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The effects of pretreatment with glycyrrhizin and glycyrrhetinic acid on the retrorsine-induced hepatotoxicity in rats

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Abstract

A wide variety of medicinal herbs contain hepatotoxic pyrrolizidine alkaloids (PAs), and often cause acute and chronic liver damages in man. Liquorice, a known antihepatitis, is commonly used with PA-containing herbs concurrently, and hepatotoxicity induced by such combined uses was not pronounced. The present study is to investigate effects of glycyrrhizin (GL) and 18β-glycyrrhetinic acid (GA), the major biologically active ingredients of liquorice, against PA-induced hepatotoxicity in rats.

Single dose (35 mg/kg, i.p.) of retrorsine (RET), a typical potent hepatotoxic PA, was given to rats to induce liver injury. A single dose pretreatment with GL or GA prior to retrorsine challenge did not show hepatoprotection. However, when rats were pretreated with either GL (200 mg/kg/day, i.p.) or GA (10 mg/kg/day, i.p.) for three consecutive days prior to retrorsine exposure, the elevated serum GOT and GPT levels induced by retrorsine were significantly reduced. Serum levels of transaminases almost returned to normal (GOT: 56 ± 2 (control), 104 ± 5 (RET), 64 ± 3 (GL + RET) and 59 ± 3 (GA + RET). GPT: 40 ± 2 (control), 90 ± 7 (RET), 45 ± 2 (GL + RET) and 45 ± 4 (GA + RET) SF units/ml). Furthermore, no extensive hepatocellular damages were observed. The results demonstrated that a three-day pretreatment with either GL or GA exhibited protective effect on retrorsine-induced liver damage in rats.

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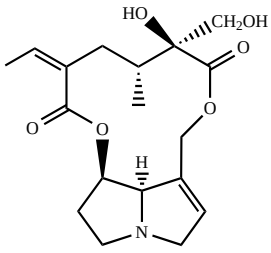
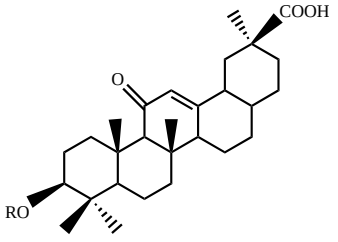
1. Introduction

Pyrrolizidine alkaloids (PAs) occur in a wide variety of plant species worldwide. Most naturally occurring PAs are cytotoxic and known to cause irreversible liver damage in animals (especially livestock) and man (Mattocks, 1968; Mattocks, 1986; Huxtable, 1989). PAs produce their hepatotoxicity only after being biotransformed in the liver to toxic pyrrolic metabolites. These pyrrolic metabolites then either rapidly bind cell macromolecules to produce hepatic toxicity or undergo further biotransformation into nontoxic metabolites (Mattocks, 1986; Huxtable, 1989; Mattocks and Jukes, 1990; Lin et al., 1998).

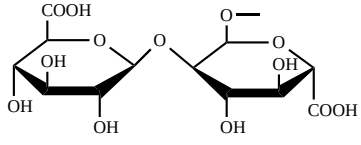
Various traditional Chinese medicines (TCM) contain PAs, for example, *Tussilago farfara* Linn and *Ligularia hodgsonii* Hook. In practice, TCM are commonly used as a combination of several herbs. Our recent investigation of the usefulness of PA-containing TCM has noted an interesting fact in that the PA-containing TCMs are always used concurrently with at least one medicinal herb, liquorice (*Glycyrrhiza glabra* Linn). Glycyrrhizin (GL) (Fig. 1) is the major biologically active component of liquorice, and its active aglycone, 18ß-glycyrrhetinic acid (GA) (Fig. 1) is also present in liquorice in a relatively small quantity. GL exhibits a number of pharmacological effects including antiinflammation (Finney and Somers, 1958; Ohuchi et al., 1981) and antihepatitis (Kiso et al., 1984; Takino, 1984). Pretreatment with glycyrrhizin has been reported



Retrorsine



Glycyrrhizin : R =



18β-Glycyrrhetinic acid : R = H

Fig. 1. Structures of retrorsine, glycyrrhizin and 18β-glycyrrhetinic acid.

to show protective action against carbon tetrachloride (CCl_4)-induced liver injury in rats (Kiso et al., 1984; Shibayama, 1989; Nose et al., 1994). However, there are no reports on the protective effects of glycyrrhizin on PA-induced hepatotoxicity.

In the present study, retrorsine (Fig. 1), one of the most potent hepatotoxic PAs (McLean, 1970; White et al., 1973), was used to induce liver injury in rats. Effects of GL and GA on retrorsine-induced hepatotoxicity in rats were examined. The results demonstrated that a three-day pretreatment with either GL or GA significantly reduced retrorsine-induced liver injury in rats.

