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Hepatoprotective and anti-hepatocarcinogenic effects of glycyrrhizin and matrine

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ABSTRACT

Matrine (Mat), a component extracted from *Sophora flavescens* Ait, has a wide spectrum of pharmacological effects. Glycyrrhizin (Gly), a major active constituent of licorice (*Glycyrrhiza glabra*) root, has various pharmacological effects. Gly and Mat are ancillary drugs used clinically in China for protection of liver function and treatment of tumors. However, habitual administration of Gly may cause adverse effects marked by the development of pseudohypercorticosteroidism. This work was designed to see whether combination use of Gly and Mat could offer better liver protective and anti-hepatocarcinogenic effects than Gly or Mat alone, and whether it could reduce the adverse effects of Gly alone by acetaminophen-induced hepatotoxicity, diethylnitrosamine-induced hepatocarcinogenesis, induction of immunosuppression, albumen-induced swelling of rat hind paws. The results showed that compared with Gly or Mat alone, Gly + Mat reduced the mortality of acetaminophen overdosed mice more effectively, attenuate acetaminophen-induced hepatotoxicity, and reduced the number and area of γ-GT positive foci, thus protecting liver function and preventing HCC from occurring. In addition, Gly + Mat had a protective effect on immunosuppression, a strong non-specific anti-inflammatory effect, and an effect of reducing the incidence of sodium and water retention.

1. Introduction

Acute and chronic liver diseases are common and frequent occurrences in China, whose pathology and pathogenesis are complex. Some patients may progress to liver cirrhosis or hepatocellular carcinoma (HCC) with poor prognosis. Current medical treatments for these liver diseases are often ineffective, and therefore efforts are being made to seek new effective medications [1]. Developing pharmacologically effective agents from natural products has become a new trend by virtue of their little toxicity or few side effects.

Matrine (Mat) (molecular formula: $C_{15}H_{24}N_2O$), a component extracted from a traditional Chinese herb, *Sophora flavescens* Ait, is widely used in the treatment of viral hepatitis, chronic liver diseases, cardiac arrhythmia and skin inflammations in China [8,9] due to its wide spectrum of pharmacological actions including anti-inflammatory [2], immunoinhibitory [3], anti-fibrotic [4], anti-arrhythmic [5], anti-tumor [6,7] and diuretic activities without causing significant toxicity or side effects [10].

Glycyrrhizin (Gly) (molecular formula: $C_{42}H_{62}O_{16}$), a triterpene glycoside and a conjugative compound of enoxolone and glucuronic acid as an active component of licorice (*Glycyrrhiza glabra*) root, has a variety of pharmacological actions including anti-inflammatory, anti-viral, antioxidative, anti-liver cancer, immunomodulatory, hepatoprotective and cardioprotective activities [11–15]. It has been used for more than 20 years in the treatment and prevention of hepatitis, chronic bronchitis, gastritis, tumor growth and immunological disorders. However, there is evidence from numerous clinical case reports and trials that conventional administration of Gly may cause pseudohypercorticosteroidism, such as sodium and water retention, hypertension and hypokalaemia [11–13,16].

Gly and Mat are ancillary drugs used clinically in China for protection of liver function and treatment of tumors. Whether combination use of Gly and Mat could produce a better clinical effect is worth of investigation. This study was designed to compare the efficacy of Gly or Mat alone and combination use of both Gly and Mat with respect to hepatoprotective, anti-hepatocarcinogenic, immunomodulatory and anti-inflammatory activities, and see whether concomitant use of the two drugs could reduce the side effects of sodium and water retention and hypokalaemia as seen in cases of using Gly alone, and potentiate the hepatoprotective and anti-hepatocarcinogenic effects of the two drugs.

Abbreviations: Mat, matrine; Gly, glycyrrhizin; GM, mixture of Gly and Mat; PCM, acetaminophen (N-acety1-p-aminophenol, paracetamol); DENA, diethylnitrosamine; 2-AAF, 2-acetylaminofluorene; CY, cyclophosphamide; γ-GT, γ-glutamyltranspeptidase; GSH, glutathione.

