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Protective effects of polydatin against CCl_4-induced injury to primarily cultured rat hepatocytes *

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Subject headings polydatin; injury, hepatocyte; $CC1_4$

Abstract

AIM To investigate the protective effects of polydatin (PD) against injury to primarily cultured rat hepatocytes induced by CCl4.

METHODS Rat hepatocytes were separated by methods of liver infusion in vivo and cultured medium (7.5×10^5 cells/mL). Two mL or 0.2mL was added into 24-well or 96-well plates respectively. Twenty-four hours after cell preculture, PD at concentrations of 10^{-7} mol/L-10^{-4} mol/L was added into each plate. At the same time injury to hepatocytes was induced by adding 10mmol/L-CCl_4. Then, 0.1mL or 1mL-culture solution was removed from the 96-well or 24-well plates at 6h, 12h, 2 4h and 48h after CCl_{14} intoxication respectively for the determination of GPT, GSH and MDA. At 48h, the survivability of rat hepatocytes was assayed by the MTT colormetric method.

RESULTS After CCl4 challenge, the release of GPT and the form ation of MDA in rat hepatocytes markedly increased and maintained at a high level in 48h, whereas PD with different concentrations could markedly inhibit this elevation with 10^{-5}mol/L PD having the strongest effects and inhibiting rate was over 50%. PD could also improve the decreased content of GSH caused by CCl_4 in accordance with the doses used. CCl_4 evidently decreased the he patocyte survivability from 91.0%±7.9% to 35.4%±3.8%. On the other hand, PD at 10^{-7}mol/L-10^{-4}mol/L could reverse this change and improve t he cell survival rates to 56.1%±5.2%, 65.8%±5.0%, 88.7%±6.8% and 75.2%±7.3%, respectively.

CONCLUSION PD at 10^{-7} mol/L-10^{-4} mol/L could protect primarily cultured rat hepatocytes against CCl4 induced injury.

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INTRODUCTION

Polygonum cuspidatum Sieb. et Zucc. (Polygonaceae) is a traditional Chinese herbal drug, with bitter taste and cold nature. It mainly acts upon the liver, gallbladder and lung meridians. It is well known that *P. cuspidatum* has various activities such as promoting blood circulation, relieving swelling and pain, eliminating phlegm, alleviating cough, clearing away heat, and removing dampness and toxin. The drug has been widely used for cardiovascular and liver diseases. Its active compounds mainly consist of free anthraquinones which include emodin, physcion and chrysophanol. Another important compound is resveratrol[1].

Polydatin (PD), 3, 4', 5-trihydroxystibene-3-β-mono-D-gluc oside, also named piceid, is the glycoside of resveratrol[1]. Some previous studies demonstrated that PD could lower the level of blood lipid, inhibit the platelet aggregation, dilate blood vessels, protect cardiocytes, reduce cerebral ischemic damage and inhibit lipid peroxidation[2-6]. However, the effects of PD on hepatocytes and its mechanisms have not been reported up to date. In this paper we report the details of protective effects of polydatin against injury to primarily cultured rat hepatocytes induced by CCl_4.

MATERIALS AND METHODS

Materials

Collagenase (type IV), 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), dexamethasone, *N*-2-hydroxyethyl-piperazine-*N*'-2'-ethane sulfonic acid (HEPES), insulin, penicillin and streptomycin were purchased from Sigma Chemical Corp (St. Louis, USA). RPMI 1640 was

a product of Gibco Life T echnologies INC (Grand Island, NY). Fetal calf serum was obtained from Institute of Hemopathy, Chinese Academy of Medical Sciences (Tianjin). PD (Purity>90%), which was isolated from the root and rhizome of *P. cuspidatum*[7], provided by the Department of Chemistry, the First Military Medical University.

Animals

Wistar rats, male, 6 weeks old, weighing 160 g-180 g, were used for hepatocyte isolation. They were provided by Laboratory Animal Center, Guangzhou Un iversity of TCM.

Isolation and culture of rat hepatocytes

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Then the liver parenchymal cells of rat were isolated by the collagenase perfusion method following the procedure of Seglen and Koji[8,9]. Simply, the portal vein of rat liver was exposed and cannulated with a teflon catheter. The liver was perfused with Ca^{2+} free solution containing NaCl 142, KCl 6.7, HEPES 10, NaOH 5.5 (mmol/L), pH 7.4, at 37℃, with a flow rate of 40 mL/min. Twelve minutes later, recirculation started with collagenase solution composed of NaCl 67, KCl 6-7, $CaCl_2 \cdot 2H_2O$5, HEPES 100, NaOH 66, collagenase 0.2 g/L, pH 7.6. Isolated cells were cultured in RPMI 1640 containing 100mL/L-fetal calf serum, 10mmol/L-HEPES, 100kU/L-penicillin and streptomycin, 10mmol/L insulin and 10mmol/L- dexamethasone. The content of hepatocytes was adjusted to 7.5×10^8 cells/ L with the above medium. Cultured medium 2mL and 0.2mL were added into 24-well and 96-well plates respectively. The cells were incubated for 4h at 37℃ under 50mL/L CO_2 in air. Non-adherent hepatocytes were eliminated by replac ing the medium, and adherent hepatocytes continued to be incubated, and the medium was changed every 24 h.

