Original Article

Protective effect of glycyrrhizin and matrine on acute vanishing bile duct syndrome induced by alpha-naphthylisothiocyanate in rats

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Aim: To explore the effects of glycyrrhizin (GL) and matrine (MT) on acute vanishing bile duct syndromes (AVBDS) in rats.

Methods: AVBDS in rats were induced by alpha-naphthylisothiocyanate (ANIT), and the effects of GL and MT on AVBDS were explored and compared with dexamethasone (DEX) by serology determination, histological assessment of liver damage and bile excretion experiments.

Results: The protection of DEX pre-treatment was directed toward cholangiocytes rather than hepatocytes. Rats remediably treated with DEX 3 h after ANIT were not resistant to ANIT toxicity. Notably, remedial treatment with DEX 12 h after ANIT enhanced ANIT toxicity. However, GL and MT attenuated both bile duct and hepatocyte damage induced by ANIT in the initial phase of impairment.

Conclusion: The nature products (GL and MT) exhibited better protection against ANIT-induced AVBDS than DEX. In the initial phase of impairment of AVBDS, the protection of GL and MT may be due partially to modifying the metabolism and excretion of ANIT and to their anti-inflammatory effects.

Key words: acute vanishing bile duct syndrome, alpha-naphthylisothiocyanate, dexamethasone, glycyrrhizin, matrine

INTRODUCTION

ACUTE VANISHING BILE duct syndrome (AVBDS) refers to a series of acute cholestatic liver diseases characterized by the destruction and loss of the intrahepatic biliary tree, occasionally accompanied by a mild degree of hepatocyte damage.1,2 The possible etiologies include drug toxic effect, virus or bacterial infection and complication of liver transplantation.1 The pathogenesis of AVBDS is not fully elucidated, but is probably a result of an immune-inflamm-mediated injury. This is suggested by eosinophilia, a history of allergy, coexistent Stevens–Johnson syndrome or other forms of skin rash, by a shortening of the latent period with repeated exposure, and evidence of lymphocyte sensitization and macrophage inhibition.3 At present, glucocorticosteroids are the main remedy for AVBDS induced by inflammation or medicine, but the effect is limited4 and glucocorticosteroids have many serious side-effects, which makes practitioners prudent in using them. Most AVBDS can progress towards biliary cirrhosis and hepatocellular insufficiency, which may require liver transplantation.5

Alpha-naphthylisothiocyanate (ANIT) is known to cause cholestasis, which is characterized by severe interlobular ducts epithelial necrosis, destruction of the interlobular ducts and a pronounced neutrophil infiltration.6,7 The inflammatory infiltration plays an important role in the amplification of ANIT-induced cholestasis.6,7 Cholestasis caused by ANIT resembles AVBDS caused by drug and chemical compound toxic effect and inflammation histologically and symptomatically.

Traditional Chinese medicine has been proven to be efficient in the remedy of liver trouble, including cholestasis. Glycyrrhizin (GL) has been clinically used for the treatment of chronic hepatitis B in China and Japan.8 After oral administration or i.v. injection in humans and in experimental animals, GL is hydrolyzed by glucuronidase of intestinal bacteria to its active principle aglycone, 18β-glycyrrhetinic acid (GA). Both GL and GA have been demonstrated to possess several
beneficial pharmacological effects, such as anti-inflammatory,9,10 antihepatotoxicity11,12 and antihepatitis effects.8 Matrine (MT), as a component of prescriptions of traditional Chinese medicine, has exhibited anti-inflammatory,13 antihepatotoxic14 and immunosuppressive effects.15

In this study, the AVBDS in rats was induced with ANIT, and the effects of GL and MT on AVBDS were explored to reveal their possible mechanism of action. The effects of these natural products were compared with those of dexamethasone (DEX). It is hoped that this study will lead to more effective therapies for AVBDS.