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2. Materials and methods

2.1. Chemicals and reagents

Chemicals and reagents included acetaminophen, diethylnitrosamine (DEN), 2-acetylaminofluorene (2-AAF), cyclophosphamide (CY) and cortisone acetate (Jiu Zhou Pharmaceutical Co. Ltd., Shanghai, China; Sigma Chemical Co., St. Louis, US); 0.1% glycyrrhizin (Lianyungang Chia Tai Tianqing Pharmaceuticals, batch number 980414); and 0.1% Mat (Shanghai No. 1 Biochem. & Pharma., batch number 9804215).

2.2. Animals and experimental design

Sexually mature Sprague–Dawley (SD) rats, Wister rats, ICR mice were obtained from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China), housed under the conditions in a temperature and light-controlled room (23–25 °C and 14 h light:10 h dark), and fed a pellet food and water ad libitum. The animal care and permission were obtained from the Committee on Ethics of Biomedicine Research of the Second Military Medical University (Shanghai, China). The animals were equally randomized to three experimental groups and 1 or 2 control groups, each group consisting of 10–15 mice/rats and allowed 1 week to adapt to their environment before treatment. Three experimental groups were set up as follows: Gly group, using 0.1% 25 mg kg^{-1} glycyrrhizin intragastrically (i.g.); Mat group, using 0.1% 25 mg kg^{-1} Mat, i.g.; and Gly + Mat group, using a mixture of 1 g L^{-1} Gly and 1 g L^{-1} Mat 0.5 mL/20 g mice weight, i.g. The control group used the equivalent amount of water for i.g.

2.3. Acetaminophen-induced hepatotoxicity and treatment protocols

Sixty ICR mice were equally randomized to four groups. According to the experimental design, each group was administered with corresponding drugs once daily for 7 consecutive days. By the end of drug administration, the animals were injected intraperitoneally (i.p.) with 500 mg kg^{-1} 2% acetaminophen to observe 48 h mortality.

2.4. Acetaminophen-induced glutathione depletion

Fifty ICR mice of equal sexes were equally randomized to five groups. Corresponding drugs were administered according to the experimental design once daily for 7 consecutive days. Then 200 mg kg^{-1} 0.8% acetaminophen was administered i.p. by the end of drug administration. The normal control group was treated only with the equivalent amount of water i.p. The animals were killed by decapitation and the livers were dissected. Total acetaminophen-induced glutathione (GSH) was measured in liver homogenates using a total glutathione quantification kit (Dojindo Laboratories, Japan).

2.5. Diethylnitrosamine-induced hepatocarcinogenesis and treatment protocols

Forty Wister rats were equally randomized to four groups, and administered with the corresponding drugs according to the experimental design once daily for 7 consecutive days. Meanwhile, DEN was administered at 100 mg kg^{-1} i.p. daily for 2 weeks. The mice were then fed with 0.015% 2-acetylaminofluorene (2-AAF) for another 2 weeks. All groups were treated with DEN and 2-AAF to induce preneoplastic lesions of liver cancer. The left and middle lobes of liver were surgically resected on 7 days after feeding of the 2-AAF-containing food. Rats were killed by decapitation at day 3 after operation, and then the right lobes of liver were resected and fixed with ice-cold acetone/alcohol (1:1, v/v). Liver tissue specimens were paraffin-embedded for 24 h at 52–54 °C, and then stained with γ-glutamyltranspeptidase (γ-GT) according to a modified Rutenberg method. The number (foci/cm^2) and area (mm^2/cm^2) of γ-GT positive foci per square centimeter of the liver area and the average area of each focus (m^2/foci) were calculated with pathology-image analysis software.

2.6. Induction of immunosuppression and treatment protocols

Fifty ICR mice were equally randomized to five groups. Each group was administered with the corresponding drugs according to the experimental design for 13 consecutive days, during which 25 mg kg^{-1} 0.125% cyclophosphamide was administered i.p. for 7 consecutive days after the mice were treated for 4 days as above. Animals in the normal control group were injected with the equivalent amount of water for injection. Blood was drawn from the orbital cavity of the mice 24 h after the last drug administration. T lymphocyte subsets ($CD4^+T$ and $CD8^+T$) from peripheral blood were measured by flow cytometric assay. The ratio of $CD4^+T$ and $CD8^+T$ and leukocyte number were calculated.

