Original Article

Matrine improves 17α-ethinyl estradiol-induced acute cholestasis in rats

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Aim: To explore the effects of matrine (MT) on acute intrahepatic cholestasis induced by 17α-ethinyl estradiol (EE) in rats.

Methods: Acute intrahepatic cholestasis in rats were induced by EE, and the effects of MT on acute intrahepatic cholestasis were explored and compared with ursodeoxycholic acid (UDCA) by serum biochemical determination and bile excretion experiments.

Results: The serum biochemical and bile biochemical results indicated that MT and UDCA had notable hepatoprotective effects by counteracting cholestasis induced by EE. The bile flow and the bile excretion of glycocholic acid (GC, a substrate of bile salt export pump [Bsep]), ketoprofen glucuronide (KPG) and rhodamine 123 (Rh123, a substrate of multidrug resistance protein 1 [MDR1]) decreased by EE, were significantly improved after administration of MT.

Conclusion: MT exhibited potential protection against EE-induced acute intrahepatic cholestasis.

Key words: bile excretion, ethinyl estradiol, intrahepatic cholestasis, matrine

INTRODUCTION

Cholestasis is the clinical, biochemical and histological manifestations of defective bile transport from the liver to the intestine. The enterohepatic circulation of bile acids enables the absorption of fats and fat-soluble vitamins from the intestine and allows the elimination of cholesterol, toxins and metabolic by-products such as bilirubin from the liver. Cholestasis is one of components in many liver diseases, such as cholelithiasis, intrahepatic cholestasis of pregnancy (ICP), primary biliary cirrhosis (PBC) and primary sclerosing cholangitis.1

Although there are many treatments of cholestatic liver diseases and their associated complications, most chronic cholestatic symptoms can progress towards biliary cirrhosis and hepatocellular insufficiency. The ultimate treatment is liver transplantation.1 Until now, ursodeoxycholic acid (UDCA) has been the only US Food and Drug Administration-approved treatment for PBC, and also the current mainstay for intrahepatic cholestasis therapy. It is increasingly being used to treat all cholestatic conditions because of improving serum liver chemistries.2–5 Unfortunately, multiple clinical trials demonstrated UDCA treatment cannot prolong the survival time of PBC patients.6 Given these circumstances, developing safe and effective therapies for the treatment of intrahepatic cholestasis is urgent.

In the clinical situation, intrahepatic cholestasis has a range of causes including drug or xenobiotic toxicity, viral or bacterial infection, as a complication of pregnancy and liver transplantation.7 17α-ethinyl estradiol (EE), a synthetic estrogen, induces intrahepatic cholestasis which is characterized by reducing the liver’s capacity to excrete bile salts and organic solutes.7,8 EE was commonly used to study the mechanisms of cholestasis. Traditional Chinese medicines have proved to be effective in the treatment of liver disorders, including cholestasis. Matrine (MT) (Fig. 1), as an alkaloid monomer extracted from a traditional Chinese herb Radix Sophorae Flavescentis, has exhibited hepatoprotective,9 anti-inflammatory10 and immunosuppressive effects.11 MT injection has been extensively used clinically in Asia for the treatment of...
liver diseases. However, there are few multiple clinical trials and experimental studies on the effectiveness of MT in intrahepatic cholestasis. The known hepatoprotective effects of MT, warrants its investigation as potential treatment for EE-induced intrahepatic cholestasis in rats.

The etiology of cholestasis is varied, but the predominant mechanism in many forms of intrahepatic cholestasis is altered canalicular transport. The canalicular membrane is enriched in adenosine triphosphate-binding cassette (ABC) transporters that function as export pumps for bile acids and a variety of organic solutes. Multiple molecular defects in disorders of canalicular transport regulation have been identified. The fact that alterations in these canalicular transports give rise to severe cholestatic liver disease reveals their importance in maintaining bile flow and healthy liver function.12 In this study, the serum biochemical and bile biochemical markers of cholestasis were investigated. Meanwhile, to estimate the function of canalicular transport, we utilized ketoprofen (KP) as a model drug to study the bile excretion of its glucuronide conjugate metabolite, ketoprofen glucuronide (KPG). The primary route of elimination of KP is glucuronidation, once KP has undergone glucuronidation, the resulting KPG is mainly excreted through the bile.13,14 The transport of glucuronide conjugates into the extracellular space and is mediated by members of the family of multidrug resistance-associated protein (Mrp).12,15 To estimate whether the effect of MT affected canalicular hydrophobic and cationic transporter, the transport activity of the model substrates of multidrug resistance protein 1 (Mdr1), rhodamine 123 (Rh123), was evaluated in vivo. UDCA was also used as the clinical available comparator in this study.

