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Phenidone, a dual inhibitor of cyclooxygenase and lipoxygenase, inhibits carbon tetrachloride-induced acute liver injury in rats

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Abstract : This study was carried out to find whether phenidone (1-phenyl-3-pyrazolidinone), a cyclooxygenase as well as a lipoxygenase inhibitor, exhibits the preventive effect on carbon tetrachloride (CCl_4)-induced acute liver injury in rats. Rats were pretreated with phenidone at a dose of 50 or 200 mg/kg (*p.o.*) once daily for 3 consecutive days before CCl_4 administration. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured. Malondialdehyde (MDA) production was determined as an index of lipid peroxidation in the liver and serum. The histopathological changes in the liver were also examined in each group. The reduction in body weights was significantly inhibited in the phenidone-treated group than in the CCl_4 control group. Significant increase in the relative liver weights of the phenidone-treated groups was observed compared with either the vehicle or CCl_4 groups. Elevation of serum AST and ALT activities occurred after CCl_4 treatment was significantly attenuated by the pretreatment with phenidone. The elevation of MDA levels in liver and serum were completely inhibited in phenidone-treated groups. The protective effects on phenidone-treated groups were confirmed histopathologically. These results suggest that phenidone may be a useful protector through modulation of hepatic inflammation in CCl_4-induced acute liver injury.

Keywords : ALT, AST, carbon tetrachloride (CCl_4), malondialdehyde, phenidone

Introduction

Carbon tetrachloride (CCl_4) is a well-known hepatotoxicant inducing liver injury in experimental animals. When the metabolism of CCl_4 is initiated by NADPH-dependent cytochrome P-450 enzyme, the trichloromethyl radicals ($\bullet CCl_3$) are produced in liver microsomes and react with O_2 to form trichloromethyl peroxyl radicals ($Cl_3COO\bullet$) that cause membrane lipid peroxidation [5].

The cleavage products of the lipid peroxides, namely malondialdehyde, 4-hydroxy-2-pentenal, 2,4-hexadienal, and 4-hydroxy-2-nonenal have the toxic mechanisms of causing the breakdown of the smooth endoplasmic reticulum (sER) structure, decreased activity of the sER enzyme, and the inhibition of the protein synthesis which leads to fatty liver connected with hepatocellular necrosis [1]. The CCl_4 also causes direct and acute toxicity in the liver and results in the central lobular necrosis of the liver histopathologically.

Cyclooxygenase (COX) and/or lipoxygenase (LOX), two major pro-inflammatory enzymes that mediate the arachidonate cascade pathway, are believed to be involved in the progression of liver injury by CCl_4 [3]. When CCl_4-induced liver damage occurs, the levels of leukotrienes mediated by LOX are known to increase at an early stage while prostaglandins mediated cyclooxygenase-2 (COX-2), the isoform of inducible type, increases at a later stage during hepatic inflammation [3]. Recently, a strategy of combination therapy with inhibitors of COX and LOX has been found to improve hepatic function [2].

Phenidone (1-phenyl-3-pyrazolidinone) is known to exhibits a dual inhibitory action of COX and LOX in various inflammatory diseases. Previously, we had reported that phenidone has a protective action on

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various neurotoxic or neuroinflammatory disease models in *in vitro* and *in vivo* studies [4, 6, 10]. Therefore, in this study, we examined whether pretreatment of phenidone exhibited protective effects on CCl_4-induced hepatotoxicity through alleviation of lipid peroxidation in rats.

Materials and Methods

Experimental animals

As many as 12 female and male Sprague-Dawley rats which were ten weeks old were purchased from Orient Bio (Korea). Rats were housed in plastic cages and maintained at 23 ± 2°C for 12 h / 12 h light-dark cycle. The feed was 5L79, rat formula, manufactured by PMI nutrition in USA. Feed and water were given *ad libitum* before fasting. The rats were fasted for 20 h before CCl_4 injection, but water was supplied *ad libitum* during the whole experiment period. The rats were sacrificed 24 h after CCl_4 injection. All animal studies were performed in accordance with the National Institute of Health (USA) Guide for the Humane care and Use of Laboratory Animals.

