Protective Effect of Glycyrrhizin, Glycyrrhetic Acid and Matrine on Acute Cholestasis Induced by \( \alpha \)-Naphthyl Isothiocyanate in Rats

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Abstract

\( \alpha \)-Naphthyl isothiocyanate (ANIT) is a known hepatotoxicant that causes acute cholestatic hepatitis characterized by the infiltration of neutrophils around bile ducts and necrotic hepatocytes. The effects of glycyrrhizin (GL), 18β-glycyrrhetinic acid (GA), matrine (MT), oxymatrine (OMT), salvianolic acid B (SAB), silymarin (SI) and dexamethasone (DEX) on ANIT-induced acute cholestasis in rats were investigated. Serological and histological data demonstrated that the administration of GL, GA or MT all protected against hepatocyte injury and cholestasis induced by ANIT. Furthermore, the bile flow and the accumulative bile excretion of ketoprofen glucuronide (KPG), that were significantly suppressed by ANIT, were preserved in rats administered GL or GA. DEX protected against acute cholestasis but did not protect against hepatocyte necrosis and elevated serum alanine aminotransferase levels following ANIT administration. Rats administrated OMT, SAB or SI were not resistant to ANIT toxicity. In summary, the protective effect of DEX is directed toward cholangiocytes rather than hepatocytes whereas the natural products, GA, GL and MT, exhibit significantly better protective effects against ANIT-induced liver damage including the protection of hepatocytes as well as cholangiocytes.

Abbreviations

ANIT: \( \alpha \)-naphthyl isothiocyanate
GL: glycyrrhizin
GA: 18β-glycyrrhetinic acid
MT: matrine
OMT: oxymatrine
SAB: salvianolic acid B
SI: silymarin
DEX: dexamethasone
KPG: ketoprofen glucuronide
RP-HPLC: reversed-phase high-performance liquid chromatography
ALT: alanine aminotransferase
Tbil: total bilirubin
ALP: alkaline phosphatase
\( \gamma \)-GT: \( \gamma \)-glutamyl transpeptidase

Introduction

Cholestasis can be defined as the clinical, biochemical and histological manifestations of defective bile acid transport from the liver to the intestine. Cholestasis mostly results from inflammatory and destructive processes affecting the intrahepatic or extrahepatic biliary tree. Although there are treatments of cholestatic liver diseases and their associated complications, most chronic cholestatic conditions can progress towards biliary cirrhosis and hepatocellular insufficiency which may require liver transplantation [1].

Traditional Chinese medicines have proved to be effective in the treatment of liver disorders, including cholestasis. Glycyrrhizin (GL) has been clinically used for the treatment of chronic hepatitis B in China and Japan. After oral or intravenous administration in humans and in experimental animals, GL is hydrolysed by glucuronidases of intestinal bacteria to its active principle aglycone, 18β-glycyrrhetinic acid (GA); the hydrolysis occurring either prior to absorption or following biliary excretion, respectively. It has been demonstrated that both GL and GA possess several beneficial pharmacological effects, such as anti-inflammatory [2], [3], hepatoprotective [4], [5], antitoxin and antiviral actions [6].
and interferon inducibility [7]. Matrine (MT), as a component of traditional Chinese medicine prescriptions, has exhibited anti-inflammatory [8], hepatoprotective [9], immunosuppressive [10] and anticancer effects [11]. The pharmacological properties of oxymatrine (OMT) resemble those of MT, including anti-inflammatory [12], antitumor and antiviral actions [13], as well as hepatoprotective and immunomodulatory effects [14]. The water-soluble extract of Salviae Miltiorrhizae (SM) contains phenolic compounds that are effective in protecting liver microsomes, hepatocytes and erythrocytes against oxidative damage [15]. Recent reports have demonstrated that long-term administration of the water-soluble extract of SM reduced hepatic fibrosis induced by both carbon tetrachloride (CCL₄) and bile duct ligation [16], [17], and ameliorated the portal hypertensive state in bile duct ligated rats [18]. The major effective component of the water-soluble extract of SM is salvianolic acid B (SAB). Silimarin (SI), a flavonoid isolated from milk thistle, exerted a radical scavenger effect that prevented lipid peroxidation and subsequent hepatic damage in CCL₄- and ethanol-induced hepatotoxicity [19], [20]. SI has been extensively used clinically in Europe and Asia for the treatment of liver diseases.