2.7. Albumen-induced swelling of rat hind paws

Fifty SD rats were equally randomized to five groups: three experimental groups, a positive control group and a negative control group. Each group was administered with the corresponding drugs according to the experimental design once daily for 7 consecutive days. The positive control group was administered with 50 mg kg^{-1} cortisone acetate i.p., and the negative control group with the equivalent amount of water for injection. Fresh albumen 0.05 mL/claw was injected subcutaneously to the left hind claw 1 h after pretreatment, the size of which was measured at 0.5, 1, 2, 3, 4 and 5 h after albumen injection.

2.8. Determination of urine output and accumulative excretion of K^+, Na^+, and Cl^-

Forty ICR mice were equally randomized to four groups. Each group was administered with the medications according to experimental design once daily for 7 consecutive days. The mice were housed in metabolic cages 7 days after treatment. Urine was collected for 9 h to determine the output and levels of K^+, Na^+ and Cl^-.

2.9. Statistical analysis

Data were expressed as mean ± SD. All continuous variables were tested for normality by Kolmogorov–Smirnov test. Statistical comparison between groups was performed using the non-parametric Mann–Whitney-*U*, χ^2 or Dunnett's *t*-test. The comparison between two groups was performed using the Student's *t*-test. SAS (SAS Institute, Cary, NC) software package was used in the analysis. A P value ≤ 0.05 was accepted as the level of significance.

3. Results

3.1. Effects on acetaminophen-induced hepatotoxicity in mice

The results showed that combination use of Gly and Mat significantly attenuated the development of acetaminophen-induced hepatotoxicity in mice ($P < 0.05$) compared with the control group, and also increased 48 h-mortality by 20% and 26.7% compared with Gly alone and Mat alone, respectively. Gly + Mat had a bet-

Table 1
Effects on acetaminophen-induced hepatotoxicity in mice.

Group/treatment		n	Death number	Mortality (%)
Control	PCM	15	12	80.0
Gly	PCM + Gly	15	8	53.3
Mat	PCM + Mat	15	9	60.0
Gly + Mat	PCM + Gly + Mat	15	5*	33.3*

* Compared with the control group, $P < 0.05$.

Table 2
Effects on acetaminophen-induced GSH depletion ($n = 10$, μmol/g liver tissues, $\bar{x} \pm$ SD).

Group/treatment		Hepatic GSH
Normal control	Normal sodium (NS)	204.5 ± 42.8
Model control	PCM	41.6 ± 8.2
Gly	PCM + Gly	84.3 ± 19.2
Mat	PCM + Mat	68.1 ± 15.3
Gly + Mat	PCM + Gly + Mat	123.0 ± 24.4**

** Compared with Gly group and Mat group, $P < 0.01$.

ter effect in protecting liver function than Gly or Mat alone (Table 1).

3.2. Effects on GSH depletion

The results showed that acetaminophen treatment caused significant GSH depletion compared with the normal control group ($P > 0.05$), and that Gly + Mat significantly diminished the subsequent acetaminophen-induced GSH depletion in mice ($P < 0.01$) compared with the Gly or Mat alone. Gly + Mat had a better effect in protecting liver function than Gly or Mat alone (Table 2).

3.3. Effects on diethylnitrosamine-induced hepatocarcinogenesis in rats

The results from Table 3 indicate that Gly + Mat reduced the diethylnitrosamine-induced liver/body weight ratio increment markedly. The number and area of γ-GT positive foci were 7.1 ± 3.9 and 0.9 ± 0.7 respectively in Gly + Mat group, versus 21.7 ± 17.3 and 3.6 ± 2.7 respectively in control rats. The differences between the two groups were statistically significant ($P < 0.01$) (Table 2). Gly + Mat protected the rats against DEN-induced toxicity and inhibited DEN-induced precancerous lesions of liver cancer. These protective effects were better than those of Gly or Mat alone.

