Full Length Research Paper

Inhibitory effect of *Dioscorea panthaica* on CCl$_4$-induced liver fibrosis in rats

Xinmei Chen$^{1,3}$, Guilong Gao$^2$, Wenmin Luo$^2$, Guiping Wang$^3$, Wenling Zheng$^1$, Qiang Jia$^{2*}$ and Wenli Ma$^1$

$^1$Institute of Genetic Engineering, Southern Medical University, Guangzhou, 510515, PR China.
$^2$Institute of Biology, Guizhou Academy of Sciences, 1 Tongying Road, Guiyang, 550009, PR China.
$^3$Guangzhou Medical College, Guangzhou, 510182, PR China.

Accepted 18 November, 2011

*Dioscorea panthaica* (Dioscorea) is an herb commonly used in traditional Chinese medicine but the effects of DPE on liver fibrosis have not yet been reported. It was undertaken to evaluate the protective effects of the aqueous extracts of *D. panthaica* Prain et Burkil (DPE) on carbon tetrachloride (CCl$_4$)-induced liver fibrosis in rats and to elucidate the mechanisms underlying these protective effects in rats. Liver fibrosis was produced by hypodermics injection of CCl$_4$ (3 ml/kg body weight, 4:6 in olive oil, twice per week) after eight weeks. The rat model of liver fibrosis was used to assess the effect of daily oral administration of DPE on the indexes of liver fibrosis. Histological and hepatic hydroxyproline examination revealed that DPE significantly arrested the progression of liver fibrosis. The decrease of the hepatic glutathione and the increase of the lipid peroxidative products, indicating the altered redox state during the development of CCl$_4$-induced liver fibrosis, were partially normalized after DPE treatment. Additionally, DPE may reduce $\alpha$-smooth muscle actin (SMA) protein level and HSC activation of CCl$_4$-treated rats. Oral administration of DPE may significantly reduce CCl$_4$-induced liver fibrosis in rats, probably through the reduction of HSC activation.

Key words: *Dioscorea panthaica*, oxidative stress, liver fibrosis.

INTRODUCTION

Liver fibrosis is a dynamic and sophisticatedly regulated wound healing response to hepatocellular injury. Carbon tetrachloride (CCl$_4$) is a xenobiotic used extensively to induce oxidative stress. Chronic CCl$_4$ treatment is frequently used in rats to produce an experimental model to study liver fibrosis (Knight et al., 2007; Watanabe et al., 2007). Liver fibrosis induced by CCl$_4$ is associated with the exacerbation of lipid peroxidation and the depletion of antioxidant status (Knight et al., 2007; Muriel, 1998). It is known that CCl$_4$ induced liver toxicity is associated with excessive generation of free radicals that cause oxidative damage to a number of molecules in the hepatocytes. In this regard, reduction of oxidative stress may be a potential and effective therapeutic strategy for prevention and treatment of liver fibrosis (Popovic et al., 2008; Desai et al., 2010).

*Dioscorea panthaica* (Dioscorea) is an herb commonly used in traditional Chinese medicine. The chemical analysis of *D. panthaica* has been reported (Li et al., 2006; Jing et al., 2009). It has been verified that an aqueous extract of saponins from *D. panthaica* exhibited anti-hypercholesterolemia activity in rats (Ma et al., 2002). Moreover, steroidal saponins from *D. panthaica* exhibited cytotoxic activity against tumor cells (Dong et al., 2004). However, to the best of our knowledge, the effects of DPE on liver fibrosis have not yet been reported until now. Therefore, the present study was undertaken to evaluate the protective effects of DPE on CCl$_4$-induced liver fibrosis and to elucidate the mechanisms underlying these protective effects in rats.

MATERIALS AND METHODS

Chemicals, reagents and plant materials

The raw materials were collected from the herbal market...
Animals and treatment

The male Wistar rats (180 to 200 g) were housed in conventional cages (20 to 22°C, 12 h light–dark cycle) with free access to water and rodent chow. All procedures including laboratory animal use were performed according to the guidelines of institutional animal care. Furthermore, the Sun Yat-Sen University was referred as the committee for the care and use of laboratory animals. Rats were randomly divided into six groups. Groups 1 (normal control) and 2 (induction control) were received water for eight weeks. Group 3 (induction control) were received colchicine (0.1 mg/kg, p.o. daily) for 8 weeks. All animals except Groups 1 were administrated with CCl4 (3 ml/kg body weight, 4:6 in olive oil, twice per week). At 24 h after the final injection of CCl4, a laparotomy was performed and blood was drawn from the abdominal aorta under ether anesthesia. The serum was stored at −80°C after separation until assayed as described below. Liver samples were collected and frozen in liquid nitrogen (Gandhi et al., 1995).