METHODS

Materials

GL AND MT were purchased from Xi’an Fujie Biotech Co, Ltd (Xi’an, China). GL 10 mg/mL was dissolved in absolute ethanol then diluted in normal saline. MT (2 mg/mL) was dissolved in normal saline. DEX (dexamethasone sodium phosphate injection, 5 mg/mL) was purchased from Sangdong Lukang Pharmaceutical Group Co, Ltd (Jinan, China). ANIT 10 mg/mL was dissolved in peanut oil. Ketoprofen (KP) was obtained from Sigma (St. Louis, MO, USA). KP (8 mg/mL) was dissolved in saline by adding adequate 2 mol/L sodium hydroxide solution and then adjusting the pH to 7–8 with 0.5 mol/L hydrochloric acid. Ketoprofen glucuronide (KPG) was isolated from human urine and purified by semipreparative high-performance liquid chromatography (HPLC) and used to prepare standards for calibration curves. The identity of the isolated material was confirmed by mass spectrometry (fast atom bombardment) and by alkaline hydrolysis to KP.16 Acetonitrile was HPLC grade; tetrabutylammonium bromide and potassium dihydrogen phosphate were high purified grade; all other reagents were the highest purity commercially available.

Animals

Male Sprague–Dawley rats weighing 190 g–240 g were obtained from South-east University (Nanjing, China), and were housed in plastic cages on aspen-chip bedding in temperature controlled conditions (18–21°C) and humidity (55 ± 5%) with a 12-h light/12-h dark cycle. Rats were allowed free access to rat food and tap water.

Experimental protocol

For the cases of pre-treatment, rats in different groups (four in each group) were treated with GL (100 mg/kg b.w., q12 h, i.p.), MT (10 mg/kg b.w., q12 h, i.p.), or DEX (10 mg/kg b.w., q12 h, i.p.) for two consecutive days, respectively. ANIT (50 mg/kg b.w., i.g.) was given at 30 min after the second administration of the medicines (GL, MT or DEX). In the cases of remedial treatment, rats in different groups (four in each group) were administered with ANIT 3 h or 12 h prior to the first treatment of GL, MT or DEX. Dosage and dosing interval were similar to the cases of pre-treatment. In overall cases, 12 h after the last dose of the medicines (GL, MT, or DEX), just 36 h after ANIT administration, bile excretion experiments were carried out to measure the bile flow and the accumulative bile excretion of KPG. Blood samples were collected from the femoral artery, and livers were removed from the animals immediately after sacrifice. Normal controls and ANIT-alone controls were conducted, respectively.

Serology determination

Serum total bilirubin (Tbil), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (γ-GT) levels were determined using a commercially available clinical test kit with a chemistry analyzer system (Beckmax coulter synchron clinical system Lx 20).

Histological assessment of liver damage

Liver portions from the experimental animals were excised and fixed in 10% neutral formalin. Three or four paraffin sections (4–5 µm thick) per liver were prepared and stained with hematoxylin–eosin. Section preparation and assessment was conducted by professional pathologists (AiFeng Zhang et al., Department of Pathology, South-east University, China).

Bile excretion experiments

The rats were anesthetized with urethane (1 g/kg, i.p.). The bile duct was cannulated with PE10 polyethylene tubing (inner diameter, 0.28 mm; outer diameter, 0.61 mm; Becton, Dickinson and Co., Franklin Lakes, NJ, USA). After the operation, KP (20 mg/kg b.w.) was injected i.v. via the jugular vein. Bile specimens were collected into preweighed Eppendorf tubes containing 100 µL 1 mol/L potassium dihydrogen phosphate at 0–30, 30–60, 60–90, and 90–120 min. The sampling tubes were kept on ice during sampling. For 20 µL bile samples, 80 µL mobile phase and 20 µL internal standard solution (5 µg/mL naproxen dissolved in methanol) were added, and 20 µL mixture was injected for HPLC analysis.

The HPLC system consisted of a Model LC-10ATvp pump, a Model SPD-10 A UV detector, and a Model SCL-
10Avp system controller (Shimadzu, Japan). The chromatographic separation was performed using an ODS analytical column (200 × 4.6 mm I.D. 5 µm). The mobile phase consisted of acetonitrile and 0.05 mol/L phosphate buffer (pH 5.6) including 5 mmol/L tetra-butylammonium bromide (35:65), the solution was filtered using 0.45 mm nylon membrane prior to use and delivered at a flow rate of 1 mL/min, the detector wavelength was 232 nm, and the oven temperature was 30°C.

Statistical analysis
Statistical evaluation of the data was performed by SPSS 10.0. All results were expressed as mean ± standard deviation (SD) of the mean. Analysis of variance (one-way ANOVA) with least significant difference (LSD) and Dunnett’s for post-hoc analysis was used to compare results between different groups. A P-value of less than 0.05 was considered statistically significant.