2. Methods

2.1. Chemicals

Retrorsine, glycyrrhizin, 18β-glycyrrhetinic acid, glutathione (GSH), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), glutamate pyruvate transaminase (GPT) and glutamate oxaloacetate transaminase (GOT) kits were all purchased from Sigma Chemical Company (St. Louis, MO, USA). Ellman's reagent was freshly prepared as 1.5 mg/ml of DTNB in 0.5% $NaHCO_3$. Retrorsine (15 mg/ml) was dissolved in 0.1 M HCl followed by neutralization with an equivalent volume of 0.1 M NaOH and then diluted in normal saline. GL (20 mg/ml) was dissolved in saline and GA (2 mg/ml) was formulated in 1% Tween 80.

2.2. Protocol

Male Sprague Dawley rats (body weight, 200–250 g) were supplied by the Laboratory Animal Services Centre at The Chinese University of Hong Kong. Animals were placed in a controlled environment (50% relative humidity, temperature of 25°C and dark/light cycles) and maintained on standard rat chow.

Rats in four groups (six in each group) were intraperitoneally administered with different doses of retrorsine for the determination of dose-dependent liver injury. While, rats in other five groups were given with a same dose of retrorsine (35 mg/kg, i.p.), but sacrificed at different time intervals of 2, 12, 24, 48 and 72 h after dosing, in order to determine the time influence in liver injury.

For a single dose pretreatment, rats in two groups (10 in each group) were administered with either GL (200 mg/kg, i.p.) or GA (10 mg/kg, i.p.) 30 min prior to retrorsine (35 mg/kg, i.p.). Rats in another group were given GL (200 mg/kg, i.p.) with retrorsine (35 mg/kg, i.p.) simultaneously. Controls with normal saline, GL, and GA were conducted separately. In the case of multiple dose pretreatment, rats in different groups (10 in each group) were pretreated with GL (200 mg/kg/day, i.p.) or GA (10 mg/kg/day, i.p.) for three consecutive days, respectively. Retrorsine (35 mg/kg, i.p.) was then given at 30 min after the last dose of GL or GA. Various controls with a three-day treatment of GL, GA, and normal saline were also performed. All treated animals, except those for the

determination of time influence, were sacrificed at 24 h after retrorsine administration. Blood samples were collected by cardiac puncture, and livers were removed from the animals immediately after sacrifice.

2.3. Determination of serum transaminases and hepatic glutathione levels

Serum GPT and GOT levels were determined by a Sigma standard method using a commercially available kit with a Pharmacia-LKB 4060 ultraviolet–visible spectrophotometer (Reitman and Frankel, 1957). Sigma-Frankle unit (SF units/ml) was utilized for the measurements of GOT and GPT levels. One SF unit will form 4.82×10^{-4} μmol glutamate/min at pH 7.5 and 25°C. Hepatic GSH contents were measured by a standard spectrophotometric method using Ellman's reagent (Akerboon and Sies, 1991) with a modification of the buffer solution for sample preparation (White, 1976).

2.4. Histological assessment of liver damage

Liver portions from the treated animals were excised and fixed in 10% phosphate buffered formalin. Three or four paraffin sections (5 μm thick) per liver were prepared and stained with hematoxylin and eosin. Section preparation and assessment were conducted by professional pathologists (Victorian Veterinary Pathology Services, Vict., Australia).

2.5. Statistical analysis

All results were expressed as mean $\pm$ standard error of the mean. Analysis of variance (ANOVA) with Bonferroni/Dunn for post hoc analysis was used to compare results between different groups. A probability (p) of less than 0.05 was considered statistically significant.

3. Results

3.1. Retrorsine-induced hapatotoxicity

Single intraperitoneal dose of retrorsine induced a dose-dependent hepatic damage in rats (Table 1). A dose of 35 mg/kg, representing 83% of the acute LD_{50} to rats (White, 1976), produced remarkable liver damage indicated by significant elevations of serum levels of transaminases, the biochemical markers for the determination of liver injury (Woodman, 1981). Elevation of serum GOT and GPT levels occurred at 2 h and reached a maximum at 24 h after retrorsine administration (Table 1). Hepatic glutathione contents were also significantly increased after administration with a single dose of 30 mg/kg or higher dosages of retrorsine (Table 1). Moreover, morphological changes were extensive at 24 h after

Table 1
Serum GOT and GPT levels and hepatic GSH contents in rats measured after a single intraperitoneal dose of retrorsine