Measurement of serum aminotransferase levels

A 50 µl sample of blood were treated with EDTA and citrate-theophylline-adenosine-dipyridamole and centrifuged at 1500 × g for 15 min at 4°C to obtain plasma, and then stored at −80°C until LPA and measurement. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined using commercial kits produced by Institute of Shanghai Biological Products affiliated with the Ministry of Health, as previously described (Umezu-Goto et al., 2002; Macey et al., 2002; Kishimoto et al., 2003).

Hepatic hydroxyproline concentration

Liver collagen concentration was determined by measuring hydroxyproline content in liver samples (Sakaida et al., 1996). Briefly, liver samples were homogenized and hydrolyzed in 6 N HCl at 110°C for 18 h. After filtration of the hydrolysate through Millipore filter, chloramine T was added to a final concentration of 2.5 mM. The mixture was then treated with 410 mM para-dimethyl-amino-benzaldehyde and incubated at 60°C for 30 min. The concentration of hydroxyproline in each sample was determined spectrophotometry at 560 nm using a standard curve generated from known quantities of hydroxyproline. Each liver sample was measured in triplicate, and the mean value of hydroxyproline was used for analysis. Results were expressed as micrograms per gram of fresh tissue.

Pathological examination

After formalin fixation, tissue samples were sliced, embedded in a standard manner and stained with Sirius red. Fibrosis was graded: grade 0, normal liver; grade 1, increase of collagen without the formation of septa; grade 2, formation of incomplete septa from the portal tract to the central vein (that is septa that do not interconnect with each other); grade 3, complete but thin septa that interconnect with each other, so as to divide the parenchyma into separate fragments; grade 4, same as grade 3 with the exception of thick septa (complete cirrhosis) (Ruwart et al., 1989). To avoid sampling errors, all biopsies were obtained from the same lobe and the semi-quantitative grades assigned without knowledge of the sample treatment (Hsieh et al., 2008).

Immunohistochemistry

The immunohistochemical stains for α-smooth muscle actin (SMA) proteins were carried out using anti-α-SMA antibodies (Santa Cruz, CA, USA) (Zhen et al., 2007).

Hepatic glutathione and thiobarbituric acid-reactive substance measurements

The glutathione (GSH) concentrations in the liver homogenate were determined with a GSH-400 colorimetric assay kit. In brief, samples were mixed with 0.1 M of sodium phosphate buffer (pH 7.5) containing 5 µM EDTA, 0.6 mM of 5, 5-dithiol-bis (2-nitrobenzoic acid), and 0.2 mM NADPH and glutathione reductase. The mixture was incubated for 2 min at room temperature. The absorbance of the product was measured at 412 nm. GSH content was determined using a standard curve generated from known concentrations of GSH. The results were expressed as µM/mg protein. Lipid peroxidation was measured by the thiobarbituric acid reaction method (Fraga et al., 1998). The liver homogenate was prepared with 2 ml of 50 mM potassium phosphate buffer, pH 7.4, and thiobarbituric acid-reactive substances (TBARS) were determined. The fluorescence of the samples was detected at an excitation wavelength of 515 nm and an emission wavelength of 555 nm in a F4500 fluorescence spectrophotometer with 1, 1, 3, 3-tetramethoxypropane being used as the TBARS standard (Zhen et al., 2007).

Statistics

Values are expressed as the mean ± S.E.M. of at least three separate experiments. Results were analyzed by one way analysis of variance followed by the Student–Newman–Keul’s test. Differences with P < 0.05 were considered significant.

RESULTS

Effect of DPE on liver function tests

Generally, the serum activities of AST and ALT were regarded as biochemical markers for the liver injury. Compared to the normal group, the serum AST and ALT levels were significantly increased in the liver fibrosis group. However, an obvious reduction in these parameters was observed from three DPE-treated groups (Table 1).