**METHODS**

**Materials and preparation of solutions**

α-ETHINYL ESTRADIOL, KP, KPG and Rh123 were purchased from Sigma-Aldrich (St Louis, MO, USA). EE (5 mg/mL) was dissolved in propylene glycol. MT was purchased from Xi’an Fujie Biotech (Xi’an, China). The purity of MT was 98%. MT (2 mg/mL) was dissolved in normal saline. Ursofalk (250 mg UDCA per capsule, Dr Falk Pharma, Freiburg, Germany) suspended in normal saline at a final concentration of 10 mg/mL. KP (8 mg/mL) was dissolved in saline by adding adequate 2 M sodium hydroxide solution and then adjusting the pH to 7–8 with 0.5 M hydrochloric acid. Rh123 for i.v. administration was dissolved in 10 mL of a mixture containing 5% (v/v) ethanol/normal saline, at a final concentration of 0.2 mg/mL. All other reagents were of the highest analytical grade and used as supplied.

**Animals**

Male Sprague–Dawley rats (180–210 g) were obtained from Southeast University (Nanjing, China), and were housed in plastic cages on aspen-chip bedding under conditions of controlled temperature (18–21°C) and humidity (55 ± 5%) with a 12:12 h light : dark cycle. Rats were allowed free access to rat chow and tap water. All studies were approved by the Animal Study Committee of China Pharmaceutical University.

**Experimental protocol**

Rats were randomly assigned to four experimental groups (12 rats/group). Rats in three of the groups were administered either MT (30 mg/kg i.p. every 12 h), UDCA (100 mg/kg p.o. once a day) for 5 consecutive days. EE (5 mg/kg s.c. once a day) was daily co-administered with the test compounds (MT or UDCA) for 5 consecutive days. The remaining two groups were used as either non-treated controls or EE-only controls.

One day after the administration of the last dose of EE (i.e. on the sixth day), bile excretion experiments were conducted, as described below, to measure the bile flow, the bile excretion of bilirubin and glycocholic acid (GC) and the accumulative bile excretion of either KPG or Rh123. Following this, blood samples were collected from the femoral artery for biochemical determination.

**Bile excretion experiments**

Bile excretion experiments started at approximately 09.00 hours to minimize influence of circadian varia-
tions. The rats were anesthetized with urethane (1 g/kg i.p.) and maintained under this condition throughout the experiment. The bile duct was cannulated with PE10 polyethylene tubing (inner diameter, 0.28 mm; outer diameter, 0.61 mm; Becton Dickinson, Franklin Lakes, NJ, USA). Bile flow was determined by gravimetry, assuming a density of the bile of 1.0 g/mL. The bile concentration of bilirubin was determined using a commercially available clinical test kit with a chemistry analyzer system (Synchron Clinical System Lx 20; Beckman Coulter, Fullerton, CA, USA). The concentration of GC was determined using a commercially available clinical test kit with a photoelectricity γ-radioimmunity analyzer system (GC-911; Keda, Shanghai, China).

Excretion study of KPG (n = 6)
After the operation, KP (20 mg/kg) was injected through the femoral vein. Bile specimens were collected in fractions at 0–30, 30–60, 60–120, and 120–240 min into Eppendorf tubes containing 100 μL of 1 mM potassium dihydrogen phosphate.13 The sample tubes were kept on ice during bile collection. The quantity of KPG in bile was analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) immediately after collection as described by us previously.13

Excretion study of Rh123 (n = 6)
After the operation, Rh123 (0.2 mg/kg) was injected through the femoral vein. Bile specimens were collected in fractions at 0–15, 15–30, 30–45, 45–60, 60–90, 90–120, 120–150 and 150–180 min into Eppendorf tubes. The sample tubes were kept on ice and protected from light during bile collection. The quantity of Rh123 in bile was analyzed by a spectrofluorophotometer (RF-540; Shimadzu, Kyoto, Japan), using an excitation wavelength of 485 nm and an emission wavelength of 527 nm.

