Basic Investigation

Dangfei Liganning Capsules (当飞利肝宁胶囊) Attenuate the Susceptibility of Rat Nonalcoholic Fatty Liver to Carbon Tetrachloride Toxicity

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Objective: To test whether nonalcoholic hepatic steatosis sensitizes carbon tetrachloride (CCl4)–induced liver injury, and to assess the therapeutic effect of Chinese medicine extracts of Dangfei Liganning capsules (当飞利肝宁胶囊) and their potential underlying mechanisms.

Methods: Male Wistar rats were fed a high-fat diet to induce nonalcoholic fatty liver disease (NAFLD) or a normal diet (N). Eight weeks later, a nonlethal dose of CCl4 was applied intraperitoneally. From the start, HF-CCl4 rats were administered daily Dangyao extracts (D), Dangfei Liganning capsules (DF), or Diammonium Glycyrrhizinate (G) intragastrically. Rats were sacrificed 48 h after CCl4 administration. In addition to serum biochemistry, liver histopathology was observed using hematoxylin-eosin (HE) and oil red O staining, and hepatic levels of triglyceride (TG), malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), caspase-3 activation and cytochrome P450 (CYP2E1) expression were assessed.

Results: There was almost no response to the nonlethal dose of CCl4 in the N control group. However, the HF group demonstrated massive steatosis, and elevated levels of serum ALT and AST, liver MDA, CYP2E1, and caspase-3 activation, whereas the levels of GSH and SOD were significantly decreased. All indexes assessed were dramatically worse in the HF-CCl4 group compared to the HF group, in addition to the more severe steatosis, hepatocyte ballooning, and inflammatory infiltration apparent in the centrilobular area. The medicines we tested affected the pathological changes in HF-CCl4 rats to differing degrees: DF and G led to improvements in all of the above examined indexes, including an obvious improvement in histopathology, and DF improved serum ALT and MDA levels more markedly than G, whereas D extracts produced only mild liver injury attenuation.

Conclusion: Liver with NAFLD is more sensitive to hepatotoxicity; furthermore, the disrupted balance of oxidative stress and anti-oxidant defense contributes to the underlying mechanisms. Dangfei Liganning capsules potentially decrease this toxic susceptibility and alleviate liver injury in non-alcoholic fatty liver.

Keywords: NAFLD; liver injury; CCl4; Dangfei Liganning capsules; oxidative stress

Nonalcoholic fatty liver disease (NAFLD) refers to a wide spectrum of liver damage that ranges from simple steatosis to nonalcoholic steatohepatitis (NASH), advanced fibrosis, and cirrhosis.1,2 It is the most common chronic liver disease and is a major cause of elevation of liver enzymes worldwide. Currently, the prevalence is estimated to be 20%–30% in Western countries,12%–15% in Asian countries, and it is rapidly increasing in parallel with the upward trends in obesity and type 2 Diabetes.3,4 The “2 hit” hypothesis proposed in 1998 is widely accepted to explain the pathogenesis in which insulin resistance and oxidative stress are key factors in NAFLD development.5

Simple nonalcoholic fatty liver (NAFL) is usually ignored and not considered to be a disease because affected patients do not present with identifiable signs and symptoms or recognizable etiological agents. However, previous experiments have demonstrated that enhanced liver sensitivity to acute toxic injury in NAFLD,6,7 leads to severe liver dysfunction. A clinical investigation demonstrated that NAFLD patients were more susceptible to acute hepatotoxicity due to pharmacological agents taken for other illnesses and that presence of NAFLD was an independent risk factor in

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determining drug-related acute hepatitis, with an odds ratio of 3.95. Therefore, elucidation of the underlying mechanisms of this increased toxic sensitivity of NAFLD liver is warranted in order to develop novel therapies to prevent development of severe liver disease. Dangfei Liganning capsules (DF 鼬 ATF庂olfT) contain a mixture of alcohol extracts from the Chinese traditional herbal medicines Dangyao (Herba Swertiae) and Shuifeiji (Fructus Silybi). These capsules have been applied in a clinical setting to protect liver function in various liver diseases and have been demonstrated to lessen liver damage induced by drugs for tuberculosis.9-13

The purpose of this study is to initially determine whether NAFLD sensitizes the liver to hepatotoxicity and to investigate the underlying mechanisms, as well as to investigate whether Dangfei Liganning capsules could attenuate susceptibility and protect the liver from heavy injury and determine the underlying mechanisms involved.