RESULTS
ANIT-induced AVBDS
In ANIT- Alone Rats, ANIT (50 mg/kg i.g.) produced profound hepatotoxicity at 36 h after administration, as evidenced by increases in serum marker for hepatocyte injury: more than 12-fold increases in ALT activity. Serum total bilirubin levels, ALP and γ-GT activity are markers for AVBDS. ANIT alone significantly increased serum total bilirubin levels (about fivefold), ALP (approx twofold) and γ-GT activity (about fourfold). (Fig. 1). In addition, rats treated with ANIT alone showed significant loss of body weight (Table 1).

Histologically, ANIT-induced liver injury showed sinusoid congestion, inflammatory cell infiltration and a severe demolishment or loss of the interlobular ducts, associated with a mild degree of hepatocyte damage (Table 2 and Fig. 2).

Within two hours, the bile flow was extremely suppressed in ANIT-intoxicated rats compared to the normal control group (2.49 ± 1.50 mL/kg b.w. vs. 9.36 ± 0.495 mL/kg b.w., P < 0.01) and the bile excretion of KPG was nearly completely inhibited in ANIT-intoxicated rats (3.42% ± 2.17% vs. 77% ± 6.9%, P < 0.01) (in Figs 2,3).

Effects of GL, MT and DEX on ANIT-induced AVBDS
Pre-treatment
Pre-treatment with GL or MT exhibited protective effects against ANIT-induced liver damage, as demonstrated by the significant reduction of the elevated serum levels of total bilirubin, ALT, ALP and γ-GT, and the serology in pre-treatment groups resembled that in the control (Fig. 1). In addition, pre-treatment with MT antagonized the loss of body weight caused by ANIT (Table 1). Although DEX pre-treatment significantly reduced the elevated serum levels of total bilirubin, ALP and γ-GT, it did not reduce the elevated serum levels of ALT (Fig. 1).

As shown in Figure 2 and Table 2, histological evaluations provided basic support for the protective effects of pre-treatment with GL or MT on ANIT-induced AVBDS. Consistent with serology, the pathological changes in hepatocellular degeneration and necrosis and inflammatory cell infiltration did not occur, and only mild bile duct injury was observed in rats also receiving pre-treatment with GL or MT. However, pre-treatment with DEX did not reduce hepatocyte necrosis caused by ANIT. Thus, the protective effect of pre-treatment with DEX is directed towards cholangiocytes rather than hepatocytes, consistent with the serology.

Notably, the observations of bile excretion experiments provided further support for the results obtained from serological and histological findings. The accumulative bile flow extremely suppressed by ANIT was significantly ameliorated in GL, MT or DEX pre-treatment groups (Fig. 3). The accumulative bile excretion of KPG nearly completely inhibited by ANIT was significantly ameliorated in GL, MT or DEX pre-treatment groups (Fig. 4). After 20 mg/kg of KP was administrated, the accumulative bile excretion of KPG in 30, 60, 90, and 120min is shown in Figure 5.

Remedial treatment
For rats with remedial treatment of GL or MT 3 h after ANIT administration, the serology and morphological parameters of liver injury were reduced significantly. (Figs 1,2 and Table 2). Only a mild degree of hepatocyte degeneration, mild to moderate interlobular duct epithelial degeneration and necrosis and inflammatory cell infiltration were observed, and the bile excretion inhibited by ANIT was significantly ameliorated. However, remedial treatment with GL or MT 12 h after ANIT exhibited few protective effects against ANIT-induced liver damage, and rats with remedial treatment of DEX 3 h after ANIT were not resistant to ANIT toxicity. The elevated serum Tbil, ALT, ALP and γ-GT levels and inhibition of bile excretion induced by ANIT were sustained (Figs 1,3,4) and extensive morphological changes, including demolishment or loss of the interlobular ducts and a mild to moderate degree of hepatocyte damage were observed (Fig. 2 and Table 2).
Figure 1 Effects of pre-treatment and remedial treatment with GL, MT or DEX on the level of serum bilirubin (a), ALT (b), ALP (c) and γ-GT (d) 38 h after ANIT administration in male rats. (Data are mean ± sampling distribution of the mean [S.D.M.] of four rats.) *P < 0.05, **P < 0.01 compared with the control animals, #P < 0.05, ##P < 0.01 compared with the rats receiving ANIT alone.
Figure 2 Effects of pre-treatment and remedial treatment with GL, MT or DEX on morphological changes of rat liver obtained 38 h after ANIT administration (HE strained 200× magnification).
DISCUSSION