Rats treated with N-naphthyl isothiocyanate (ANIT), a potent hepatotoxicant, are commonly used to study the mechanisms of cholestasis and the therapeutic effects of medicines. ANIT is known to cause intrahepatic cholestasis which is characterized by interlobular bile duct epithelial degeneration and necrosis and a pronounced neutrophil infiltration [21].

In the clinical situation, intrahepatic cholestasis has a range of causes including drug or xenobiotic toxicity, viral or bacterial infection, and as a complication of liver transplantation. At present, the pathogenesis of intrahepatic cholestasis is not fully elucidated but inflammatory injury represents a significant component of the disease process [1]. Similarly, the infiltration of neutrophils plays an important role in the amplification of ANIT-induced acute cholestatic hepatitis [21], [22].

Glucocorticosteroids are the current mainstay of therapy for acute intrahepatic cholestasis but their effects are limited despite possessing potent anti-inflammatory properties [1]. Furthermore, the side-effect profile of glucocorticosteroids makes them a less than ideal option for the treatment of this disease. Hence, there is a need to develop safe and effective therapies for the treatment of acute intrahepatic cholestasis. The known anti-inflammatory and hepatoprotective effects of GL, GA, MT, OMT, SAB and SI warrant their investigation as potential remedies. To date, there are few reports on the effectiveness of GL, GA, MT, OMT, SAB and SI on ANIT-induced intrahepatic cholestasis in rats.

### Materials and Methods

#### Materials

GL, GA, MT, OMT, SAB and SI were purchased from Xi'an Fuji Biotech Co, Ltd. (Xi'an, China). The purities of GL, GA, MT, OMT and SAB were 98%, 98%, 98%, 98% and 90%, respectively. SI contained a mixture of four isomeric flavonolignans: silybin (28%), isosilybin (12%), silydianin (16%) and silychristin (12%). GL (10 mg/mL) was dissolved in absolute ethanol then diluted in normal saline. GA (2 mg/mL) was dissolved in 0.1 mol/L sodium hydroxide solution followed by neutralization with 0.1 mol/L hydrochloric acid and then diluted in normal saline. MT (2 mg/mL), OMT (10 mg/mL) and SAB (10 mg/mL) were all dissolved in normal saline. SI (10 mg/mL) was suspended in 0.5% sodium carboxymethylcellulose. DEX (dexamethasone sodium phosphate injection, 5 mg/mL) was purchased from Sanbang Pharmaceutical Co, Ltd (Jinan, China). ANIT (10 mg/mL) was dissolved in arachis 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 adjusted 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 [23]. Acetoinitrile 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 (190–240 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.

#### Experimental protocol

Rats were randomly assigned to nine experimental groups (n = 4). Rats in seven of the groups were administered either GL (100 mg/kg i.p.), GA (10 mg/kg i.p.), MT (10 mg/kg i.p.), OMT (100 mg/kg i.p.), SAB (100 mg/kg i.p.), SI (100 mg/kg i.p.), or DEX (10 mg/kg i.p.) every 12 h for two consecutive days. ANIT (50 mg/kg i.g.) was administered 30 min after the second dose of the test compound (GL, GA, MT, OMT, SAB, SI or DEX). The remaining 2 groups were used as either non-treated controls or ANIT-only controls. Thirty-six hours post ANIT administration, bile excretion experiments were conducted, as described below to measure the bile flow and the cumulative bile excretion of KPG. Following this, blood samples were collected from the femoral artery and the livers removed for histological assessment.