MATERIALS AND METHODS

Drugs and Reagents
DF was provided by Sichuan Medco Pharmaceutical Company (四川美大康药业有限公司, Shifang, Sichuan, China); the compound in the capsules is a mixture of alcohol extracts from Dangyao (Herba Swertiae) and Shuifeiji (Fructus Silybi). Dangyao extract (D) was also provided by this company. Diammonium Glycyrrhizinate Enteric-coated capsules (G, 甘草酸二铵肠溶胶囊) are the product of Chia Tai Tianqing Pharmaceutical Company (正大天晴药业股份有限公司, Lianyungang, Jiangsu, China). The dose used for rats was seven times that of a human adult.

Mouse monoclonal anti-CYP2E1 and rabbit polyclonal anti-UCP2 were the products of Abcam Inc., mouse monoclonal anti-cleaved caspase-3 was purchased from Cell Signaling Technology, Inc., and PVDF membrane and ECL reagents were from Millipore Corporation. Chemistry reagents were purchased from Sigma Aldrich Company or Sinopharm Chemical Reagent Company.

Animals and Experimental Design
Seventy male Wistar rats were purchased from Slac Laboratory Animal Company, with initial weight of 170±10 g. The rats were housed at 24±2 °C, 55±10% humidity with a 12-hour photoperiod. Rats were fed with high-fat diet (HF, 88% normal chow + 10% lard + 2% cholesterol) or a standard normal diet (N) for 8 weeks. Next, 33% CCl4 (1.2 dissolved in corn oil for injections, 0.5 mL / 100 g body weight) was applied intraperitoneally. From the initiation of this experiment, some of the HF-CCl4 rats were administrated intragastrically each day with Dangyao extract D, DF, or G, respectively. Animals were sacrificed 48 h following CCl4 administration, and liver and serum samples were obtained and immediately frozen in liquid nitrogen, then stored at -80 °C until analysis.

Measurement of Serum Biochemistry
Serum was separated via centrifugation, and activity levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using HITACHI 7170S biochemistry analysis equipment.

Liver Histopathology Assessment
Similar portions of liver tissue were fixed by immersion in 10% neutral formaldehyde for 24 h. Four µm slices of paraffin-embedded sections were routinely processed for hematoxylin-eosin (HE) staining. Frozen sections fixed in 4% neutral parafomaldehyde were stained with oil red O and utilized to confirm liver steatosis. Liver histology was examined under a light microscope.

Measurement of Liver Tissue Triglyceride
A total of 200 mg of liver tissue was minced and added to a mixture of alcohol and acetone for homogenization; samples were then centrifuged at 10,000 rpm/min for 20 s and stored overnight at 4 °C. After centrifugation, sample supernatants were collected and analyzed for hepatic triglyceride content in accordance with the kit manual instructions from Nanjing Jiancheng Bioengineer Institute.

Determination of Liver Malondialdehyde, Glutathione and Superoxide Dismutase
Samples of 10% liver homogenate (500 mg liver tissues in 4.5 mL 0.9% NaCl) were prepared, and protein concentrations were examined using the Bradford method. Hepatic malondialdehyde (MDA) and reduced Glutathione in the homogenate were measured using colorimetric microplate assays in accordance with the kit manual instructions of Beyotime Institute of Biotechnology. Liver SOD was determined using the SOD assay kit from Dojindo Laboratories. The assay kit measured the mitochondrial activity via production of a water-soluble formazan dye upon reduction with superoxide anion; the rate of this reduction was linearly correlated to xanthine oxidase (XO) activity and was inhibited by SOD. Thus, the rate of XO inhibition as determined by a colorimetric method was used to reflect the serum SOD levels in this study.

Western Blot Analysis of Hepatic CYP2E1 Expression and Caspase-3 Activation
Liver tissues were homogenized in RIPA buffer and protein concentrations were determined using the BCA method. The whole liver protein extracts were resolved by SDS-PAGE gel electrophoresis and transferred to a PVDF membrane. After blocking, immunoblotting was performed for each primary antibody against CYP2E1 (1:1000), cleaved caspase-3 (1:500), and GAPDH (1:1000) as internal loading control, then incubated with the secondary antibodies against rabbit or mouse. The blots were developed using the ECL western blotting system and analyzed by computerized densitometry (Tanon, Labworks 4.6 software).

Statistical Analysis
Data were expressed as the mean ± standard deviation (SD). Comparisons were made among the groups using one-way ANOVA, followed by Tukey-Kramer’s post test to assess the specific pairs of groups using Graph-Pad Prism5 and SPSS16.0 software. A P value of less than...
0.05 was considered to represent a statistically significant result.