Preparation of materials

Phenidone, CCl_4, thiobarbituric acid (TBA), and sodium dodecyl sulfate (SDS) were purchased from Sigma (USA). Acetic acid and KCl were purchased from Showa Chemical (Japan) and Yakuri Pure Chemicals (Japan), respectively. Olive oil was purchased from Public Market in Chuncheon (CJ, Korea).

Instruments

Spotchem EZ SP-4430 (Arkray, Japan), T10 basic homogenizer (IKA Ultra-Turrax, Germany), and Spectra Max Plus Spectrophotometer (Molecular Devices, USA) were used for every biochemical assay.

Grouping of the experimental rats

The rats were divided into four groups. Each group was classified as follows: control group (vehicle), CCl_4 group, CCl_4 + phenidone (50 mg/kg) group, and CCl_4 + phenidone (200 mg/kg) group. Each group consisted of six rats, three females and three males. A 1 : 1 (v/v) mixture of CCl_4 and olive oil was injected (2 mL/kg) intraperitoneally to the rats of the experimental groups. Phenidone was administered orally once a day for 3 days before CCl_4 injection. Rats were fasted and sacrificed 24 h after CCl_4 injection.

Preparation of serum and liver homogenate

The rats were anesthetized using urethane (ethyl carbamate, 1,5 g/kg, *i.p.*) for the sampling of blood and liver. Blood samples were collected from the abdominal aorta. The samples were kept in the refrigerator for 1 h and centrifuged at 3,000 rpm for 15 min. The supernatant was used as serum.

The blood in the liver was eliminated by washing immediately with refrigerated saline. The saline on the surface of the liver was removed using gauze and the relative liver weights per 10 g of body weight measured. Similar portion of tissues removed from livers were analyzed for malondialdehyde (MDA). Liver tissues were placed in ten-time refrigerated 1.15% KCl-10 mM phosphate buffer (pH 7.4) in order to be homogenized.

Measurement of MDA in the liver tissue

The MDA production induced by the injection of carbon tetrachloride was measured by the procedure of Ohkawa *et al.* [7]. Briefly, 200 μL of the liver homogenates was put into each test tube and 8.1% SDS solution was added and mixed. After 1.5 μL of the 20% acetic acid was added and mixed, 1 μL of the 1.2% TBA solution was added and heated in the water bath for 1 h. The tubes were cooled to room temperature and centrifuged for 10 min at 2,000 rpm. The absorbance was measured at 532 nm. The concentration of the lipid peroxidates was expressed as MDA nmol per gram liver weight.

Measurement of the serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and MDA

Serum AST and ALT were evaluated by Spotchem EZ SP-4430. The serum was diluted if the value of the measurement was out of range. Serum MDA was quantified using thiobarbituric acid reactive substances Assay kit (ZeptoMetrix, USA). The 100 μL serum supernatant was put into the test tubes and 100 μL SDS solution was added and mixed. Simultaneously, a 2.5 mL TBA/ Buffer Reagent was mixed and heated at 95°C in a water bath for 60 min and cooled for 10 min in an ice box. It was centrifuged for 15 min at 3,000 rpm and the absorbance value of the supernatant was measured at 532 nm.

Histological examination

To observe for microscopic change in the liver tissue, parts of the liver were fixed in 10% formaldehyde soon after the removal of the liver. The fixed tissues were washed, dehydrated according to the increase of alcohol concentration, and embedded in 60°C paraffin. The embedded tissues were sliced to strips of 3 μm thickness (RM2145; Leica, Germany) and stained using hematoxylin-eosin. The tissues were then observed using the optical microscope (BX51; Olympus, Japan) and liver damage was estimated by Pérez Tamayo's method [8].

Statistical analysis

Statistical analysis was carried out by one-way analysis of variance with application of Duncan's multiple range tests using SAS 9.1 program (SAS, USA). Data were expressed as mean ± SE. A value of $p < 0.05$ was considered to be statistically significant.

Results

Change in body weight and relative liver weight

A reduction in body weight was shown due to the long fasting for 20 h before CCl_4 injection from the beginning of the experiment upto the sacrifice. The CCl_4 group revealed the maximum decrease as –20.0 g, whereas the 200 mg/kg phenidone treatment group showed the lowest decrease as –6.8 g. Both CCl_4- and phenidone-treated groups showed significant difference, but there was no significant difference between the 50 mg/kg and 200 mg/kg phenidone treatment group (Table 1). When the weights of the removed livers were compared with the relative weights per 10 g of BW, there was no significant difference between the control group and the CCl_4 group, and the relative liver weights of the phenidone-treated group significantly increased compared to those of the control group and CCl_4 group (Table 1).