Dose (mg/kg)	GOT[a] (SF units/ml)	GPT[a] (SF units/ml)	GSH[a] (μmol/g)	Time (h)	GOT[b] (SF units/ml)	GPT[b] (SF units/ml)
Control	55.7 ± 1.6	40.3 ± 1.8	5.6 ± 0.1			
25	50.3 ± 2.9	44.0 ± 6.2	5.1 ± 0.4			
30	$83.1 \pm 8.2^{*}$	$71.9 \pm 5.4^{**}$	$7.9 \pm 0.2^{***}$			
35	$108.2 \pm 6.5^{***}$	$86.2 \pm 4.5^{***}$	$7.8 \pm 0.5^{***}$			
40	$112.3 \pm 9.1^{***}$	$99.6 \pm 7.6^{***}$	$8.2 \pm 0.3^{***}$	control	55.7 ± 1.6	40.3 ± 1.8
				2	$69.6 \pm 1.7^{*}$	36.9 ± 2.0
				12	$96.8 \pm 11.2^{***}$	42.5 ± 3.7
				24	$103.1 \pm 6.4^{***}$	$88.1 \pm 5.7^{***}$
				48	$86.4 \pm 6.3^{**}$	$53.7 \pm 6.0^{*}$
				72	$69.6 \pm 3.2^{*}$	$55.5 \pm 8.1^{*}$

[a] Measurements were conducted at 24 h after retrorsine administration.[b]Dose of retrorsine was 35 mg/kg.Data expressed as mean ± standard error of the mean ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ difference from the control.

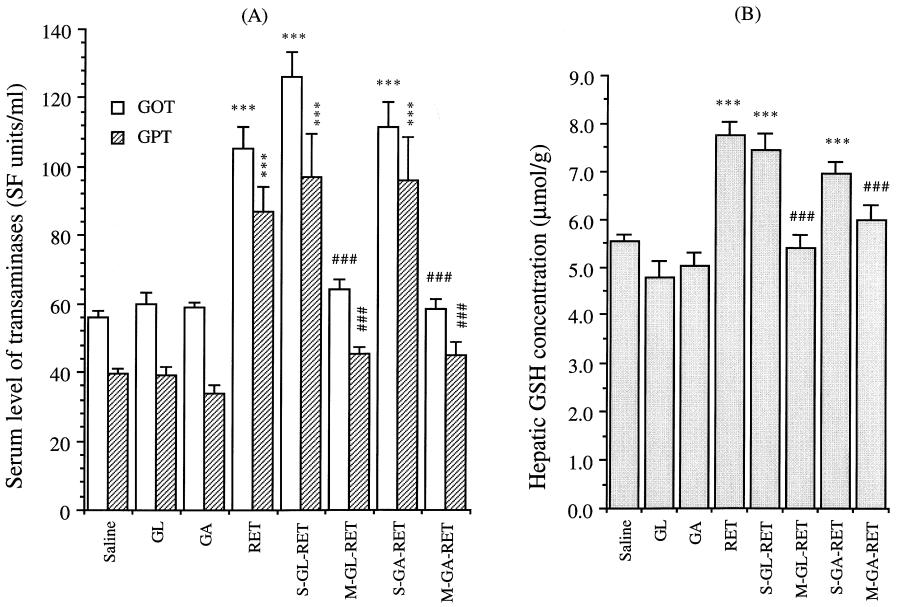


Fig. 2. Effects of a single dose and a three-day pretreatment with glycyrrhizin (GL) or 18β-glycyrrhetinic acid (GA) on the elevated serum GOT and GPT levels (a) and hepatic GSH contents (b) at 24 h after retrorsine (RET) administration ($n = 10$). S-GL-RET: single dose pretreatment with GL prior to retrorsine; M-GL-RET: multiple dose pretreatment with GL prior to retrorsine; S-GA-RET: single dose pretreatment with GA prior to retrorsine; M-GA-RET: multiple dose pretreatment with GA prior to retrorsine. $^{***}p < 0.001$ difference from the control. $^{\#\#\#}p < 0.001$ difference from the retrorsine dosed rats.

a single dose of retrorsine (Table 2). Significantly, vacuolated hepatocytes in the periportal area, apoptotic bodies and hepatocellular necrosis were observed. Basophilic granules in periacinar hepatocytes, which represent endoplasmic reticulum in the control liver, were lost in the liver of rats treated with retrorsine. Although a mild degree of variation in cell and nuclear size of hepatocytes are normal features of rat livers, such variations were markedly extensive in the livers of the treated rats. Consequently, a single dose of 35 mg/kg of retrorsine was chosen for the induction of liver injury. Measurements of hepatic GSH, serum GPT and GOT levels, and assessments of hepatic morphological changes were conducted at 24 h after retrorsine chanllege for the determination of effects of pretreatment with GL and GA on retrorsine-induced hepatotoxicity in rats.