CCl_4-induced hepatocytes injury

After pre-culture for 24 h, the hepatocytes were exposed to fresh medium containing 10mmol/L-CCl_4 and various concentrations of PD. At 6, 12, 24 and 48 h after CCl_4 intoxication, 0.1 mL and 1 mL culture solution were removed from 96-well and 24-well plates respectively for determination.

Measurement of glutamic pyruvic transaminase (GPT)

The kits of GPT analysis, provided by the Shanghai Institute of Biological Products of Ministry of Health, were used to measure the activity of GPT in 0.1 mL- culture medium.

Determination of reduced glutathione (GSH) and malondialdehyde (MDA)

Utilizing the kits of GSH analysis and the kits of MDA analysis, all purchased from Nanjing Jiancheng Bio-engineering Institute, the content of GSH in 1mL culture medium and the level of MDA in 0.1mL culture medium we re measured.

Cell survivability assay

The survivability of rat hepatocytes was assayed by the MTT colormetric method[10]. At 48h after CCl_4 challenge, 20 μL/well- MTT stock solution (5 g/L) was added into each well of 96-well plates. The cells were continuously incubated for another 4 h before 0.1 mL/well dimethyl sulfoxide was added to all wells and mixed thoroughly to dissolve the brown-black crystals. The plates were read on microplate reader, using a test wavelength of 570 nm with a reference wavelength of 655 nm.

Statistical analysis

The results were expressed as $\bar{x} \pm s$ and significant difference was a ssessed by Student's *t* test.

RESULTS

Effects of PD on GPT activity in culture medium

The concentration of GPT in culture medium significantly increased after CCl_4 challenge, and maintained at a high level in 8 h (Table 1). Furthermore, a progressively elevated trend existed with time-dependence. PD could significantly inhibit the level of GPT in accordance with the doses used. Especially, PD 10 μmol/L had the strongest effects and the inhibiting rate was over 50%.

Table 1 Effect of PD on GPT activity in culture medium ($\bar{x} \pm s, n = 8$)

Group	c/(mol/L)	GPT(U) 6 h	12 h	24 h	48 h
Normal		13.5±2.5[b]	13.8±3.1[b]	13.7±5.6b	14.1±3.3b
Control		72.3±14.1	79.7±10.3	85.4±9.2	88.3±19.6
PD	10^{-7}	60.3±17.1[a]	62.0±15.6[a]	68.8±17.5[a]	71.4±20.5[a]
PD	10^{-6}	55.0±10.3[a]	58.3±16.7[a]	64.1±13.6[a]	69.1±19.2[a]
PD	10^{-5}	30.6±10.6[b]	38.3±5.5[b]	42.5±7.0[b]	45.0±7.6[b]
PD	10^{-4}	42.1±7.8[a]	47.5±9.8[a]	56.8±11.3[a]	59.2±10.7[a]

[a]$P<0.05$, [b]$P<0.01$, *vs* CCl_4-treated control group.

Effects of PD on GSH content in culture medium (Table 2).

The content of GSH in culture medium decreased obviously as compared with that in normal hepatocytes after 6 h incubation with CCl_4 (Table 2). On the other hand, PD of various concentrations could

improve GSH in a dose-dependence manner, and 10 μmol/L PD showed a most signifi cant activity.

Table 2 Effects of PD on GSH content in culture supernatant ($\bar{x}\pm s$, n = 8)

Group	c/(mol/L)	GSH (ng/L) after CCl_4 challenge			
		6 h	12 h	24 h	48 h
Normal		9.8±0.8[b]	10.1±0.8[b]	10.4±0.7[b]	10.6±1.2[b]
Control		4.2±0.6	4.1±0.7	4.1±0.3	3.8±0.6
PD	10^{-7}	5.0±0.3	5.4±0.5	5.6±0.9	6.1±1.0[a]
PD	10^{-6}	5.3±0.8	5.6±0.9	6.4±0.6[a]	6.8±1.1[a]
PD	10^{-5}	8.4±1.2[b]	5.9±1.3[a]	7.7±0.8[a]	9.0±1.2[b]
PD	10^{-4}	6.7±0.4[a]	6.1±1.0[a]	6.8±0.7[a]	7.6±0.9[a]

[a]P<0.05, [b]P<0.01, *vs* CCl_4 treated control group.

Effects of PD on MDA formation in rat hepatocytes

CCl_4 challenge obviously elevated the MDA formation in rat hepatocytes, with a marked rise in time-dependence manner, whereas MDA formation of rat hepatocytes decreased significantly at various concentrations of PD as compared with that in CCl_4 control group, and it reached minimum value at 10^{-5} mol/L and slightly elevated when PD concentration was up to 10^{-4}-mol/L (Table 3).