Effect of phenidone on serum AST and ALT activities

Serum AST and ALT activities were highly elevated 24 h after CCl_4 treatment compared with control rats. Pretreatment with phenidone at a dose of 50 or 200 mg/kg significantly decreased AST and ALT activities to 61.8%, 32.5% and 35.3%, 15.3% of CCl_4-treated group, respectively (Table 2).

Effect of phenidone on liver and serum MDA

Liver and serum MDA, as a marker of lipid peroxidation, were increased about two-fold after CCl_4 treatment. The MDA formation of liver and serum induced by CCl_4 was completely inhibited by phenidone at doses of 50 and 200 mg/kg (Table 3).

Histopathological changes in liver

In the control group, liver tissues showed a normal morphology. The degree of pathologic lesion in the

Table 1. Changes of body weight (g) and relative liver weight (g/10 g body weight)

Group / Profile	Change of body weight (g)	Liver weight
Control (vehicle)	-20.0 ± 9.3^{ab}	0.32 ± 0.01^{b}
CCl_4	-29.0 ± 4.6^{b}	0.34 ± 0.01^{b}
CCl_4 + phenidone (50 mg/kg)	-8.4 ± 4.3^{a}	0.40 ± 0.02^{a}
CCl_4 + phenidone (200 mg/kg)	-6.8 ± 2.6^{a}	0.43 ± 0.01^{a}

CCl_4: carbon tetrachloride. Values are expressed as mean ± SE for 6 rats. [a,b]Values not sharing a common letter are significantly different ($p < 0.05$).

Table 2. Effects of phenidone on serum biochemical profile in CCl_4-induced hepatotoxicity

Group / Profile	AST (U/L)	ALT (U/L)
Control (vehicle)	118.0 ± 5.1^{c}	34.0 ± 7.9^{c}
CCl_4	964.0 ± 34.5^{a}	693.0 ± 53.1^{a}
CCl_4 + phenidone (50 mg/kg)	595.4 ± 111.0^{b}	244.6 ± 45.7^{b}
CCl_4 + phenidone (200 mg/kg)	313.6 ± 72.0^{bc}	106.0 ± 21.0^{c}

AST: aspartate aminotransferase, ALT: alanine aminotransferase. Values are expressed as mean ± SE for 6 rats. [a,b,c]Values not sharing a common letter are significantly different ($p < 0.05$).

Table 3. Effects of phenidone on liver and serum malondialdehyde (MDA) levels in CCl_4-induced hepatotoxicity

Group / Profile	MDA nmol/g liver	MDA nmol/mL serum
Control (vehicle)	75.2 ± 2.8[b]	17.9 ± 6.1[a]
CCl_4	121.6 ± 10.3[a]	31.3 ± 7.4[a]
CCl_4 + phenidone (50 mg/kg)	72.9 ± 9.4[b]	14.4 ± 7.5[a]
CCl_4 + phenidone (200 mg/kg)	79.5 ± 1.8[b]	10.8 ± 4.2[a]

Values are expressed as mean ± SE for 6 rats. [a,b]Values not sharing a common letter are significantly different ($p < 0.05$).

