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

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

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23 **Abstract:**

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

40

41 **Keywords:** Glutamine; Obstructive cholestasis; Bsep; Bile acid; FXR; Mrp2

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44 **Introduction**

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

52 The pathogenesis of cholestasis may result from the dysfunctions of hepatic transport
53 systems, such as the impairment of bile formation or bile flow in the hepatocyte (Marin et al.
54 2015; Zollner and Trauner 2006). Bile acids (BAs) are synthesized by hepatocytes from
55 cholesterol in the liver, mainly involved in regulating bile flow in the hepatocytes and
56 cholangiocytes through stimulating some signaling pathways (Kullak-Ublick et al. 2000;
57 Mazuy et al. 2015; Zollner et al. 2006). The intracellular ligand-activated nuclear receptor
58 (NR), farnesoid X receptor (FXR, NR1H4) as well as various transporters and enzymes have
59 been considered to play pivotal roles in regulating BAs homeostasis in the liver, of which
60 FXR is the master regulator of hepatic BAs synthesis and secretion (Ding et al. 2015; Suchy
61 and Ananthanarayanan 2006). BAs are firstly synthesized by the transcription of the
62 rate-limiting enzyme cholesterol 7 α -hydroxylase (CYP7a1) in hepatocytes (Trauner et al.
63 2005); Then in the liver, an orphan nuclear hormone receptor named small heterodimer
64 partner (Shp; NR0B2) is transcriptionally upregulated by BAs- FXR interaction, which

65 reversely represses Cyp7a1 activity (Zhou and Hylemon 2014); In addition, the activation of
66 FXR can negatively regulate the expression of Na⁺/taurocholate cotransporting polypeptide
67 (Ntcp), resulting in inhibiting the uptake of BAs into the hepatocytes (Stieger 2011); while
68 increase bile acid efflux by two ATP-binding cassette (ABC) transporters bile salt export
69 pump (Bsep) and multidrug resistance-associated protein 2 (MRP2) to induce BAs excretion
70 from the bile canalicular membrane into the canalicular lumen (Stieger and Beuers 2011).

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

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

83 **Materials and Methods**

84 **Obstructive cholestasis rat model build**

85 Adult male Sprague-Dawley rats weighing 250-300 g were purchased from the

86 Laboratory Animal Center of Xi'an Jiaotong University, Xi'an, China [License: SCXK (Shan)
87 2007-001] and housed at a constant room temperature ($24 \pm 2^{\circ}\text{C}$) and humidity (50%) with an
88 alternating 12h light/dark cycle. Rats were fed with a standard diet of commercial rat chow
89 and tap water *ad libitum*. Obstructive cholestasis animal models were built using a modified
90 method in previous study(Li and Chung 2001). Rats were subjected to laparotomy twice. All
91 procedures were performed under anesthesia with 3% pentobarbital sodium (1.0 ml/kg).
92 Obstructive cholestasis was induced by double ligation of the common bile duct and Sham
93 group (SH) was produced by separating bile duct locally but not ligated. To minimize tissue
94 handling and dissection the duodenum was extracted with an ophthalmic muscle hook and the
95 common bile duct isolated using very fine forceps. After bile duct ligation (BDL), all SD rats
96 were randomly divided into 4 groups ($n = 10$) and treated as follows: Sham + normal saline
97 (SH + NS) group, the rats in SH group were orally administrated with 0.9% normal saline at
98 the dose of 10 mg/kg per day for 14 consecutive days; BDL + NS group, the rats with BDL
99 were orally administrated with 0.9% normal saline at the dose of 10 mg/kg per day for 14
100 consecutive days; BDL+ Gln group, rats with BDL were given Gln (Sigma, MO, USA) at the
101 dose of 200 mg/kg orally per day for 14 consecutive days; BDL+UDCA group, rats with BDL
102 were given ursodeoxycholic acid (UDCA, Sigma) at dose of 50 mg/kg orally per day for 14
103 consecutive days. After final dose of injection, rats were fasted for the following 24 h, the
104 animals were then succumbed and serum and liver tissue samples were obtained immediately.
105 This study was in accordance with the provisions of the Declaration of Helsinki and approved
106 by Animal Research Ethics committee of Xi'an Jiaotong University.

