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Protective effect of oleanolic acid against chemical-induced acute necrotic liver injury in mice[1]

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AIM: To evaluate the protective effect of oleanolic acid (OA) against necrotic liver injury. **METHODS:** Mice were pretreated with OA (200 μmol · kg^{-1}, sc for 3 d), and subsequertly administered hepatotoxicants. Liver damage was asessed by quantifying serum activities of alanine aminotransferase and iditol (sorbitol) dehydrogenase, as well as by histopathological examination. **RESULTS:** OA pretreatment dramatically diminished CCl_4-, bromobenzene-, acetaminophen-, phalloidin-, and cadmium-induced liver injury, and decreased the hepatotoxicity of *D*-galactosamine plus endotoxin, thioacetamide, furosemide, and colchicine. However, OA had no effect on the toxicity of dimethylnitrosamine, α-amanitin, chloroform, and allyl alcohol. **CONCLUSION:** OA protects against many, but not all, hepatotoxicants, and the hepatoprotective effect of OA may involve multiple mechanisms.

KEY WORDS oleanolic acid; liver; carbon tetrachloride poisoning; acetaminophen; bromobenzenes; phalloidine; cadmium poisoning; galactosamine; endotoxins; thioacetamide

Oleanolic acid (OA) is a triterpenoid compound that exists widely in food, medicinal herbs and plants[1]. The hepatoprotective effect of OA was first reported in China[2]. OA protected against CCl_4-induced acute liver injury[2,3], and was used to treat liver disease in humans[1,2]. To evaluate the hepatoprotective effects of OA, and to obtain clues for the mechanism of protection, we used 13 different hepatotoxicants to determine the effect of OA on chemical-induced necrotic liver injury in mice.

MATERIALS AND METHODS

Chemicals and mice OA was obtained from Guiyang Pharmaceutical Co (China), with approximate 99 % purity. Bromobenzene, thioacetamide, and allyl alcohol were obtained from Aldrich Chemical Co (Milwaukee WI). Acetaminophen, dimethylnitrosamine, furosemide, phalloidin, α-amanitin, colchicine, *D*-galactosamine (*D*-GalN), and lipopolysaccharides (LPS, endotoxin) were obtained from Sigma Chemical Co (St Louis MO). Chloroform, CCl_4, and cadmium chloride were obtained from Fischer Chemical Co (Fair Lawn NJ). All other chemicals were of reagent grade. CF-1 mice ♂ 26 ± *s* 2 g were housed in an environmentally controlled room at 21 ± 1 C with a 12-h light/dark cycle. Food and tap water were provided *ad lib*.

Experimental design Mice were pretreated with OA (200 μmol · kg^{-1}, sc, suspended in 2 % Tween 80 in saline), or vehicle for 3 d. One hour after the last injection, the mice were injected with one of the following hepatotoxicants: CCl_4 (15 μL · kg^{-1}, ip), bromobenzene (0.7 mL · kg^{-1}, ip), acetaminophen (500 mg · kg^{-1}, ip), furosemide (250 mg · kg^{-1}, ip), thioacetamide (120 mg · kg^{-1}, ip), dimethylnitrosamine (45 mg · kg^{-1}, ip), allyl alcohol (90 mg · kg^{-1}, ip), or chloroform (1.0 mL · kg^{-1}, *po*). The effects of OA on liver injury produced by these hepatotoxicants were determined 24 h after their administration. However, the effects of OA on phalloidin (1.5 mg · kg^{-1}, ip)- and colchicine (200 mg · kg^{-1}, ip)-induced hepatotoxicity were measured 6 — 8 h, the

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hepatotoxicity of $CdCl_2$ (3.7 mg · kg^{-1}, iv), and *D*-GalN (500 mg · kg^{-1}, ip) together with LPS (150 μg · kg^{-1}, ip), were determined 15 h, whereas its effect on α-amanitin (0.5 mg · kg^{-1}, ip) was determined 48 h after administration of hepatotoxicant. Furosemide was suspended in 2 % Tween 80 in saline, bromobenzene, and chloroform were dissoved in corn oil, and all the other chemicals were prepared in saline. All treatments were in the volume of 10 mL · kg^{-1}.

Blood and liver samples Mice were decapitated. A piece of the liver was put into 10 % formalin (buffered with NaH_2PO_4 29 and Na_2HPO_4 45.8 mmol · L^{-1}, pH 7.4). Serum was prepared by allowing blood samples to coagulate on ice for 2—4 h followed by centrifugation (10 000 × *g*, 5 min).