DPE decreased liver fibrosis in CCl4-treated rats

Liver fibrosis of the rats was evaluated by two histological methods. Hematoxylin and Eosin (H and E) staining and
Table 1. Effects of DPE on serum ALT and AST activities, and hydroxyproline content in liver fibrotic rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
<th>Hydroxyproline content (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>-</td>
<td>130.5 ± 28.5</td>
<td>36.8 ± 6.1</td>
<td>151 ± 11</td>
</tr>
<tr>
<td>CCl&lt;sub&gt;4&lt;/sub&gt;</td>
<td>-</td>
<td>279.8 ± 63.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>185.2 ± 36.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>561 ± 25&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Colchicine</td>
<td>0.1</td>
<td>105.4 ± 15.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>67.3 ± 13.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>251 ± 17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DPE -treated</td>
<td>50</td>
<td>198.2 ± 25.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>112.3 ± 21.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>427 ± 17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DPE -treated</td>
<td>100</td>
<td>174.0 ± 35.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>91.6 ± 27.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>362 ± 20&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DPE -treated</td>
<td>200</td>
<td>124.5 ± 27.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>80.2 ± 22.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>311 ± 19&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.05 vs. normal control group.  <sup>b</sup> P < 0.05 vs. CCl<sub>4</sub> group.

**Figure 1.** Hepatic histopathology during development of carbon tetrachloride (CCl<sub>4</sub>) induced liver injury with or without concurrent DPE treatment. Liver sections stained with haematoxylin and eosin (H&E) (×100). (A) Normal control. (B) CCl<sub>4</sub> group. Light microscopy examination showing large cirrhosis septa formation. (C) Colchicine group. (D) DPE-treated group (100 mg/kg). H&E staining showing that collagen fiber bundles were markedly reduced. Original magnification ×200.

Masson’s trichrome staining, and both methods showed the same pattern. The histological analysis of the livers from normal control rats indicated normal architecture (Figures 1A and 2A). An experimental design involving CCl<sub>4</sub> administration was used to produce general liver morphological changes and liver fibrosis evidenced by both qualitative and quantitative histopathological examinations. Representative photographs of liver morphology are shown in Figures 1B and 2B, in contrast to normal rat liver morphology (Figures 1A and 2A). CCl<sub>4</sub>-induced liver fibrosis was evidenced by disruption of tissue architecture, extension of fibers, large fibrous septa formation, pseudolobe separation, and collagen accumulation. These alterations were remarkably reduced in the liver sections of the rats that received both DPE and CCl<sub>4</sub> treatment for 8 weeks (Figures 1D and 2D). It was verified that the scoring of the DPE-treated group had obviously been improved compared with the liver fibrosis group.

In parallel to the observed improvement of liver histology, liver fibrosis was also quantified by measurement of hepatic hydroxyproline levels (Table 2).
Figure 2. Liver sections stained with Masson’s trichrome (×200). (A) Normal control. (B) CCl$_4$ group. Light microscopy examination showing large fibrous septa formation, and the same with hematoxylin and eosin (H&E) staining. (C) Colchicine group. (D) DPE-treated group (100mg/kg). Masson’s trichrome staining also showed that collagen fiber bundles were markedly reduced. Original magnification ×200.

Table 2. The pathological score of CCl$_4$-induced liver fibrosis in rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>CCl$_4$</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Colchicine</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>DPE -treated</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>DPE -treated</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>DPE -treated</td>
<td>200</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>-</th>
<th>+</th>
<th>++</th>
<th>+++</th>
<th>++++</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCl$_4$</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Colchicine</td>
<td>2</td>
<td>6</td>
<td>5</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>DPE -treated</td>
<td>2</td>
<td>6</td>
<td>5</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>DPE -treated</td>
<td>2</td>
<td>6</td>
<td>5</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

Grade definition of the histological findings; (-) normal, (+) very slight, (++) slight, (+++) moderate, (++++) severe. The value indicated the number of animals in each grade.

There was a significant increase in hepatic hydroxyproline levels in rats with CCl$_4$-induced liver fibrosis compared to normal controls (Table 2), and administration of DPE prevented the increase in hepatic hydroxyproline content in CCl$_4$-treated rats (Table 2). Hepatic stellate cells (HSCs) are recognized as the primary cellular source of matrix components in chronic liver disease. Activated HSCs are the major source of matrix proteins in diseased liver (Ookhtens and Kaplowitz, 1998). Accordingly, we evaluated α-SMA, an indicator of HSCs activation, by immunohistochemical staining in liver samples. In normal control livers, α-SMA positive cells were present in portal veins and hepatic arteries (Figure 3A). At the 8th weeks after CCl$_4$ treatment, α-SMA-positive cells occupied most of the liver fibrosis tissue (Figure 3B). Concurrent DPE treatment for 8 weeks significantly reduced the number of α-SMA-positive cells in the liver fibrosis areas (Figure 3C), and,
Figure 3. Hepatic immunohistochemical stain for α-smooth muscle actin (α-SMA): (A) Normal control group. α-SMA-positive cells are restricted to the portal vein and hepatic artery walls only. (B) CCl4 group. Abundant α-SMA staining in the liver injury areas indicate the presence of activated hepatic stellate cells. (C) DPE-treated group (100mg/kg). The number of α-SMA positive cells in the liver injury areas is reduced significantly. Original magnification ×200.

