of FXR agonist CDCA. In the past decade, FXR has been regarded as a potential therapeutic target in the treatment of cholestatic liver disorders. Therefore, we proposed that the role of Gln in FXR agonism may also contribute to its anticholestatic effect.

In conclusion, we suggested that the involvement of Bsep and Mrp2 played crucial roles in the process of Gln treatment in obstructive cholestasis, its effect might via activating FXR pathway, and resulted in suppressing accumulation of TB, DB, and TBA in the liver through increase in hepatic efflux, which might give new insight into this hepatic disease and obtain effective therapeutic strategies.
Acknowledgement

We would like to thank Dr. Yiming Li for his insightful comments and suggestions.

Conflict of interest and disclosure

The authors declare that no competing interest exists.


Table 1 PCR primer sequences and the length of the amplified products

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**Table 2** The results of liver histopathological examination grade

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Figure Legends

Fig. 1. Detection of biochemical indicator levels in four rat groups with different treatments. Serum ALT (A), AST (B), ALP (C), γ-GTP (D) activity as well as serum TC (E), DBIL (F), TBIL (G) and TBA (H) levels were detected with automatic biochemical analyzer. Data are the mean ± SD. **P < 0.01 vs. SH+NS; ##P < 0.01 vs. BDL+NS.

Fig. 2. The images of representative H&E stained liver sections (200×magnification) were shown from SH+NS, BDL+NS, BDL+Gln, and BDL+UDCA groups.

Fig. 3. Immunohistochemical staining of Bsep and Mrp2 protein distributions in liver tissues of BDL rats with Gln treatment (400×magnification; bar = 50 µm).(A) Representative images of immunohistochemical staining for Bsep and Mrp2. (B) IOD values of Bsep and Mrp2 in rats liver tissue. The IOD values were examined were analyzed by microscope in six random fields. Data are the mean ± SD. **P < 0.01 vs. SH+NS; ##P < 0.01 vs. BDL+NS.

Fig. 4. Alternation of BAs regulatory gene expression changes in BDL rats with Gln treatment. (A) Protein levels of FXR, Bsep, Mrp2, Shp, Ntcp, and Cyp7a1 change detected was by western blot. (B) Relative protein expression levels detected in (A). (C) mRNA levels of FXR, Bsep, Mrp2, Shp, Ntcp, and Cyp7a1 change was detected by RT-PCR. Data are the mean ± SD. **P < 0.01 vs. SH+NS; ##P < 0.01 vs. BDL+NS.

Fig. 5. In vitro evidence on FXR activation by Gln. (A) FXR knockdown abrogated the regulation of Bsep and Mrp2 by Gln in rat primary hepatocytes. (B) Relative protein expression levels detected in (A). (C) Luciferase reporter assay performed in HepG2 cells with FXR expression vector and Bsep promoter reporter. Data are the mean ± SD. *P < 0.05,
**P < 0.01 vs. vehicle; ##P < 0.01 vs. Gln treatment alone.
Fig1.TIF

67x27mm (600 x 600 DPI)
Fig2. TIF

68x59mm (600 x 600 DPI)
Fig3. TIF

117x81mm (600 x 600 DPI)