**RESULTS**

**Histopathology**

According to liver tissue HE staining results (Figure 1), rats fed a HF diet exhibited massive steatosis in liver tissue, with abundant accumulation of fat droplets in hepatocytes, whereas the liver tissues from rats fed a normal diet exhibited no signs of steatosis. There was almost no pathologic change in the N-CCl₄ group aside from very mild steatosis that was observed in some samples. In contrast, liver damage was more severe in the HF-CCl₄ group compared to the HF group: in addition to the more diffuse hepatosteatosis observed, there was evidence of infiltration of a mixed population of inflammatory cells in the centrilobular regions, as well as numerous areas of ballooning hepatocyte degeneration characterized by cell swelling, rarefied cytoplasmic content, Mallory-Denk bodies, and small apoptotic bodies. DF and G intervention markedly protected the liver from histopathological injuries, including almost no inflammatory infiltration and a decrease in the degree of hepatosteatosis. D also lessened the degree of hepatitis, although it functioned less effectively than DF and G.

Liver tissue samples stained with oil red O (figure is not shown) also confirmed the hepatosteatosis results seen in the HF group which was more diffuse in the HF-CCl₄ group and was alleviated by DF and G treatment.

![Figure 1. Histopathology of liver tissues from rats of groups: N (A), N-CCl₄ (B), HF (C), HF-CCl₄ (D), D (E), DF (F) or G (G). Sections were stained with HE, original magnification is 200×. CV represents centrilobular vein. Black arrow indicates hepatocyte ballooning degeneration with Mallory–Denk body, arrow head shows a hepatic apoptotic body, and white arrow shows infiltration of inflammatory cells.](image)

**Serum Aminotransferase**

Compared with N, the serum ALT or AST levels were elevated in the HF group ($P<0.01$). The two aminotransferases between N and N-CCl₄ exhibited almost no difference, whereas there were significant changes between HF and HF-CCl₄ ($P<0.01$). In comparison with HF-CCl₄, both aminotransferases were dramatically decreased in the DF and G groups ($P<0.01$), whereas only AST was reduced in the D group ($P<0.01$). The ALT level of the DF group was even lower than the HF group without toxic stimulation ($P<0.05$). (Data shown in Table 1 and Figure 2).

**Table 1. Serum ALT and AST in the Groups ($\bar{x} \pm s, n=10$)**

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>32.92±4.17**#/#</td>
<td>41.79±7.16**##</td>
</tr>
<tr>
<td>N-CCl₄</td>
<td>39.84±4.43**##</td>
<td>50.17±1.61**</td>
</tr>
<tr>
<td>HF</td>
<td>57.33±10.01**</td>
<td>65.18±19.43**</td>
</tr>
<tr>
<td>HF-CCl₄</td>
<td>82.90±15.80##</td>
<td>84.14±7.90##</td>
</tr>
<tr>
<td>D</td>
<td>71.71±6.68</td>
<td>45.32±11.73**##</td>
</tr>
<tr>
<td>DF</td>
<td>43.28±3.63**#</td>
<td>38.42±13.35**##</td>
</tr>
<tr>
<td>G</td>
<td>49.64±12.84**</td>
<td>31.97±7.27**##</td>
</tr>
</tbody>
</table>

Notes: Compared with HF-CCl₄ group, *$P<0.05$, **$P<0.01$; compared with HF group, #$P<0.05$, ##$P<0.01$. 
Liver Triglyceride, Malondialdehyde, Glutathione, and Superoxide Dismutase
The triglyceride (TG) content mirrored the degree of hepatic steatosis, and the HF diet appeared to induce dramatically greater TG accumulation in the liver compared to the N diet \((P<0.01)\). The TG level exhibited almost no change between N and N-CCl4, whereas between HF and HF-CCl4 there was a significant difference \((P<0.01)\). Treatment with DF or G reduced the TG level efficiently with HF-CCl4 or HF equally \((P<0.01)\), so did D \((P<0.01\) vs HF-CCl4, \(P<0.05\) vs HF). (Data shown in table 2 and figure 3A).

Compared with N, MDA and SOD increased, whereas GSH decreased significantly in the HF group \((P<0.01)\). No changes were observed in these indexes between N and the N-CCl4 group, but MDA was elevated and GSH markedly reduced in the HF-CCl4 group compared to the HF group \((P<0.01)\), and the SOD activity exhibited a decreasing trend. Treatment by DF, G \((P<0.01)\) and D \((P<0.05)\) reduced the MDA level, and DF and G significantly reverted the reduced GSH and SOD levels \((P<0.01)\), whereas D only exhibited a slight effect. (Data shown in table 2 and figure 3B, C, D).