107 Histological and biochemical assessment

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

117 Immunohistochemistry and morphometry

118 The immunohistochemical staining was performed on sections of liver samples. The
119 sections were incubated overnight at 4°C with the anti-Bsep antibody (rabbit polyclonal
120 antibody against Bsep, bs-1954R, Boisynthesis Biotechnology, Beijing, China) at a dilution of
121 1:200, and the anti-Mrp2 antibody (mouse monoclonal antibody against Mrp2, SC-59611,
122 Santa Cruz, CA, United States) at a dilution of 1:300, respectively. Following several rinses in
123 phosphate buffered solution; the sections were incubated with the biotinylated goat anti-rabbit
124 IgG antibody or biotinylated goat anti-mouse IgG antibody (Boisynthesis Biotechnology,
125 Beijing, China) for 30 min at 37°C. Finally, the sections were colored with DAB at room
126 temperature for 1-15 min, counterstained with hematoxylin for 30 s, dehydrated through
127 gradient ethanol, cleared in xylene and then mounted with permount. Images were obtained

128 using a light microscope (Eclipse 50i, Nikon, Tokyo, Japan). Immunoreactivity of Bsep and
129 Mrp2 in rat liver was morphometrically identified by the Image Pro Plus 6.0 image analysis
130 software system (Media Cybernetics, MD, United States).

131 **Quantitative real time (qRT-PCR) analysis**

132 Total RNA was extracted from 100 mg snap-frozen liver tissue using RNAiso plus
133 reagent (Takara, Dalian, China) according to the manufacturer's instructions. The quality and
134 quantity of RNA were assessed using 1% agarose gel electrophoresis and spectrophotometric
135 analysis of 260/280 ratios. RNA was reversely transcribed with oligo primer using
136 PrimeScript™ RT reagent Kit (Takara, Dalian, China). The primers of genes were also
137 designed by Takara, and no significant homologous sequence was discovered using
138 NationalCenter for Biotechnology Information, The Basic Local Alignment Search Tool
139 (NCBI BLAST). The housekeeping gene β -actin was used as an internal reference gene to
140 normalize the transcript levels. The primer sequences of FXR, Bsep, Mrp2, Cyp7a1, Ntcp and
141 Shp were listed in Table 1. qRT-PCR was performed on a Step one plus real-time PCR system
142 (ABI, CA, United States) using the SYBR® Premix Ex Taq™ II reagent Kit (Takara, Dalian,
143 China) under the following conditions: 30 s at 95°C followed by 40 cycles of 5 s at 95°C and
144 30 s at 60°C. The relative expression of the target gene was represented by the $2^{-\Delta\Delta CT}$ value of
145 the tested sample.

146 **Western blot analysis**

147 The proteins were extracted using Protein Extraction Kit (Promega, WI, United States).
148 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 (M2III-5), 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 0.1 μ M Dexamethasone (Sigma, MO, USA) and incubated for another 12 h before treatment. The viability of hepatocytes was measured

170 by trypan blue exclusion and only cells with viability more than 90% could be used for
171 further study.

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

173 Cultured primary rat hepatocytes were seeded onto 24-well plate at a density of 1×10^5
174 cells per well. 12 h later, the cells were treated with vehicle (0.1% DMSO) or siRNA. For
175 RNA silencing experiment, siRNA specific targeting at mouse FXR sequence AGG TTC CTT
176 TCT ATG TTT ATA TC were designed as follows: sense: 5'- UAU AAA CAU AGA AAG
177 GAA CCU -3'; Anti-sense: 3'- GUU CCU UUC UAU GUU UAU AUC-5' (GenePharma,
178 Shanghai) and transiently transfected to cultured primary rat hepatocytes using Lipofectamine
179 2000 (Invitrogen, US) and incubated for 24 h. After that, 100 μ M Gln were added to the
180 medium for another 24 h. the cells were harvested for western blot analysis.

181 **Dual luciferase reporter assay**

182 FXR expression plasmid was constructed by cloning the FXR gene sequence into
183 pCI-neo mammalian expression vector (Promega). Bsep luciferase reporter vector was
184 constructed by cloning the upstream DNA fragment of promoter region into the luciferase
185 vector pGL4.14 [Lcu2/Hygro] (Promega) (Sang et al. 2008). For vitro transfection study,
186 HepG2 cells were seeded in 24-well plates at a density of 1×10^5 cells/ well in DMEM media
187 supplemented with 10% FBS and cultured for 24h. Then, the cells were cotransfected with
188 FXR expression plasmid, Bsep promoter luciferase reporter vector and the null-Renilla
189 luciferase plasmid as an internal control. After transfection for 24 h, cells were treated with
190 vehicle (0.1% DMSO), 100 μ M CDCA (Sigma, MO, USA) or 10, 40, 70, 100 μ M Gln for 24

191 h. the luciferase activities were measured with a Dual-luciferase Reporter assay System
192 (Promega) according to manufacturer's instructions. The firefly luminescence was
193 normalized to the Renilla luminescence signal, and the ratio of treatment over control served
194 as fold activation.