Hepatotoxicity Serum activities of alanine aminotransferase (ALT) and iditol (sorbitol) dehydrogenase (IDH), as well as histological examination were used as means for assessing liver damage. The activity of ALT was measured using a commercial kit (Sigma 59-UV, St Louis MO), and IDH was measured as described previously[4]. The fixed liver samples were processed by standard histological technics and stained with hematoxylin and eosin. The slides were examined by light microscopy, and the extent of liver necrosis was scored[5]: 0= absent, 1+= necrosis of less than 6 % of the parenchymal hepatocytes, 2+= necrosis 6 %—25 % of hepatocytes, 3+ = necrosis 26 %—50 % of hepatocytes, and 4+ = necrosis more than 50 % of hepatocytes.

Statistics Comparison between control and OA-treatment groups was made by *t* test. Liver histopathological data were analyzed by Kruskal-Wallis nonparametric test.

RESULTS

Effect of OA against liver injury produced by chemicals that require P450 activation The liver damage produced by some of these chemicals was so severe that mortality occurred; for example, 22 % of the control mice given thioacetamide and 35 % given bromobenzene died, wheras no mortality was seen in OA-pretreated mice.

As a result of parenchymal cell death, liver specific enzymes, such as ALT and IDH, were released into blood, and thus markedly elevated (20 to 150 times for ALT, 10 to 40 times for IDH). OA pretreatment diminished dramatically bromobenzene-, acetaminophen-, and CCl_4-induced liver injury ($P<0.01$), as indicated by approxinatelly 80 % decreases in elevated serum ALT and IDH activities, as well as by decreasing parenchymal cell necrosis. The widespread necrosis produced by bromobenzene, was ameliorated by OA (Fig 1A, B, Plate 1). OA pretreatment also decreased thioacetamide and furosemide hepatotoxicity ($P<0.01$); the elevated serum enzyme activities were decreased approximately 65 % by OA. However, OA had no effect on chloroform- and dimethylnitrosamine-induced liver injury ($P>0.05$) (Tab 1).

Effect of OA against liver injury produced by mushroom toxins and colchicine α-Amanitin and phalloidin are 2 major toxins from *Amanitia phalloides*. OA-preteatment had no protective effect against α-amanitin-induced hepatotoxicity($P>0.05$), but it diminished dramatically phalloidin-induced liver injury ($P<0.01$). Phalloidin caused severe liver congestion and necrosis (Fig 1C), and 25 % of the control mice died from hepatic failure within 8 h. OA pretreatment prevented phalloidin-induced mortality, liver congestion and necrosis (Fig 1D), as well as decreased phalloidin-induced elevation of serum enzyme activities by more than 90 %. Colchicine also produces liver injury within 8 h, similar to that observed after phalloidin. OA pretreatment also decreased chochicine-induced liver injury ($P<0.01$), as indicated by a 75 % decrease in elevated serum enzyme activities produced by colchicine (Tab 1).

Effects of OA against liver injury produced by other types of hepatotoxicants

Tab 1. Effect of oleanolic acid (OA) on liver injuries. $\bar{x}\pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* control.

	Group	Mice	Mortality/%	Serum ALT/U·L^{-1}	Serum IDH/kU·L^{-1}	Necrosis/grade
Saline	Control	8	0	55±11	340±50	0
	OA	8	0	54±9^a	335±55^a	0
Bromobenzene	Control	26	35	2840±2550	5980±4700	2.36±0.86
	OA	21	0	560±720^c	1070±1330^c	1.06±0.82^c
Acetaminophen	Control	20	0	4750±2970	9180±5250	2.85±0.88
	OA	10	0	370±260^c	1060±520^c	1.01±0.67^c
Carbon tetrachloride	Control	13	0	1840±1900	7590±7200	2.08±0.64
	OA	8	0	420±480^c	1140±750^c	0.75±0.71^c
Thioacetamide	Control	23	22	7050±3700	12900±6400	3.46±0.85
	OA	18	0	2120±2200^c	4440±4500^c	1.53±0.93^c
Furosemide	Control	15	0	8360±6960	12100±9900	3.35±0.65
	OA	15	0	2800±3250^c	4207±5200^c	1.63±0.86^c
Chloroform	Control	16	13	820±910	2890±1680	1.50±0.52
	OA	15	0	810±890^a	2670±810^a	1.47±0.50^a
Dimethylnitrosamine	Control	11	0	3170±2900	5060±5390	3.45±0.70
	OA	10	0	2240±1530^a	3470±2070^a	3.10±0.63^a
α-Amanitin	Control	19	5	1820±2050	4280±3590	1.60±0.74
	OA	15	0	1570±1280^a	3830±2840^a	1.56±0.72^a
Phalloidin	Control	12	25	5590±5650	10500±8700	3.22±1.08
	OA	11	0	290±170^c	800±440^c	0.77±0.46^c
Colchicine	Control	18	0	4680±4170	5700±4780	1.98±0.95
	OA	15	0	1240±1200^c	1500±930^c	1.13±0.45^c
$CdCl_2$	Control	18	22	7520±6310	11600±8300	2.71±1.27
	OA	10	0	710±510^c	1300±1100^c	0.90±0.32^c
Allyl alcohol	Control	10	10	2720±3740	3850±4920	1.73±1.30
	OA	10	10	2250±2970^a	3080±3550^a	1.57±0.96^a
D-GalN/LPS	Control	23	35	9680±8190	21100±14400	3.46±0.45
	OA	22	0	3210±3900^c	6750±7810^c	1.55±0.53^c