Biochemical determination
Serum total bilirubin (T. Bil), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) levels were determined using a commercially available clinical test kit with a chemistry analyzer system (Synchron Clinical System Lx 20; Beckman Coulter).

Statistical analysis
All measurement data are expressed as mean ± standard deviation. One-way ANOVA with least significant difference (LSD) and Dunnett’s for post-hoc analysis was used to compare results between different groups. Statistical significance was defined as P < 0.05.

RESULTS
In rats that received only EE, serum markers for cholestasis were significantly elevated: GC and ALP increased approximately 7- and 2.5-fold, respectively (Fig. 2a,b). In contrast, EE treatment failed to modify serum T. Bil levels (data not shown). In line with the serum biochemical findings, the bile excretion of bilirubin (Bil) remained and GC significantly decreased (Fig. 3a,b). The bile flow (0–60 min) was significantly suppressed in the EE-only treated group compared with the non-treated control group (2.01 ± 0.527 vs 3.63 ± 0.454 mL/kg, P < 0.01. Fig. 4). Similarly, the accumulative bile excretion of KPG (0–4 h) and Rh123 (0–3 h) were significantly inhibited in EE-treated rats (43.55% ± 6.338% vs 89.69% ± 8.78%, P < 0.01; 8.36 ± 2.027 vs 12.20 ± 1.648 μg/kg, P < 0.01; respectively) (in Figs 5,6a,b).

Treatment with MT exhibited protective effects against EE-induced liver damage as demonstrated by a significant antagonizing the EE-induced elevation of serum GC and ALP (Fig. 2a,b). The bile excretion of GC inhibited by EE was recovered to a normal level (Fig. 3b). The bile flow (Fig. 4), accumulative bile excretion of KPG and Rh123 (Figs 5,6) were significantly higher in MT treated rats compared to those administered with EE only.

However, UDCA was different in antagonizing the elevation of ALP and the decrease of KPG bile excretion caused by EE compared with MT (Figs 2b,5).

DISCUSSION
In this study, rats exposed to EE had significant elevations in serum GC and ALP. In rats receiving MT or UDCA, the biochemical indicators of cholestasis associated with EE were significantly ameliorated. These results suggested that both MT and UDCA treatment have protective effects against EE-induced cholestasis.

Estrogens are well known to cause reversible intrahepatic cholestasis in susceptible women during pregnancy, administration of oral contraceptives, and postmenopausal replacement therapy. Cholestasis induced by administration of estrogen to rodents, is a model commonly used to gain mechanistic and therapeutic insights in this condition. This cholestasis has been linked to a reduction of activity and/or expression or alteration of distribution of several transporters,
including: (i) the bile salt export pump (Bsep);\textsuperscript{16,17} (ii) Mdr1a/1b;\textsuperscript{18–20} (iii) Mrp;\textsuperscript{18} and (iv) the Na\textsuperscript{+}/taurocholate co-transporting polypeptide (Ntcp).\textsuperscript{16,21}

It is suggested that fine tuning of the response of the canalicular membrane transports may well be a novel and potential means to treat intrahepatic cholestasis, a condition where effective therapeutics currently do not exist.\textsuperscript{12} The function of transporters located in the hepatocyte canalicular membrane can be evaluated by bile excretion experiments \textit{in vivo}. In the current study, we analyzed the potential protective effects of MT on EE-induced acute intrahepatic cholestasis by evaluating the improvement in the bile excretion of GC (one marker of cholestasis and a substrate of Bsep), KPG (glucuronide conjugate metabolite) and Rh123 (Mdr1 substrate) \textit{in vivo}, accompanied by the liver biochemical assessment.

The suppression of bile flow and accumulated bile excretion of GC, KPG and Rh123 by EE were significantly ameliorated in MT administration rats. The data indicated the improvement of transport of Bsep sub-

![Figure 2](image1.png)

\textbf{Figure 2} Effects of matrine (MT) and ursodeoxycholic acid (UDCA) on the level of serum glycocholic acid (GC) (a), alkaline phosphatase (ALP) (b) and alanine aminotransferase (ALT) (c) in 17α-ethyl estradiol (EE) administered male rats. Bar 1, non-treated control; bar 2, EE-only control; bar 3, MT + EE; bar 4, UDCA + EE. Data are mean± standard deviation (\( n = 12 \)) A 5-day treatment with MT exhibited protective effects on the EE-induced elevation of serum GC, ALP. *\( P < 0.05 \), compared with the EE-only control.