195 **Statistical analysis**

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

199 **Results**

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

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

210 **Levels of Serum Hepatic Biomarkers in Rats**

211 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 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

233 architecture with only mild bile duct proliferation and slight inflammatory cell infiltration in
234 comparison to SH + NS rats. Thus, consistent with the results of serum biomarkers detection,
235 the histological examination demonstrated that Gln supplementation alleviated BDL-induced
236 liver damage.

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

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

247 **Alternation of bile acids regulatory molecules of obstructive cholestasis involved in Gln** 248 **treatment**

249 To further verify the hepatoprotective effect of Gln on obstructive cholestasis was related
250 to the restoration of bile flow system. Western blot and RT-PCR were used to investigate the
251 alterations of hepatic key molecules including FXR, Bsep, Mrp2, Cypa1, Ntcp and Shp in
252 livers of rats with different treatments. The mRNA and protein levels of FXR, Bsep, Mrp2
253 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

275 cotransfected with FXR expression plasmid and FXR target gene Bsep promoter reporter
276 vector. As demonstrated in Figure 5C, CDCA (100 μ M), a characterized FXR agonist,
277 significantly increased the luciferase activity of FXR reporter gene compared to vehicle group
278 ($P < 0.01$), while Gln (at doses of 10, 40, 70 and 100 μ M) also significantly increased the
279 luciferase activity of FXR at a dose-dependent manner ($P < 0.05$, $P < 0.05$, $P < 0.01$, $P < 0.01$,
280 respectively), which directly demonstrated the positive regulatory role of Gln on FXR
281 activation.

282 Discussion

283 Bile acids (BAs) as the major composition of bile, plays a pivotal role in lipometabolism
284 (Geier et al. 2007; Morgan et al. 2016). The impairment of bile secretion and flow could lead
285 to cholestasis syndrome such as lesions, primary sclerosing cholangitis, hepatocellular and
286 cholangiocellular secretory defects (Matsuzaka et al. 2015). Furthermore, Tarantino et al.
287 have reported that acute drug-induced liver injury (DILI) in patients suffering from
288 non-alcoholic fatty liver disease (NAFLD) could present as a cholestatic form (Tarantino et al.
289 2007), because it's well known that NAFLD patients with biochemical cholestasis have a
290 histological picture of bile damage as Sorrentino et al. suggested (Sorrentino et al. 2005).
291 Previous researches have demonstrated that if the activation of FXR is blocked, liver
292 cholestasis could be aggravated by increasing the endogenous synthesis of BAs while
293 decreasing bile acid efflux (Li and Guo 2015; Trauner et al. 1998). Current studies also
294 reported that knockout of the expression of Bsep could induce the occurrence of cholestasis in
295 rats (Funk et al. 2001). Moreover, the dysregulated expressions of Bsep and Mrp2 contributed

296 to the onset of related cholestatic diseases such as intrahepatic cholestasis of pregnancy (ICP)
297 in adults and Dubin-Johnson syndrome (Cui et al. 2009; Kajihara et al. 1998; Wendum 2010).
298 Donner et al. also declared that zonal downregulation of Bsep in obstructive cholestasis was
299 associated with portal inflammation and is mediated by TNF-alpha and IL-1beta (Donner et al.
300 2007).

301 Aldemir et al. has reported that Gln could reduce the incidence of bacterial translocation
302 and preserve intestinal mucosal integrity in common bile duct ligated rats (Aldemir et al.
303 2003). Xu et al. demonstrated that preconditioning with Gln significantly improved hepatic
304 structure and function by inhibiting reactive oxygen species, inflammation, and hepatocyte
305 apoptosis after ischemia/reperfusion injury in rats with obstructive jaundice (Xu et al. 2014).
306 Alecrim et al. indicated that Gln supplementation had no positive effect on colonic scar
307 strength in rats with extrahepatic biliary obstruction (Alecrim et al. 2015). Thus, it is urgent to
308 deeply reveal the hepatoprotective mechanism underlying Gln on obstructive cholestasis. In
309 the present study, Gln apparently mitigated serum transaminases in OJ-induced intrahepatic
310 cholestasis rats such as ALT, AST, ALP, γ -GTP and bile acids TC, TBIL, DBIL, TBA levels,
311 which were commonly used as crucial criteria for the diagnosis of hepatobiliary disorders
312 (Knight 2005; Lalisang 2012; Scheig 1996). Furthermore, H&E experiment revealed that
313 obstructive cholestasis rats with Gln treatment remarkably alleviated severe necrotic and
314 degenerative changes of hepatobiliary cells, the bile duct proliferation, and the inflammatory
315 cell infiltration, indicating the hepatoprotective effects of Gln involved in obstructive
316 cholestasis.