Table 3 Effects of PD on MDA formation in rat hepatocytes ($x\pm s$, n = 8)

Group	c/(mol/L)	GSH (ng/L) after CCl4 challenge			
		6 h	12 h	24 h	48 h
Normal		4.0±0.4[b]	4.5±0.6[b]	4.8±0.4[b]	4.6±0.7[b]
Control		15.5±1.8	16.0±2.7	17.5±2.1	19.0±2.4
PD	10^{-7}	13.1±2.0	13.8±3.3	13.0±4.3[a]	14.5±1.8[a]
PD	10^{-6}	11.4±1.7[a]	12.0±1.8[a]	12.1±3.1[a]	12.5±2.0[a]
PD	10^{-5}	6.5±1.2[b]	6.7±1.2[b]	7.5±2.3[b]	8.2±2.7[b]
PD	10^{-4}	8.7±3.5[b]	8.9±2.8[b]	9.8±2.6[b]	10.3±3.0[b]

[a]P<0.05, [b]P<0.01, *vs* CCl_4 treated control group.

Effects of PD on cell survivability in primary culture rat hepatocytes

The results of MTT assay showed that normal hepatocytes had high level of cell viability (91.0% ± 7.9%) and CCl_4 induced marked decrease of hepatoc ytes survivability (35.4% ± 3.8%, $P < 0.01$ *vs* normal group), whereas the level of cell survivability could be significantly enhanced by PD at the concentrations of 10^{-7} mol/L - 10^{-4} mol/L to 56.1% ± 5.2% ($P < 0.05$, *vs* CCl_4-treated control group), 65.8% ± 5.0% ($P < 0.05$), 88.7% ± 6 .8% ($P < 0.001$) and 75.2% ± 7.3% ($P < 0.01$) respectively. It reached a maximum value at 10^{-5} mol/L and slightly declined when the concentration of PD was up to 10^{-4} mol/L (Figure 1).

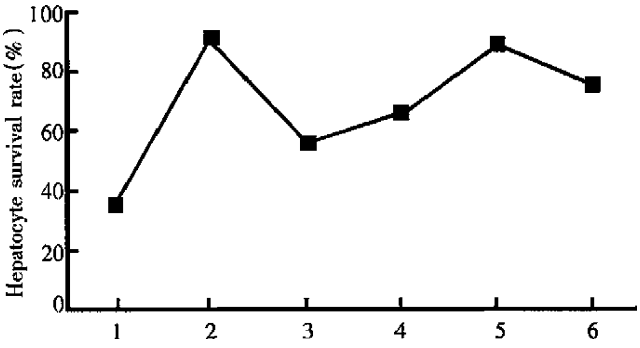

Figure 1 Effects of PD on cell survivability in primary culture rat hepatocytes.
1. CCl_4-treated control group; 2. normal hepatocytes; 3. PD 10^{-7} mol/ L; 4. PD 10^{-6} mol/ L; 5. PD 10^{-5} mol/ L; 6. PD 10^{-4} mol/ L.

DISCUSSION

P. cuspidatum has been used to treat some chronic liver diseases such as hepatitis and hepatocirrhosis. We have been trying to search for hepatoprotective compounds of *P. cuspidatum*. Our previous *in vitro* studies showed that emodin, another active compound, had a hepatoprotective effect[11]. The present *in vitro* study also indicated that PD had a protective effect against CCl_4 induced injury to primarily cultured rat hepatocytes. Since the extraction and isolation of PD are relatively simple and have a high content of 1.23% in the root of *P. cuspidatum*[7], we may take these advantages to further study its mechanisms of hepatoprotective effect and develop a new drug from it.

CCl_4 is a well-known example of a chemical that produces free radical-mediated liver injury. It generates CCl_4 by the activation of liver cytochrome P-450, initiating lipid peroxidation of bio-membranes[12]. In the presente xperiment, it was found that CCl_4 induced both the increase of GPT in supernat ant and the elevation of MDA in rat hepatocytes. However, administration of 10^{-7} mol/ L - 10^{-4} mol/L PD could partly reduce GPT and MDA. Therefore, there may be two possible mechanisms contributing to the hepatoprotective actions of PD. One is that PD inhibits further production of lipid peroxidation in rat hepatocytes, and the other is that it inhibits the destructive action of lipid peroxidation on liver cells.

GSH is an important endogenous anti-oxidant substance. The decrease of GSH content may be due to increased GSH consumption as it participates in the detoxifica tion system for the metabolism of CCl_4, and results in an enhanced susceptibility of hepatocytes to CCl_4 toxicity[13]. Our results showed that CCl_4 obviously decreased GSH content in the

hepatocytes, but PD could partly reverse it. This suggested that the nature of PD protecting-SH compounds (such as GSH) from CCl4 injury may be the third mechanism of its hepatoprotection.

It is interesting that PD of 10^{-5} mol/L was more effective than that of 10^{-4} mol/L, at the same time, the hepatoprotective action of PD was in dose dependence at concentrations of 10^{-7} mol/ L - 10^{-5} mol/ L. Its mechanisms of action need to be further studied.

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