3.4. Effects on cyclophosphamide-induced immunosuppression

Table 4 shows that Gly + Mat significantly increased the number of leucocytes in peripheral blood and the percentage of CD4 and CD8 T-1ymphocyte subsets in CY-induced immunosuppressive model mice ($P < 0.05$) compared with the control group ($P > 0.05$) compared with the CY group. Gly + Mat modulated cellular immunity and attenuated cyclophosphamide-induced immunosuppression. These effects were better than those of Gly or Mat alone (Table 4).

Table 4
Effects on T lymphocyte subsets from peripheral blood in the CY-induced mouse model ($n = 10$, $\bar{x} \pm$ SD).

Group/treatment		Leucocyte (10^9/L)	$CD4^+/CD8^+$
Normal control	Normal sodium (NS)	9.4 ± 1.8**	1.97 ± 0.38**
Model control	CY	4.6 ± 0.9	1.03 ± 0.21
Gly	CY + Gly	5.7 ± 1.1	1.31 ± 0.28
Mat	CY + Mat	5.5 ± 1.2	1.23 ± 0.27
Gly + Mat	CY + Gly + Mat	8.2 ± 1.7**	1.77 ± 0.34**

** Compared with the CY control group, $P < 0.01$.

3.5. Inhibitory effects on albumen-induced swelling of rat hind paws

The results indicate that Gly + Mat displayed a more significant inhibitory effect on albumen-induced swelling of rat hind paw between 0.5 and 5 h of treatment than did the negative control group ($P < 0.01$) or Mat ($P < 0.05$) alone. In addition, Gly + Mat had a non-specific anti-inflammatory effect (Table 5).

3.6. Effects on urine volume and accumulative excretion volume of K^+, Na^+, Cl^- in mice

Table 6 shows that 9 h accumulative urine volume decreased markedly after administration of Gly for 1 week, and meanwhile the K^+ level increased and Na^+ level decreased. There was no significant difference in urine volume, Na^+ and K^+ changes between Gly + Mat group and control group ($P > 0.05$), indicating that Gly + Mat did not produce such side effects as hypokalaemia (Table 6).

4. Discussions

The hepatoprotective effects of Gly + Mat were examined using an acetaminophen-induced hepatotoxicity animal model. Acetaminophen (N-acetyl-p-aminophenol, paracetamol, PCM) is a clinically common analgesic and antipyretic OTC drug. However, overdosage of acetaminophen may cause hepatic necrosis, nephrotoxicity, extrahepatic pathology, or even death in humans and experimental animals [17]. For many years, P450-dependent biotransformation of PCM to its reactive metabolite, N-acetyl-p-benzoquinone imine (NAPQI) has been clearly identified as the mechanism mediating PCM-induced hepatotoxicity. NAPQI requires glutathione (GSH) for detoxification by forming its GSH-adduct. Once intracellular stores of GSH are depleted, excess NAPQI may react with cellular proteins, including mitochondrial proteins, causing hepatocellular necrosis [18–20]. GSH, also known as g-L-glutamyl-L-cysteine-glycine, is a ubiquitous tripeptide that functions as an important intracellular radical scavenger. Cellular redox potential is largely determined by GSH content, accounting for more than 90 °C of cellular non-protein thiols [21]. GSH deple-

Table 3
Number and area of GGT-positive liver foci in non-initiated and DEN-initiated male Wistar rats ($n = 10$, $\bar{x} \pm$ SD).

Group/treatment		Effective number of rats	Liver/body weight (g/100 g)	GGT + foci		
				Number (foci/cm²)	Area (mm²/cm²)	mm²/foci
Control	DEN + 2-AAF	10	3.0 ± 0.5	21.7 ± 17.3	3.6 ± 2.7	0.21 ± 0.18
Gly	DEN + 2-AAF + Gly	10	2.6 ± 0.4	15.5 ± 10.4	2.4 ± 1.6	0.16 ± 0.12
Mat	DEN + 2-AAF + Mat	10	3.1 ± 0.6	20.3 ± 15.8	3.2 ± 2.2	0.20 ± 0.23
Gly + Mat	DEN + 2-AAF + Gly + Mat	10	2.3 ± 0.5*	7.1 ± 3.9**	0.9 ± 0.7*	0.10 ± 0.11**

* Compared with the control group, $P < 0.05$.
** Compared with the control group, $P < 0.01$.