OA pretreatment decreased $CdCl_2$-induced mortality and liver injury ($P<0.01$); liver congestion and necrosis produced by $CdCl_2$ were ameliorated by OA, and the elevated serum enzyme activities were decreased by 90 %. In contrast, OA had no protective effect against allyl alcohol-induced liver injury ($P>0.05$). When *D*-GalN was combined with endotoxin (*D*-GalN/LPS), fulminant hepatitis occurred as indicated by widespread parenchymal cell neocrosis and congeation (Fig 1E), and 35 % mortality was seen in the control mice. OA pretreatment prevented *D*-GalN/LPS-induced mortality, and decreased elevated serum enzyme activities, as well as parenchymal cell necrosis and congestion (Fig 1F, Tab 1) ($P<0.01$).

DISCUSSION

A number of chemicals can induce liver injury and are referred to as hepatoxicants. Hepatic injury depends not only on the chemicals involved, but also on the period of exposure. After acute exposure, the major lesions are steatosis, cholestasis and necrosis, whereas cirrhotic and neoplastic changes are usually the result of chronic exposure[6]. Necrosis is the most common lesion induced by acute exposure to hepatotoxic chemicals. Necrosis is a serious lesion because it is not spontaneously reversible (as opposed to steatosis), and often leads to fibrosis[6]. As a consequence, control

of acute necrosis has become a major goal of hepatic therapy, and intensive efforts are being made to develop anti-necrotic hepatoprotective agents. Therefore, to determine whether the necrosis produced by various hepatotoxicants can be prevented by OA is of pharmacological and toxicological significance.

Necrosis produced by hepatotoxicants is an extremely complex phenomenon. First of all, many hepatotoxicants must be activated in the liver, especially by the cytochrome P 450 system, to form reactive metabolites that produce liver injury in experimental animals and humans[7,8]. In our studies, CCl_4, acetaminophen, bromobenzene, thioacetamide, furosemide, dimethylnitrosamine and chloroform are all cytochrome P450-dependent hepatotoxicants[7,8]. OA pretreatment protects mice aganst five of these hepatotoxicants, but has no effect on the hepatotoxicity of chloroform and dimethylnitrosamine. Therefore, the hepatoprotective effect of OA may be attributed, but not entirely, to the suppression of P450. As various hepatotoxicants are metabolized by different P450 isozymes, whether OA suppresses specific forms of P450 requires further investigation.

CCl_4 is a well known example of a chemical that produces free radical-mediated liver injury[9]. In contrast, chloroform ($CHCl_3$) is different from CCl_4 in that its major metabolite is phosgene, which binds covalently to tissue proteins[7]. OA protects mice against the hepatotoxicity of CCl_4, but not $CHCl_3$, which supports the suggestion that $CHCl_3$ produces liver injury via mechanism(s) different than does CCl_4[10].

Acetaminophen and bromobenzene are well known examples of chemicals that produce hepatotoxicity by covalent binding via their reactive metabolites, *N*-acetyl-*p*-benzoquinoneimine (NAPQI) and bromobenzene-3,4-oxide, respectively[11]. The arylation of liver macromolecules by a reactive metabolite of furosemide is also causally related to the development of hepatic necrosis[5]. OA pretreatment protects against the hepatotoxicity of all three of these hepatotoxicants, suggesting that (1) the metabolic activation of these toxicants is suppressed, (2) the covalent binding of their reactive intermediates is reduced, and/or (3) the chain of events following the initial toxic insult is inhibited by OA.

Thioacetamide and dimethylnitrosamine are both carcinogens. TA is activated by P450 mediated *S*-oxidation, whereas dimethylnitrosamine is activated by P450 and non-P450 enzymes to methyldiazonium hydroxide and diazomethane[7]. Both thioacetamide and dimethylnitrosamine produce acute liver necrosis. OA pretreatment decreased the hepatotoxicity of thioacetamide but not of dimethylnitrosamine. The results suggest that either the bioactivation or the mechanism(s) of toxicity for dimethylnitrosamine may be different from that for thioacetamide.