![Figure 3](image2.png)

\textbf{Figure 3} The bile excretion of bilirubin (Bil) and glycocholic acid (GC) in different treatment groups. Bar 1, non-treated control; bar 2, EE-only control; bar 3, matrine (MT) + 17α-ethyl estradiol (EE); bar 4, ursodeoxycholic acid (UDCA) + EE. Data are mean± standard deviation (\( n = 12 \)). EE treatment did not modify the bile excretion of Bil. The bile excretion of GC inhibited by EE was recovered to normal level in MT or UDCA treatment rats. *\( P < 0.05 \) compared with the EE-only control.
strate, glucuronide conjugates and Mdr1 substrate in MT administration rats. As mentioned above, multiple hepatocyte membrane transporters associated with intrahepatic cholestasis have been altered in EE-reduced cholestasis. Activation/inactivation of canalicular transporters was proposed as a potential explanation for the modulation of canalicular secretory function. MT beneficial protection effects on EE-induced cholestasis may be based on the improvement of the canalicular membrane transporters function impaired by the EE. The results of the biliary excretion experiments are in line with the results obtained from the liver biochemics. This striking profile demonstrates that MT affords marked hepatoprotection in this established model of intrahepatic cholestasis.

Ursodeoxycholic acid, used in the treatment of several cholestatic liver diseases, has beneficial effects on patients with ICP by improving pruritus, hypercholanemia, and other biochemical parameters and by prevent-

Figure 4 Effects of matrine (MT) and ursodeoxycholic acid (UDCA) on the bile flow over 60 min in EE administered male rats. Bar 1, non-treated control; bar 2, 17α-ethinyl estradiol (EE)-only control; bar 3, MT + EE; bar 4, UDCA + EE. Data are mean± standard deviation (n = 12). The bile flow suppressed by EE was significantly ameliorated by a 5-day treatment with MT and UDCA. **P < 0.01 compared with the EE-only control.

Figure 5 Accumulative bile excretion of ketoprofen glucuronide (KPG) over 240 min in different treatment groups (n = 6). Error bars are omitted for clarity. The accumulative bile excretion of KPG inhibited by 17α-ethinyl estradiol (EE) was significantly ameliorated by a 5-day treatment with matrine (MT). **P < 0.01 compared with the EE-only control.

Figure 6 Effects of matrine (MT) and ursodeoxycholic acid (UDCA) on the accumulative bile excretion of rhodamine 123 (Rh123) over 180 min following the i.v. injection of 0.2 mg/kg Rh123 to 17α-ethinyl estradiol (EE)-treated and control rats (n = 6). (a) Error bars and significance marks are omitted for clarity. (b) Bar 1, non-treated control; bar 2, EE-only control; bar 3, MT + EE; bar 4, UDCA + EE. Data are mean ± standard deviation. The accumulative bile excretion of Rh123 inhibited by EE was significantly ameliorated by a 5-day treatment with MT and UDCA. **P < 0.01 compared with the EE-only control.
ing prematurity in these pregnancies.\textsuperscript{3,5} Its beneficial effect on reversion of EE-induced cholestasis is based on the improvement of the biliary secretory function impaired by the EE. And being a polar bile acid, UDCA may act by decreasing the hydrophobicity and toxicity of the bile. In this study, UDCA failed to improve the bile excretion of KPG that may be partly due to its inhibition of the glucuronoyltransferase.\textsuperscript{2,2}

Similarly, the effect of MT on acute cholestasis induced by \( \alpha \)-naphthylisothiocyanate (ANIT) in rats was investigated recently, and the results showed that MT exhibited notable protective effects against ANIT-induced liver damage including the protection of hepatocytes as well as cholangiocytes.\textsuperscript{2,3} The mechanism/s of action by which MT exerts its hepatoprotective effect is unclear. However, given that it is effective in counteracting the decrease of bile flow and bile excretion of GC, KPG and Rh123 caused by EE, the modulation of bile secretory function is a potential mechanism that warrants further investigation. Nevertheless, whether the improved bile secretory function is the predominant mechanism or the result of its hepatocyte protective effects against EE exposure has not directly tested.

**ACKNOWLEDGMENTS**

THE RESEARCH WAS founded by National Natural Science Foundation of China (no. 30472060).

**REFERENCES**