Table 5
Inhibitory effect on albumen-induced swelling of rat hind paws ($n = 8$; $\bar{x} \pm$ SD).

Group/treatment		0.5	1	2	3	4	5
Control	NS	0.96 ± 0.19	0.90 ± 0.20	0.83 ± 0.19	0.71 ± 0.15	0.65 ± 0.13	0.58 ± 0.12
Cortisone	Cortisone	0.58 ± 0.14**	0.50 ± 0.12**	0.46 ± 0.09**	0.40 ± 0.10**	0.33 ± 0.07**	0.27 ± 0.06**
Gly	Gly	0.78 ± 0.18*	0.72 ± 0.15*	0.65 ± 0.14*	0.58 ± 0.12*	0.50 ± 0.12*	0.41 ± 0.10*
Mat	Mat	0.98 ± 0.20	0.90 ± 0.20	0.81 ± 0.19	0.73 ± 0.15	0.68 ± 0.13	0.60 ± 0.13
Gly + Mat	Gly + Mat	0.70 ± 0.16**	0.62 ± 0.13**	0.58 ± 0.13**	0.49 ± 0.10**	0.41 ± 0.10**	0.36 ± 0.08**

* Compared with the negative control group, $P < 0.05$.
** Compared with the negative control group, $P < 0.01$.

tion has been found to either precede the onset of apoptosis or render cells more sensitive to cell death [22]. PCM hepatotoxicity is related to excessive oxidative stress mainly caused by electrophile and highly reactive metabolite of PCM (NAPQI) under the action of liver CYP450 oxidase [23]. In fact, overdose of PCM is the most frequent cause of drug-induced liver failure in the United States [24]. Therefore, PCM-induced hepatotoxicity is commonly used to screen and evaluate liver protectants. As expected, a single dose of PCM (200 mg/kg, .i.p.) had significant hepatotoxicity, as evidenced by mortality and hepatic GSH level.

Previous studies showed that Gly could reduce acetaminophen-induced hepatotoxicity in rats by preventing lipid peroxidation [25,26], and inhibit peroxide-induced toxicity in rat hepatocytes [27]. Serological and histological data demonstrated that administration of Gly or Mat protected against hepatocytic injury [28–30]. The present study was designed to see whether Gly + Mat could offer better hepatoprotective effects. The results showed that Gly + Mat attenuated the development of acetaminophen-induced hepatotoxicity in mice, and had better effects in protecting the liver than did Gly or Mat alone. Gly + Mat significantly reduced acetaminophen-induced intracellular GSH depletion, probably by attenuating lipid peroxidation and decreasing the hepatic GSH content, which suggests that Gly + Mat increases the hepatic pool of GSH and reduces oxidative stress.

The initiation–promotion or two-stage hepatocarcinogenesis model resembles the early events of the latent period of human carcinogenesis. Chemically induced rat liver carcinogenesis, especially one initiated by the environmental carcinogen DENA, has been considered as one of the best-characterized experimental models of carcinogenesis, allowing screening of potential anticancer compounds on various phases of neoplastic development [31]. It is known that DENA causes the development of HCC through various stages with formation of preneoplastic foci, neoplastic nodules, and ultimately HCC nodules of various sizes [32]. DENA has been shown to induce tumors in rodents that closely mimic a subclass of human HCC [33]. It was found that DENA/2-AFF (diethylnitrosamine 100 mg/kg i.p. at the beginning of the experiment) decreased the number of γ-GGT positive foci (foci/cm^2), the covered area (mm^2/cm^2) and the average area of each focus (m^2/foci), which is consistent with the report of Toledo et al. [34].

Although previous studies have shown that either Gly or Mat has anticarcinogenic activities in liver cancer in vitro and in vivo [6,16,35], whether Gly + Mat could mutually potentiate these activities is unclear. In this study, we analyzed the inhibitory effect of Gly + Mat on the appearance of early hepatic preneoplastic events in a two-stage carcinogenic model combining DENA and 2-AFF. The results showed that GM significantly reduced the size, volume and number of preneoplastic liver lesions. This finding first demonstrated that continuous administration of Gly + Mat during DEN/2-AAF treatment in male Wistar rats had a better protective effect on preneoplastic liver pathology than did Gly or Mat alone. However, the molecular mechanism of this anti-cancer effect needs to be further clarified in future studies.