#### 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; Recton Dickinson; Franklin Lakes, NJ, USA). After the operation, KP (20 mg/kg) was injected via the jugular vein. Bile specimens were collected over 30 min intervals (0–30, 30–60, 60–90, and 90–120 min) into preweighed Eppendorf tubes containing 100 µL of 1 mol/L potassium dihydrogen phosphate. The sample tubes were kept on ice during bile collection. The quantity of KP and KPG in bile was analyzed by reversed-phase HPLC (RP-HPLC) immediately after collection. Eighty µL of mobile phase and 20 µL of internal standard solution (5 µg/mL naproxen dissolved in methanol) were added to 20 µL of the collected bile, and 20 µL of the mixture were injected for RP-HPLC analysis. The RP-HPLC system consisted of a Model LC-10A TVp pump, a Model SPD-10A 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 tetrabutylammonium bromide (35:65), the solution was filtered using a 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.

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 (Beckman Coulter SynchroN Clinical System Lx 20; Beckman Coulter; Fullerton, CA, USA).

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 and eosin. Section preparation and assessment were conducted by an independent professional pathologist (AiFeng Zhang, Department of Pathology, Southeast University, China). Twenty portal areas in each rat liver were assessed and the number of bile ducts counted. Loss or destruction of bile ducts was quantified using the ratio: \( r = (A - n)/A \) where \( A = \) the mean number of bile ducts in control rats and \( n = \) the number of bile ducts in each experimental rat.

Statistical analysis
Statistical evaluation of the data was performed using SPSS 10.0. All results are expressed as mean ± standard deviation of the mean. Analysis of variance (one-way ANOVA) with LSD/Dunnett for post hoc analysis was used to compare results between different groups. A probability (p) of less than 0.05 was considered statistically significant.

Supporting information
Morphological figures of rat liver under different treatments are available as Supporting Information.

Results

In rats that received only ANIT, profound hepatotoxicity was observed 36 h after administration, with a greater than 12-fold increase in serum ALT activity compared to controls. Similarly, serum markers for cholestasis were significantly elevated: Tbil by approximately 5-fold, ALP by approximately 2-fold, and γ-GT by approximately 4-fold (Fig. 1A, B, C and D). In addition, rats treated with ANIT alone had significant decreases in body weight (Table 1). Histologically, ANIT-induced liver injury was characterized by sinusoid congestion, neutrophil infiltration, interlobular bile duct epithelial cell degeneration and necrosis, bile duct destruction/loss, and a mild degree of hepatocyte damage (Table 2). The bile flow (0–120 min) was significantly suppressed in ANIT-treated rats compared with the untreated control group (2.49 ± 1.50 mL/kg vs. 9.36 ± 0.495 mL/kg, \( p < 0.01 \)). Similarly, the bile excretion of KPG was almost completely inhibited in ANIT treated rats (3.42% ± 2.17% vs. 77% ± 6.9%, \( p < 0.01 \)) (Fig. 1A and Fig. 1B).

Treatment with GL, GA or MT exhibited protective effects against ANIT-induced liver damage as demonstrated by a significant inhibition of the ANIT-induced elevation of serum Tbil, ALT, ALP and γ-GT (Fig. 1A, B, C and D). In addition, the two-day treatment with MT prevented the decrease of body weight caused by ANIT (Table 1). While DEX treatment did confer some protection against ANIT-cholestasis it did not influence the effects of ANIT on serum levels of ALT.

As shown in Table 2, histological evaluations confirmed the hepatoprotective effects of a two-day treatment with GL, GA or MT.
Bile flow

\[
\begin{array}{ccccccccc}
\text{bile flow (mL/kg b.w.)} & 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 \\
\hline
10 & # & # & # & # & # & & & & \\
5 & # & # & # & & & & & & \\
0 & & & & & & & & & \\
\end{array}
\]

Fig. 2 Effects of GL, GA, MT, OMT, SAB, SI and DEX on the bile flow over 120 min at 36 h after ANIT administration in rats. (Bar 1: control; Bar 2: ANIT alone; Bar 3: GL + ANIT; Bar 4: GA + ANIT; Bar 5: MT + ANIT; Bar 6: OMT + ANIT; Bar 7: SAB + ANIT; Bar 8: SI + ANIT; Bar 9: DEX + ANIT). Data are mean ± S.D. n = 4. The accumulation of bile flow extremely suppressed by ANIT was significantly ameliorated by a two-day treatment with GL, GA, MT and DEX. *p < 0.05, **p < 0.01 compared with the rats receiving ANIT alone.