3.2. Single dose pretreatment

As shown in Fig. 2, a single dose pretreatment of either GL or GA at 30 min prior to retrorsine challenge did not alter the elevated serum levels of transaminase induced by retrorsine. Retrorsine raised hepatic content of GSH was

Table 2
Effects of different pretreatment with glycyrrhizin (GL) and 18β-glycyrrhetinic acid (GA) on morphological changes of rat liver obtained 24 h after retrorsine (RET) administration

Treatment	Basophilic granules in periaciner hepatocytes	Vacuolated hepatocytes in periportal area	Variation in cell and nuclear size	Apoptotic bodies within hepatocytes	Single cell necrosis
Single dose pretreatment					
Control	+ + +	+ +	+	−	−
GL	+ +	+	+	−	−
GA	+ +	−	+	−	−
RET	−	+ + +	+ + +	+ +	+ + +
GL + RET	+	+	+ + +	+ +	+ +
GA + RET	+	+	+ +	+ +	+ +
Multiple dose pretreatment					
Control	+ + +	+ +	+	−	−
GL	+ +	+ +	+	−	−
GA	+ +	+ +	+	−	−
RET	−	+ + +	+ + +	+ +	+ + +
GL + RET	+	+ +	+ +	±	±
GA + RET	+	+	+ +	+	+

Data expressed as the mean of five specimens for each treated group ($n = 5$). +, mild; + + moderate; + + +, marked; −, negative; ±, less than three livers show a mild change.

not affected either. Furthermore, similar morphological changes induced by retrorsine alone were still observed in the livers of the rats pretreated with a single dose of either GL or GA prior to retrorsine exposure (Table 2). In addition, a simultaneous administration of GL with retrorsine also resulted in significant elevations of serum levels of transaminase (128.0 ± 7.2 SF units/ml versus 56.0 ± 1.7 (control) for GOT and 101.3 ± 4.0 versus 39.6 ± 1.6 (control)). Hence, the results indicated that a single dose of active liquorice ingredients given either simultaneously with or 30 min prior to retrorsine challenge did not prevent retrorsine-induced hepatotoxicity.

3.3. Multiple dose pretreatment

Multiple dose pretreatment with GL (200 mg/kg/day, 3 days) or GA (10 mg/kg/day, 3 days) exhibited a protective effect against retrorsine-induced liver damage as demonstrated by significant reduction of the elevated serum levels of transaminases (Fig. 2). The elevation of hepatic contents of GSH stimulated by retrorsine were abolished when rats were pretreated with glycyrrhizin or its aglycone for three days prior to retrorsine challenge (Fig. 2). In addition, histological evaluations provided further support for the hepatoprotective effects of a three-day pretreatment with GL and GA against retrorsine-induced liver damage. As shown in Table 2, distinct pathological changes in liver cells did not occur, and only mild to moderate cell vacuolation and variations in hepatocyte size were observed.

4. Discussion

In the present study, GL and GA pretreatment were performed intraperitoneally at 30 min prior to retrorsine challenge. Since the bioavailability of glycyrrhizin is much higher for intraperitoneal administration than that via oral route, and its absorption reaches a maximum level within 30 min after intraperitoneal administration (Yamamura et al., 1995). For rats treated with a single dose of glycyrrhizin either simultaneously with or 30 min prior to retrorsine challenge, the elevated serum GOT and GPT levels and hepatic GSH contents induced by retrorsine was sustained (Fig. 2). Extensive morphological changes, including periportal hepatocellular necrosis and apoptosis within hepatocytes caused by most of the hepatotoxic PAs (Moore et al., 1989), were observed in the livers of treated rats (Table 2). The results suggested that a single dose of either GL or GA did not produce protective effect on retrorsine-induced hepatotoxicity in rats.