Tumor development over many years leads to reciprocal alterations in host and immune responses. Regulatory T cells have been implicated as key players in immune tolerance as well as suppression of anti-tumor responses. Different types of T cells can play different modulatory roles in immune function of immunosuppressive mice. Botanical based immunomodulators are often employed as supportive or adjuvant therapy to overcome the undesired effects of cytotoxic chemotherapeutic agents and help recovery of health. Simultaneous treatment with different extracts of these plants resulted in protection towards CP induced immunosuppression. Previous studies [2,3] have demonstrated that Gly or Mat has immunomodulatory effects. Mat is able to inhibit cell-mediated immunity and enhance non-specific immunity, which could be one of the mechanisms in treating chronic hepatitis B [37]. In the present study, we used a common CY-induced immunosuppressive mouse model to observe the immunomodulatory effect of Gly + Mat on cellular immunity. CY is a non-specific immunosuppressant agent and widely used in the treatment of tumors and some autoimmune conditions. Hou et al. [38] reported that oral administration of 10 mg/kg CY for 30 days could induce significant immunosuppressive effects in female F344 rats. In this study, we used 25 mg/kg CY i.p. for 14 days to construct an immunosuppressive model in male SD rats. It was found that the percentage of CD4$^+$ and CD8$^+$ T lymphocyte subsets was reduced significantly in the immunosuppressive model mice, as compared with the control ($P < 0.01$). The results showed that Gly + Mat significantly increased the number of peripheral leucocytes and the percentage of CD4$^+$ and CD8$^+$ T lymphocyte subsets in the immunosuppressive mouse model, suggesting that Gly + Mat modulates cellular immunity in immunosuppressive mice better than does Gly or Mat alone.

Hepatocyte death results in a sterile inflammatory response that amplifies the initial insult and increases overall tissue injury. Previous studies [29,36] showed that Gly or Mat had anti-inflammation effects, but whether combined use of Gly + Mat could produce better anti-inflammatory effects was unclear. Albumen-induced swelling of rat hind paws is a common model used to study anti-inflammatory agents due to its convenience, short trial cycle and motional observation. Our results showed that Gly + Mat had a significantly better inhibitory effect on albumen-induced swelling of rat hind paws than did Gly or Mat alone, and this anti-inflammatory effect was non-specific.

Long-term excess administration of Gly could cause some severe side effects such as sodium and water retention, hypertension and hypokalaemia [12,14,17], thus limiting its wider clinical applica-

Table 6
Effects on urine volume and accumulative excretion volume of K$^+$, Na$^+$, Cl$^-$ ($n = 10$, $\bar{x} \pm$ SD, mg).

Group/treatment		Urine volume	Na$^+$	K$^+$	Cl$^-$
Control	NS	2.1 ± 0.62	3.6 ± 1.1	4.7 ± 1.6	16.2 ± 5.1
Gly	Gly	1.5 ± 0.47*	2.7 ± 0.6*	7.3 ± 2.2	18.9 ± 4.1
Mat	Mat	2.3 ± 0.76	3.9 ± 1.4	5.1 ± 1.8	16.7 ± 4.7
Gly + Mat	Gly + Mat	2.0 ± 0.70	3.7 ± 1.2	5.0 ± 2.1	15.8 ± 5.7

* Compared with the control group, $P < 0.05$.

tions. Our study demonstrated that Gly + Mat did not have such side effects as hypokalaemia, sodium and water retention, so it is safer than Gly or Mat alone.

In conclusion, the results of the present study showed that compared with Gly or Mat alone, combined use of Gly and Mat can reduce the mortality of acetaminophen overdosed mice, attenuate the development of acetaminophen-induced hepatotoxicity in mice, and reduce the number and area of γ-GT positive foci, thus protecting liver function and preventing HCC from occurring. In addition, it has a protective effect on immunosuppression and a strong non-specific anti-inflammatory effect and an effect of reducing the incidence of sodium and water retention, without causing significant adverse effects.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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