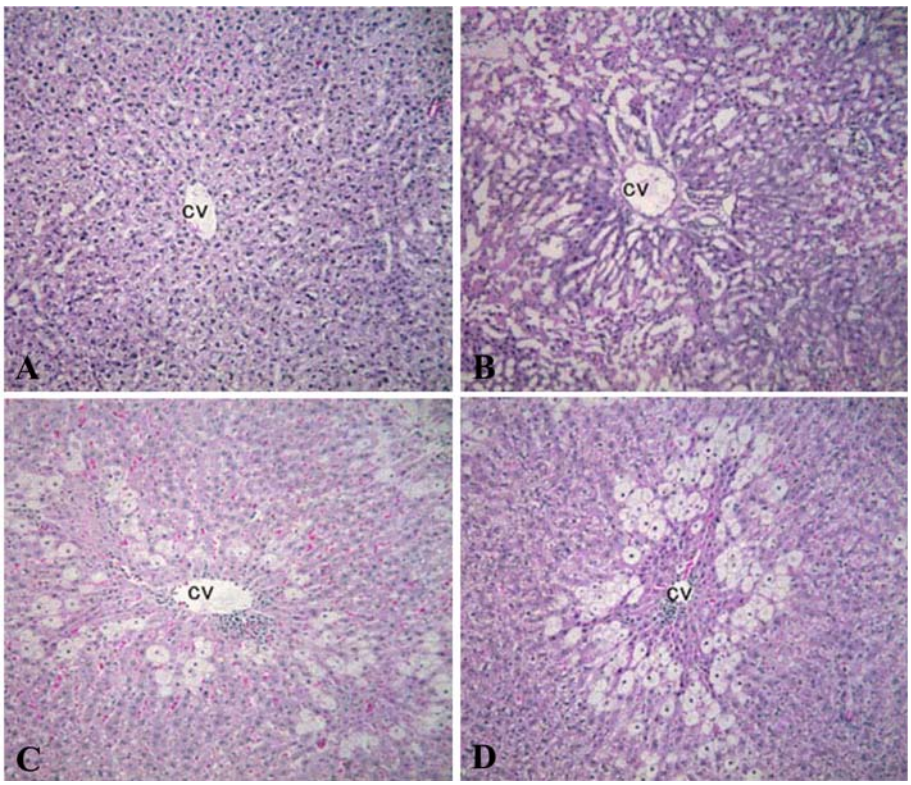

Fig. 1. Histopathological findings of liver section after carbon tetrachloride (CCl_4) intoxication and recovery by treatment with phenidone (hematoxylin and eosin staining, ×200). (A) control (vehicle) (0), (B) CCl_4 (3), (C) CCl_4 + phenidone (50 mg/kg) (2), (D) CCl_4 + phenidone (200 mg/kg) (2). Evaluation of liver damage was graded by Pérez Tamayo's method: (0 = absence; 1 = minimal, spotty necrosis; 2 = moderate necrosis in nonconfluent areas; 3 = submassive necrosis with confluent areas; 4 = massive necrosis). CV: central vein.

liver was assessed to be a normal grade 0 according to Pérez Tamayo's method [11].

In the CCl_4-treated group, centrilobular necrosis surrounding the central vein and degeneration unified with the lesions of the central vein were found, where the score was grade 3. In combined CCl_4- and phenidone-treated groups, the lesions were confined around the central vein, but not unified with adjacent lobules. Thus, it was considered as grade 2.

Discussion

In this study, we have observed that phenidone, an inhibitor of both COX and LOX, attenuated CCl_4-induced hepatotoxicity. The synthesis of arachidonic acid metabolites in the liver derived from the COX-2 and/or 5-lipoxygenase (5-LOX) pathway is known to cause CCl_4-induced liver inflammation [2]. These results suggest that phenidone could be used as a drug in various inflammatory diseases [2, 4, 6]. Recently,

the availability of COX-2 and/or 5-LOX inhibitors in various liver disease models has been recognized as a therapeutic target for modulation of hepatic inflammatory diseases. However, the exact mechanisms of these drugs have not been clarified yet. Lipid peroxidation mediated by free radicals after CCl_4 administration has been considered to play a pivotal role in acute or chronic inflammatory hepatic injury [7]. In this study, pretreatment with phenidone exerted hepatoprotective effects through potent inhibition of lipid peroxidation. Therefore, our *in vivo* results consistent with *in vitro* study have shown that phenidone has a protective effect on CCl_4-induced lipid peroxidation reported by Sen *et al*. [9]. Phenidone also exhibited dose-dependent inhibitory effects on the elevation of serum AST and ALT levels by CCl_4 administration. Our results were well confirmed by the histological observation of Pérez Tamayo's method. Phenidone has alleviated a submassive necrosis in broad areas induced by CCl_4, but there was little difference between the two concentrations. Conclusively, phenidone, a dual inhibitor of COX and 5-LOX, could contribute to recovery from liver damage through potent inhibition of lipid peroxidation in CCl_4-induced acute hepatotoxicity.

Acknowledgments

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