The typical hallmark of vanishing bile duct syndrome is a decrease in the number of interlobular ducts, termed paucity of interlobular ducts, or ductopenia. Ductopenia has classically been defined as a severe reduction of at least half of the interlobular ducts. It is clear, however, that less severe degrees of ductopenia between 0% and 50% may also occur in AVBDS. In most AVBDS instances, about a 50% destruction or loss of the interlobular ducts was observed. The ductopenia preferentially affects the small ramifications of the intrahepatic biliary tree, the interlobular ducts of about 0.03 mm in diameter or less, which are smaller than those usually destroyed in primary biliary cirrhosis. In this study, the liver of rats exposed to ANIT alone revealed diffuse alteration of interlobular ducts. The pathological change is characterized by a severe demolishment or loss of at least half of the interlobular ducts, which are lined by 4–15 cholangiocytes and have a basement membrane, accompanied by a mild degree of hepatocyte damage. These morphological changes were accompanied by significant inhibition of bile excretion.

Table 1 The body weight before and 36 h after ANIT administration in control, ANIT, pre-treatment with GL, MT or DEX rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight (g)</th>
<th>Before ANIT</th>
<th>36 h after ANIT</th>
<th>Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>206.2 ± 11.01</td>
<td>222.8 ± 10.68</td>
<td>16.6 ± 2.71</td>
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<tr>
<td>ANIT</td>
<td>215.4 ± 13.16</td>
<td>191.3 ± 9.04</td>
<td>-23.7 ± 5.95**</td>
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<tr>
<td>Pre-treatment</td>
<td></td>
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<tr>
<td>GL</td>
<td>225.1 ± 12.07</td>
<td>205.4 ± 12.12</td>
<td>-19.3 ± 4.95**</td>
<td></td>
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<tr>
<td>MT</td>
<td>212.6 ± 12.12</td>
<td>225.3 ± 11.60</td>
<td>12.5 ± 3.11****</td>
<td></td>
</tr>
<tr>
<td>DEX</td>
<td>214.6 ± 12.30</td>
<td>199.2 ± 8.10</td>
<td>-15.4 ± 7.83***</td>
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</tbody>
</table>

Data are mean of four rats. **P < 0.01 compared with control animals, ***P < 0.05, ****P < 0.01 compared with rats receiving ANIT alone.

Notably, remedial treatment with DEX 12 h after ANIT-enhanced ANIT toxicity was demonstrated by the elevated ALT and a severe loss of majority interlobular ducts, accompanied with focused hepatocyte necrosis. (Figs 1b, Table 2). Remedial treatment with GL, MT or DEX did not antagonize the decrease in body weight induced by ANIT (data not shown).

Table 2 Effects of pre-treatment and remedial treatment with GL, MT or DEX on morphological changes of rat liver obtained 38 h after ANIT administration

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hepatocellular degeneration</th>
<th>Hepatocellular necrosis</th>
<th>Sinusoid congestion</th>
<th>Inflammatory cell infiltration</th>
<th>Bile duct epithelial damage</th>
<th>Bile duct demolishment or loss</th>
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<tbody>
<tr>
<td>Control</td>
<td>-</td>
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<tr>
<td>ANIT alone</td>
<td>++</td>
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<td>Pre-treatment</td>
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<td>GL</td>
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<td>Remedial treatment</td>
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<td>3 h after ANIT</td>
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<td>GL</td>
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<td>DEX</td>
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<td>12 h after ANIT</td>
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<tr>
<td>GL</td>
<td>++</td>
<td>+</td>
<td>++</td>
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<td>MT</td>
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In histological assessment of bile duct demolishment or loss: 20 portal areas in each rat liver were overviewed and the number of bile ducts was counted. The ratio: \( r = (A - n)/A \): the mean number of bile ducts in control; \( n \): the number of bile ducts in each experimental rat; \( R \): expressed as the mean of \( "r" \) of four specimens in each group. +++ R: >80% demolishment or loss of the interlobular ducts in 20 portal areas; ++ R: 50%~80%; + R: 20%~50%; ± R: 10%~20%; ± R: <10%.

Data expressed as the mean of four specimens for each group. + mild; ++ moderate; +++ marked; – negative; ± less than two livers show a mild change.
and elevation in serum total bilirubin, ALT, ALP and γ-GT. Cholestasis caused by ANIT therefore resembles AVBDS histologically and symptomatically.