Some hepatotoxicants do not require metabolic activation. Phalloidin and α-amanitin are hepatotoxins from the mushroom *Amanitia phalloides*[12]. However, the mechanism(s) by which they produce liver injury are different. Phalloidin is a fast-acting (1-8 h) toxin, which produces liver damage by disrupting the organization of the cellular cytoskeleton[12]. In contrast, α-amanitin is a slow-acting toxin (15-48 h), and acts as a selective inhibitor of RNA polymerase II[13]. OA pretreatment protects against the hepatotoxcity of phalloidin, but not of α-amanitin. The results suggest that OA may have a protective effect on phalloidin-induced disorganization of the cytoskeleton. This suggestion is strengthened by its protective effect against a

hepatotoxic dose of colchicine, which acts similarly as phalloidin to disrupt cytoskeletal organization.

Cadmium, a heavy metal, produces acute liver necrosis by binding to critical cellular organelles or proteins, thus compromising enzyme activities in these organelles. Metallothionein, a low-molecular weight, cysteine-rich protein, has been proposed to play an important role in the detoxication of Cd. OA protection against Cd-induced liver injury is thought to be due, at least in part, to the induction of metallothionein[4].

Allyl alcohol is a P450-independent hepatotoxicant. It is biotransformed by alcohol dehydrogenase to acrolein, which causes oxidative damage to hepatocytes, thus leading to cell death[14]. However, OA is not effective in preventing allyl alcohol-induced liver damage.

D-GalN produces acute liver injury by depleting UTP and UDP-glucose. When *D*-GalN is combined with endotoxin (LPS), they act synergistically to produce fulminant hepatitis. *D*-GalN/LPS-induced liver injury is thought to be mediated by leukotrienes, oxidative stress, and/or tumor necrosis factor, as well as by activated macrophages[15]. OA protection against *D*-GalN/LPS-induced liver injury is probably due, at least in part, to the anti-inflammatory effects of OA.

In summary, the present study demonstrates that OA pretreatment protects mice against the hepatotoxity of CCl_4, bromobenzene, acetaminophen, furosemide, thioacetamide, cadmium, phalloidin, colchicine, *D*-GalN plus LPS, but had no protective effect against dimethylnitrosamine-, α-amanitin-, chloroform-, and ally alcohol-induced liver injury. The results indicate that OA protects mice from many (9/13), but not all hepatotoxicants. Multiple mechanisms may be involved for the hepatoprotective effect of OA, which requires further investigation.

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齐墩果醇酸对化学物质致小鼠急性肝损伤的保肝作用

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目的：评价齐墩果醇酸(OA)对急性肝损伤的保肝作用. **方法**：小鼠 sc OA 200 μmol·kg⁻¹三天，然后给予肝毒物. 通过病理组织学观察及测定血清丙氨酸转氨酶和艾杜糖醇脱氢酶活性来估价肝损伤. **结果**：OA 能明显减轻四氯化碳，溴苯，醋氨酚，速尿，硫代乙酰胺，鬼笔毒环肽，秋水仙硷，氯化镉，D-半乳糖胺和内毒素等所致小鼠急性坏死性肝损伤，降低这些肝毒物所引起的血清转氨酶和艾杜糖醇脱氢酶的升高，但对氯仿，二甲亚硝氨，鹅膏菌素和烯丙醇的毒性无作用. **结论**：OA 能减轻多种化学物质(但并非全部)引起的肝损伤. 其保肝机制可能是多方面的.

关键词 齐墩果醇酸；肝；四氯化碳中毒；醋氨酚；溴化苯类；鬼笔毒环肽；镉中毒；半乳糖胺；内毒素；硫代乙酰胺

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Simultaneous determination of *N*,*N*-di(*n*-butyl)doxorubicin-14-valerate and its 8 urinary metabolites by HPLC

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AIM: To develop an HPLC assay for simultaneous determination of AD202 and its metabolites and to examine metabolites of AD202 in urine of rats. **METHODS**: Reverse-phase HPLC with fluorescence detection and gradient elutionusing a Waters Chromatograph equipped with a 710 B WIFP autosampler, a power Mate SX plus computer, C_{18} Nova-pak™ (4 μm) 5 mm × 10 cm radial compression column connected with a guard micro-column, and a Waters 991 photodiode array detector for online observation of UV spectrum of related fraction. **RESULTS**: Detection limit was 1-3 ng for AD202 and 1-3 ng for its metabolites per injection. After iv AD202 20 mg·kg⁻¹, only 4.9 % dose as total anthracycline fluorescence signal was recovered in urine over 72 h. The predominant urinary metabolites were AD285 (desacyl AD202) and AD284 (*N*-mono-debutyl AD285). Six minor metabolites including aglycones and 13-keto reductive product were identified and 3 as-yet unknown metabolites were seen. Enzymatic and acid-hydrolysis failed to reveal the presence of glucuronides in urine. **CONCLUSION**: The sample analysis techniques developed in this study proved to

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