KPG

\[
\begin{array}{ccccccccc}
\text{KPG} & 1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 \\
\hline
100 & # & # & # & # & # & & & & \\
80 & # & # & # & # & # & & & & \\
60 & # & # & # & # & # & & & & \\
40 & # & # & # & # & # & & & & \\
\end{array}
\]

Fig. 3 Effects of GL, GA, MT, OMT, SAB, SI and DEX on the accumulative bile excretion of KPG over 120 min following the i.v. injection of 20 mg/kg KP, 36 h after ANIT administration in rats. (Bar 1: control; Bar 2: ANIT alone; Bar 3: GL+ANIT; Bar 4: GA + ANIT; Bar 5: MT + ANIT; Bar 6: OMT + ANIT; Bar 7: SAB + ANIT; Bar 8: SI + ANIT; Bar 9: DEX + ANIT). Data are mean ± S.D. n = 4. The accumulative bile excretion of KPG nearly completely inhibited by ANIT was significantly ameliorated by a two-day treatment with GL, GA, MT and DEX. *p < 0.05, **p < 0.01 compared with the rats receiving ANIT alone.

Discussion

In this study, rats exposed to ANIT had significant elevations in serum Thil, ALT, ALP and γ-GT. These serology findings were accompanied by morphological changes including diffuse alteration of intrahepatic bile ducts, periductular inflammation and mild hepatocyte necrosis. In rats receiving GA, GL, MT or DEX, the serological and morphological indicators of cholestasis associated with ANIT were significantly reduced. These results suggested that GA, GL, MT or DEX treatment has protective effects against ANIT-induced cholestasis.

As well as the serological and histological assessment of the effects of ANIT, the functionality of the liver and bile ducts was assessed through measuring the elimination of the non-steroidal

on ANIT-induced cholestasis, i.e., distinct pathological changes in hepatocellular degeneration and necrosis, bile duct loss, and inflammatory cell infiltration did not occur, with only mild bile duct injury observed. DEX administration did not reduce hepatocyte necrosis caused by ANIT, thus the protective effect of DEX directed toward cholangiocytes rather than hepatocytes. Again, this is in line with the serology findings. The observations from the bile excretion experiments provide further support for the results obtained from the serology and histology. The accumulative bile flow (Fig. 2) and KPG excretion (Figs. 3 and Fig. 4) were significantly higher in GL, GA, MT or DEX treated rats compared to those administered only ANIT.

In contrast, rats administered with OMT, SAB or SI were not resistant to ANIT toxicity (Fig. 1–3).

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before ANIT</td>
<td>36 h after ANIT</td>
</tr>
<tr>
<td>Increase</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>206.2 ± 11.01</td>
</tr>
<tr>
<td>ANIT alone</td>
<td>215.4 ± 13.16</td>
</tr>
<tr>
<td>GL + ANIT</td>
<td>225.1 ± 12.07</td>
</tr>
<tr>
<td>GA + ANIT</td>
<td>232.5 ± 15.00</td>
</tr>
<tr>
<td>MT + ANIT</td>
<td>212.6 ± 12.12</td>
</tr>
<tr>
<td>OMT + ANIT</td>
<td>212.9 ± 13.53</td>
</tr>
<tr>
<td>SAB + ANIT</td>
<td>219.3 ± 17.94</td>
</tr>
<tr>
<td>SI + ANIT</td>
<td>221.4 ± 10.46</td>
</tr>
<tr>
<td>DEX + ANIT</td>
<td>214.6 ± 12.30</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hepatocellular degeneration</th>
<th>Hepatocellular necrosis</th>
<th>Sinusoid congestion</th>
<th>Neutrophil infiltration</th>
<th>Bile duct epithelial necrosis</th>
<th>Bile duct loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>ANIT alone</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>GL + ANIT</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>MT + ANIT</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>OMT + ANIT</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
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<tr>
<td>SAB + ANIT</td>
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<td>+++</td>
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<tr>
<td>SI + ANIT</td>
<td>−</td>
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<td>+</td>
<td>+++</td>
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<tr>
<td>DEX + ANIT</td>
<td>−</td>
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<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

In histological assessment of bile duct demolishment or loss 20 portal areas in each rat liver were assessed and the number of bile ducts was counted. Loss or destruction of bile ducts was quantified using the ratio: *R* = (A−n)/A where A = the mean number of bile ducts in control rats and n = the number of bile ducts in each experimental rat. R is expressed as the mean of “R” of four specimens in each group: +++ = >80% demolishment or loss of the interlobular ducts in 20 portal areas; +++ = 50–80%; ++ = 20–50%; + = 10–20%; ± = <10%.