Besides histological assessment and serology determination, pharmacokinetic study is a valuable tool to evaluate the hepatocyte and bile duct functions. Glucuronidation, a phase II metabolic pathway, is an important metabolic process of xenobiotics, endobiotics and/or their phase €ni metabolites to more water-soluble compounds, which can be readily excreted from the body. A non-steroidal anti-inflammatory drug, KP, is mainly eliminated by glucuronidation. In addition, KPG is mainly excreted via the bile.16 In the present study, we selected KP as a model drug to study liver functions on the glucuronidation and bile excretion of glucuronide conjugate metabolite. Thus, the impairment of bile flow indicates directly the obstruction of the bile duct, while the bile excretion of KPG, at least in part, reflects liver functions of metabolism and excretion.

ANIT is a hepatotoxicant that causes acute cholestatic hepatitis with a severe demolishment or loss of the interlobular ducts and a mild degree of hepatocyte damage. ANIT is thought to be bioactivated in the liver by cytochrome P450s, and the bioactivated ANIT is detoxified in hepatocytes in conjugation with glutathione catalyzed by glutathione S-transferases. ANIT-glutathione complexes are transported into bile, but they are unstable and rapidly dissociate. The released ANIT then damages bile duct epithelial cells, causing inflammatory cell infiltration and cholangiolitis that leads to intrahepatic biliary obstruction.5–7 Thus, ANIT-induced hepatotoxicity involves metabolism, transport, cholestasis, inflammation, and hepatocellular damage.
In this study, the observed diverse effects between pre-treatment and remedial treatment with GL, MT or DEX on ANIT-induced AVBDS are interesting. For rats with pre-treatment of GL, MT or DEX, the serology and morphological parameters of liver injury caused by ANIT were reduced significantly. The bile flow and the accumulative bile excretion of KPG extremely suppressed by ANIT was significantly ameliorated in GL, MT and DEX pre-treatment groups. These results implied that GL, MT or DEX pre-treatment led to protective effects on ANIT-induced AVBDS. Remedial treatment with GL or MT 3 h after ANIT administration exhibited partially protective effects against ANIT-induced liver damage. Remedial treatment with GL or MT 12 h after ANIT exhibited few protective effects. However, rats remedially treated with DEX 3 h after ANIT were not resistant to ANIT toxicity. Notably, remedial treatment with DEX 12 h after ANIT enhanced ANIT toxicity, as demonstrated by the elevated ALT and a severe reduction of majority interlobular ducts, accompanied by focused hepatocyte necrosis. Pre-treatment normally may affect the metabolism and excretion of ANIT, and hence may produce protective effects. Thus, the protective effects of pre-treatment with GL, MT or DEX may be partially due to modifying the metabolism and excretion of ANIT in the body.

Furthermore, the infiltration of inflammatory cells was generally thought to play an important role in the amplification of ANIT-induced acute cholestatic hepatitis. Recent research has shown that GL and MT exhibit many pharmacological properties, principally including anti-inflammatory effects, which resembles the pharmacological characteristics of DEX. We assume that GL, MT and DEX may therefore be able to exert their hepatoprotective effects through their anti-inflammatory effect.

Notably, in this study, the protective role of DEX against AVBDS produced by ANIT was limited. Pre-treatment with DEX did not reduce the elevated serum levels of ALT and hepatocyte necrosis induced by ANIT. In brief, the protective effect of DEX pre-treatment was directed toward cholangiocytes rather than hepatocytes. Rats remedially treated with DEX 3 h after ANIT were not resistant to ANIT toxicity, and remedial treatment with DEX 12 h after ANIT enhanced ANIT toxicity. However, GL and MT attenuated both bile duct and hepatocyte damage induced by ANIT in the initial phase of impairment. Thus, the nature products (GL and MT) exhibited better protective effect against ANIT-induced AVBDS than DEX. MT pre-treatment may show the most potent hepatoprotective effects, because it antagonized the loss of body weight caused by ANIT.

In summary, the present study demonstrated that GL and MT produced obviously protective effects against ANIT-induced AVBDS in the initial phase of impairment. However, the detailed mechanisms of the protective effects on ANIT-induced AVBDS need to be clarified. Experiments to identify the mechanisms involved are now in progress.

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Protection of glycyrrhizin and matrine on AVBDS


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