Table 3. Effects of DPE on hepatic homogenate GSH level and TBARS level in liver fibrosis rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg)</th>
<th>GSH levels (µmol/g)</th>
<th>TBARS levels (µmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>-</td>
<td>6.4 ± 0.2</td>
<td>0.45 ± 0.12</td>
</tr>
<tr>
<td>CCl4</td>
<td>-</td>
<td>3.1 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.38 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Colchicine</td>
<td>0.1</td>
<td>5.2 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.87 ± 0.11&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DPE-treated</td>
<td>50</td>
<td>3.9 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.21 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DPE-treated</td>
<td>100</td>
<td>4.3 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.15 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DPE-treated</td>
<td>200</td>
<td>4.7 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.03 ± 0.18&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.05 vs.normal control group. <sup>b</sup> P < 0.05 vs. CCl4 group.

thus, low levels of collagen (Table 2).

**Effect of DPE treatment on the liver redox state**

Glutathione (GSH) constitutes the first line of defense against free radicals. As shown in Table 3, the CCl4-treated group showed a significant reduction in the GSH content (p<0.05) in the liver of rats, compared with that of the normal control. The treatment of DPE at the dosage of 1.0 mg/kg significantly recovered the GSH depletion caused by CCl4 (p<0.01).

Compared with the normal control, the concentration of TBARS was higher in the chronic liver injury group. The pretreatment with DPE at the dosage of 1.0 mg/kg was able to alleviate oxidative damage of lipids significantly (Table 3), and chronic DPE administration decreased the hepatic level of TBARS in three DPE-treated group, p<0.05, (Table 3).

**DISCUSSION**

The present study demonstrates that DPE considerably prevents the development of CCl4-induced liver fibrosis in a rat model. We can confirm our conclusion through observation of liver histology and quantitative measurement of the hepatic hydroxyproline content, which is
usually used as a marker of collagen deposition in the liver. CCl₄ is a widely used hepatotoxic agent that is known to enhance the formation of free radicals. They may cause lipid peroxidation, which produces hepatocellular damage and enhances production of liver fibrosis (Muriel, 1998). The results of the present study substantiate that treatment of rats with DPE has a markedly protective effect against CCl₄-induced hepatotoxicity in rats due to the decrease of the serum AST and ALT activities. The hepatic concentration of TBARS, an index of lipid peroxidation, increased significantly in CCl₄-administered rats (Muriel, 1998). In contrast, DPE treatment significantly suppressed the increase of TBARS formation induced by CCl₄ in rats. GSH acts as an intracellular or extracellular antioxidant in conjunction with various enzymatic processes and multiple intracellular functions include detoxification of reactive oxygen intermediates and reduction of low-molecular weight thiols, sulfides and mixed disulfides of proteins (Ookhtens and Kapiłowicz, 1998). It has been suggested that the lipid peroxides generated after intoxication are eliminated by GSH peroxidase in the presence of GSH (Meister et al., 1983). In the current study, we found that there is a significant decrease in hepatic GSH levels in CCl₄-administered rats, whereas DPE treatment significantly increased hepatic GSH levels. Taken together, these findings indicate that DPE exerts a protective effect on CCl₄-induced liver fibrosis in rats, possibly through its antioxidant action.

Our research has shown that DPE could inhibit rat liver fibrosis development, which was contemporaneous with a significant decrease of α-SMA-positive cells. Because the increase of α-SMA-positive cells is caused by the activation of HSCs, we speculate that DPE may attenuate HSC activation. Previous studies have justified that oxidative stress is associated with liver injury and activation of HSCs either directly or through paracrin stimulation by injured hepatocytes (Pietrangelo, 1998; Gianluca et al., 1998). Therefore, our data indicate that DPE reduces the oxidative stress to prevent the activation of HSCs and the resulting damages to hepatocytes.

CONCLUSIONS

In summary, our results demonstrate that DPE exhibits the beneficial effects against CCl₄-induced liver fibrosis in rats. The underlying mechanism is due to the reduction of oxidant dependent activation and proliferation of HSCs.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Science and Technology of Guizhou Province, China (J [2009]2290, NY [2009]3021 and TZJF-2009-45). This work was also supported by Guangzhou Medical College (2008A08).

REFERENCES