A three-day pretreatment with GL (200 mg/kg/day) or GA (10 mg/kg/day) exhibited protective action against retrorsine-induced hepatotoxicity. As shown in Fig. 2, serum levels of transaminase induced by retrorsine were significantly reduced and almost back to normal in the treated rats. In addition, significant

pathological changes were not observed, and only mild to moderate variations in hepatic cell size and hepatocyte vacuolation were seen (Table 2). Furthermore, a multiple dose pretreatment of GL or GA inhibited elevation of hepatic GSH content induced by retrorsine (Fig. 2). It is well demonstrated that, unlike other hepatotoxins such as paracetamol, which depletes glutathione contents at its toxic dose (Mitchell et al., 1973; Jaeschke, 1990), administration of a toxic dose of PAs significantly increases hepatic GSH levels in rats via stimulation of its biosynthesis within 24 h after PA-exposure. Glutathione conjugation with toxic pyrrolic metabolites of PAs is one of the detoxification pathways, and thus an increase in hepatic GSH contents may represent a self-defense mechanism in preventing hepatotoxicity (Mattocks and White, 1973; White, 1976; Yan and Huxtable, 1995). Result obtained from the present study showed that a toxic dose of retrorsine significantly increased hepatic GSH levels at 24 h (Table 1), and this is in good agreement with previously published observations (White, 1976; Yan and Huxtable, 1995). However, it is interesting to note that after a three-day pretreatment with both GL and GA, the elevation of hepatic GSH contents induced by retrorsine was abolished. Inhibition of increase in hepatic GSH may suggest that less toxic pyrrolic metabolties were formed and thus biosynthesis of GSH was not significantly stimulated. This result further indicated that multiple dose pretreatment with both GL and GA produced hepatoprotective action against PA-induced liver damage in rats. The present study did not attempt to compare the potency of GL with its aglycone, since different doses related to their therapeutic uses in traditional Chinese medicine practices were utilized in the experiments.

GL and GA have been previously reported to produce hepatoprotection against CCl_4-induced liver injury (Shibayama, 1989) and anti-chronic hepatitis action (Yamamura et al., 1995). In Japan, Glycyrrhizin has been clinically used for the treatment of chronic hepatitis B, and as a supplement to interferon in treating nonresponse patients undergoing interferon therapy (Suzuki et al., 1983; Fujisawa et al., 1973; Sato et al., 1996). Antihepatotoxicity of GL and its aglycone on CCl_4-induced liver injury has been reported to be due to their anti-oxidative actions (Kiso et al., 1984; Takino, 1984; Shibayama, 1989; Nose et al., 1994). Whereas, suppression of secretion of hepatic B virus surface antigen (HBsAg) from the hepatocytes by GL was suggested as the therapeutic basis for the treatment of chronic hepatitis B (Sato et al., 1996). However, the mechanism of protective effects of GL and GA against PA-induced hepatotoxicity is unknown. Previous studies have demonstrated that alteration in metabolism of retrorsine in rats significantly changed its acute toxicity (Allen et al., 1972). Levels of hepatic tissue-bound pyrrolic metabolites of PA correlated with liver toxicity (Yan et al., 1995). The formation of pyrrolic metabolites and covalent binding of such toxic metabolites to hepatic macromolecules are the key steps in causing PA-induced hepatotoxicity (Mattocks, 1986; Huxtable, 1989; Mattocks and Jukes, 1990). Therefore, inhibition of formation of toxic pyrrolic metabolites via modifications of metabolism of PAs can prevent PA-induced liver injury (Mattocks, 1986; Buhler and Kedzierski, 1986; Williams et al., 1989).

In the preset study, the observed diverse effects between a single and multiple dose pretreatment with glycyrrhizin and its aglycone on retrorsine-induced hepatotoxicity are interesting. A single dose pretreatment normally can not affect activities of metabolizing enzymes including those responsible for generation of toxic metabolites of retrorsine, and hence could not produce protection. However, a prolonged pretreatment can either induce or inhibit enzyme activities via alteration of enzyme biosynthesis. Inhibition of specific enzymes, which mediate the formation of toxic pyrrolic metabolites, will reduce liver damages. Thus, hepatoprotection of a three-day pretreatment with GL and GA may be at least partially due to modifications of metabolism of PAs in the body. Furthermore, inhibition of the retrorsine-elevated hepatic GSH level may also suggest that less toxic pyrrolic metabolites were produced after a three-day pretreatment of active liquorice ingredients. However, the detailed mechanism(s) of the effects of glycyrrhizin and its aglycone on retrorsine metabolism and of their hepatoprotective action with a multiple dose pretreatment need to be clarified. Further works aimed at investigating the effects of GL and GA with different pretreatment on the metabolic fate of retrorsine and the enzymes mediating the formation of toxic metabolites are currently in progress in our laboratories.

In summary, the present study demonstrated that a three-day pretreatment with GL and GA produced hepatoprotective effect against retrorsine-induced liver damage in rats. However, a single dose pretreatment with these active ingredients did not show such protection. Therefore, liquorice may have a potential role to play in protecting animals and humans against PA-induced hepatotoxicity if it is used appropriately.

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