Table 1 Mean rat body weight (± S.D.) before and 36 h after ANIT administration in different treatment groups. n = 4. A two-day treatment with MT prevented the decrease of body weight caused by ANIT. *p < 0.05, **p < 0.01 compared with rats receiving ANIT alone.

Table 2 Effects of different treatment with GL, GA, MT, OMT, SAB, SI and DEX on morphological changes in the rat liver obtained 38 h after ANIT administration. Data expressed as the mean of four specimens for each treated group. + mild; ++ moderate; +++ marked; − negative; ± less than two livers show a mild change.
anti-inflammatory drug KP. The primary route of elimination of KP is that of glucuronidation, a phase II metabolic pathway aimed at converting xenobiotics, endobiotics and/or their phase I metabolites to more polar compounds which can be readily excreted from the body. Once KP has undergone glucuronidation, the resulting KPG is mainly excreted via the bile [23]. Hence, in the present study, KP was used as a model drug, the elimination of which may be affected by either changes in the glucuronidation pathway or altered biliary excretion of the glucuronide conjugate metabolite. Thus, the impairment of bile flow indicates directly obstruction of the bile duct, while the bile excretion of KPG, at least in part, reflects liver functions of metabolism and excretion. However, it is known that, in general, glucuronidation is relatively insensitive to the effects of liver damage [24], [25], suggesting that reduced biliary clearance is likely to be the dominant factor where the appearance of KPG in the bile is reduced following ANIT exposure. The suppression of bile flow and accumulated bile excretion of KPG by ANIT were significantly ameliorated in GL, GA, MT or DEX administered rats. Hence, the results of the biliary excretion experiments are in line with the results obtained from the serology and histology.

Although OMT [13], [14], SAB [15], [16], [17] and SI [19], [20] have been previously reported to show hepatoprotective effects, they did not reduce ANIT toxicity in this study. ANIT is a hepatotoxin that causes acute cholestatic hepatitis characterized by interlobular bile duct epithelial degeneration and necrosis and a pronounced neutrophil infiltration. ANIT is thought to be bioactivated in the liver by cytochrome P450 s, and the bioactivated ANIT is detoxified in hepatocytes by conjugation with glutathione catalyzed by glutathione S-transfereases. ANIT-glutathione complexes are transported into bile, but they are unstable and rapidly dissociate. The released ANIT then damages bile duct epithelial cells, causing cholangiolitis that leads to intrahepatic biliary obstruction [21], [22]. Thus, ANIT-induced hepatotoxicity involves metabolism, transport, cholestasis, inflammation, and hepatocellular death. It is presumed that inflammation plays an important role in the amplification of ANIT-induced acute cholestatic hepatitis. Recent research has shown that GA, GL and MT exhibit many pharmacological properties, including anti-inflammatory effects [2], [3], [8], which resemble the pharmacological characteristics of DEX. The present study has demonstrated that GA, GL, and MT protect against the cholestasis and hepatocyte damage caused by ANIT. The mechanism(s) of action by which these natural products exert their hepatoprotective effect is(are) unclear. However, given that they have known anti-inflammatory action this is a potential mechanism that warrants further investigation.

In the present study, the protective role of DEX against ANIT appears to be somewhat limited. Treatment with DEX did not reduce the elevated serum levels of ALT and hepatocyte necrosis induced by ANIT. However, GA, GL and MT protected against both bile duct epithelial cell and hepatocyte damage providing a better overall protective effect against ANIT-induced liver damage than DEX. This is especially evident in the case of MT which not only helped to maintain liver and biliary function but also prevented the weight loss observed in all the other groups treated with ANIT.

**Acknowledgements**

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**References**