317 Obstructive cholestasis with Gln treatment remarkably increased Bsep and Mrp2
318 distributions and expressions, resulting in the restoration in bile flow and biliary bile acid
319 output (Chen et al. 2016; Kast et al. 2002). In addition, hepatic uptake of bile acids takes
320 place at the basolateral membrane of hepatocytes and is mediated through Ntcp activation,
321 which mediates Na⁺-dependent uptake of all physiological bile acid in their conjugated form
322 (Stieger 2011). Besides transporters, bile acid synthetic enzymes like Cyp7a1 play important
323 roles in bile acid homeostasis (Fu et al. 2014). In this study, Ntcp and Cyp7a1 were
324 remarkably reduced in obstructive cholestasis with Gln treatment to prevent synthesizing
325 excessive bile acids entering hepatocytes. It has been demonstrated that Bsep and Shp are
326 direct target genes of FXR and could be induced by FXR in livers; Furthermore, Shp has been
327 proved to be the upstream gene of Ntcp and Cyp7a1, which could be induced by FXR to
328 suppress the expressions of Ntcp and Cyp7a1 (Kwong et al. 2015; Li and Guo 2015).
329 Whereas, treating with Gln in BDL rats significantly reduced FXR downstream target genes
330 Cyp7a1 expression via FXR-Shp axis, lead to suppressing bile acid synthesis. Taken together,
331 Gln as the key regulatory factor for BAs may become the effective therapeutic agent against
332 obstructive cholestasis.

333 FXR plays an important role in regulating transporters and enzymes of BAs. Considering
334 the significant effect of Gln on gene expression of Bsep and Mrp, which are the PXR target
335 genes, *in vitro* experiments was performed in cultured primary rat hepatocytes, FXR siRNA
336 was applied to silence FXR expression and the result demonstrated that Mrp2 and Bsep
337 expression regulated by Gln was directly abrogated in hepatocytes. Thus, we proposed that

338 Gln mediated activation of FXR pathway against BDL-induced cholestasis. Moreover,
339 previous study has demonstrated that Bsep was predominantly regulated by FXR (Song et al.
340 2013). In this study, we speculated that Bsep upregulation in BDL rats with Gln treatment
341 might be due to FXR agonism. The dual luciferase reporter assay confirmed that Gln could
342 stimulate FXR activation dose-dependently in HepG2 cells, which was similar to the effect of
343 FXR agonist CDCA. In the past decade, FXR has been regarded as a potential therapeutic
344 target in the treatment of cholestatic liver disorders. Therefore, we proposed that the role of
345 Gln in FXR agonism may also contribute to its anticholestatic effect.

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

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359 **Acknowledgement**

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

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362 **Conflict of interest and disclosure**

363 The authors declare that no competing interest exists.

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

Gene	Forward primer(5'-3')	Reverse primer (5'-3')	Length
FXR	GCCTAGATGATGAGTCCAGC	CCAAGGTCCGATCTACCTCTA	146
Bsep	ATGTAGCAGACACGCAGCTT	CACCCTGGCACATCAAGTCT	178
Mrp2	CTTTGGTCTGTTGCCACTGC	TCGCAATTGTCAGCTGTCCT	163
Shp	GCACGATCCTCTTCAACCCA	AGGCTCACAAGGACCCAATG	136
Ntcp	TTGCGCCATAGGGATCTTCC	ATGTGGTGGTAGCTCGGTTG	112
Cyp7a1	GTGAGATGGCCGAGTAGTGG	CATGCCTAATGTCGGCACA	151
β-actin	GCGACAGCAGTTGGTTGG	ACGCGACCATCCTCCTCTTAG	200

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Table 2 The results of liver histopathological examination grade

Group	Inflammatory cell infiltration	Hepatocyte necrosis	Bile duct proliferation	Liver fibrosis
SH+NS	—	—	—	—
BDL+NS	+++	++	+++	+++
BDL+Gln	+	—	+	—
BDL+UDCA	+	—	+	—

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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 $\pm$ 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 $\times$ 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 $\times$ magnification; bar = 50 μ m). (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 $\pm$ 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 $\pm$ 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 $\pm$ SD. $*P < 0.05$,

601 $**P < 0.01$ vs. vehicle; $^{##}P < 0.01$ vs. Gln treatment alone.

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Fig1.TIF

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Fig2. TIF

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Fig3. TIF

117x81mm (600 x 600 DPI)

Fig4.TIF

143x121mm (600 x 600 DPI)

Fig5.TIF

68x42mm (600 x 600 DPI)