Hepatic CYP2E1 Protein Expression and Caspase-3 Activation Level
Western blot analyses of liver CYP450 isoform 2E1 and cleaved caspase-3 protein expression are shown in figure 4. First, compared with N, a HF diet induced an increase in CYP2E1 expression and caspase-3 activation \((P<0.01)\). No differences were observed between N and N-CCl4, whereas marked increases were shown in HF-CCl4 compared to HF \((P<0.01)\). Treatment with DF and G reverted both the elevated CYP2E1 and cleaved caspase-3 expression levels significantly \((P<0.01)\), whereas D reduced CYP2E1 expression levels \((P<0.01)\). (Data shown in Figure 4).

Table 2. Liver TG, MDA, SOD and GSH (\(\bar{x} \pm s\), \(n=10\))

<table>
<thead>
<tr>
<th>Group</th>
<th>TG (mg/g liver)</th>
<th>MDA ((\mu)mol/g protein)</th>
<th>GSH (mg/g protein)</th>
<th>SOD (inhibition%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>0.77±0.26**</td>
<td>5.30±1.17**</td>
<td>106.57±10.62**</td>
<td>0.36±0.06**</td>
</tr>
<tr>
<td>N-CCl4</td>
<td>0.93±0.12**</td>
<td>5.55±0.89**</td>
<td>104.15±11.32**</td>
<td>0.34±0.04**</td>
</tr>
<tr>
<td>HF</td>
<td>1.73±0.16**</td>
<td>8.05±1.53**</td>
<td>71.59±13.54**</td>
<td>0.25±0.04</td>
</tr>
<tr>
<td>HF-CCl4</td>
<td>2.11±0.12**</td>
<td>10.11±1.13**</td>
<td>28.10±9.07**</td>
<td>0.20±0.04</td>
</tr>
<tr>
<td>D</td>
<td>1.45±0.28**</td>
<td>8.35±1.51*</td>
<td>32.77±14.02**</td>
<td>0.23±0.06</td>
</tr>
<tr>
<td>DF</td>
<td>1.20±0.12**</td>
<td>6.50±0.67**</td>
<td>51.48±17.21**</td>
<td>0.29±0.04</td>
</tr>
<tr>
<td>G</td>
<td>1.18±0.13**</td>
<td>7.58±0.64**</td>
<td>51.96±15.77**</td>
<td>0.31±0.02**</td>
</tr>
</tbody>
</table>

Notes: Compared with HF-CCl4 group, *\(P<0.05\), **\(P<0.01\); compared with HF group, *\(P<0.05\), **\(P<0.01\).
**Figure 3.** Liver Triglyceride (A), Malondialdehyde (B), Glutathione (C), and Superoxide Dismutase (D) of the Groups. The values represent mean ± SD. *P<0.05, **P<0.01 compared to HF-CCl4 group; †P<0.05, ‡P<0.01 compared to HF group.

**Figure 4.** Immunoblot analysis of liver CYP2E1 protein expression and caspase-3 activation level, with GAPDH as the internal loading control. A representative blot from 3 replicates (n=3) is shown (A). Densitometric analysis of CYP2E1 protein normalized to GAPDH (B). Densitometric analysis of cleaved caspase-3 normalized to GAPDH (C). The values represent mean ± SD. *P<0.05, **P<0.01 compared to HF-CCl4 group; †P<0.05, ‡P<0.01 compared to HF group.
DISCUSSION

Although high fat diet (HFD) induced NAFLD animal models require a lengthy feeding period, these models resemble the pathophysiology observed in human NAFLD closely, including induced obesity, insulin resistance, and hepatic steatosis in mice or rats. In this study, serum ALT, AST, and liver TG levels are elevated in rats of the HF group, and histopathology demonstrated marked diffuse fatty droplet accumulation which was used as the hepatotoxin to study the mechanisms of liver injury. We determined the appropriate dosage of CCl4 that led to almost no observed changes in normal rats and obvious liver damage in NAFLD rats. Livers of HF rats exhibited more significant diffuse steatosis 48 h after CCl4 was applied intraperitoneally, and infiltration of inflammatory cells and ballooning degeneration in the centrilobular region was apparent. Hepatocellular ballooning is an important histological parameter in the diagnosis of nonalcoholic steatohepatitis (NASH), indicating a greater risk of disease progression. In addition, elevated levels of aminotransferases were indicative of the liver injury and severe liver dysfunction caused by CCl4. The results suggested that liver tissue affected with NAFLD is more susceptible to hepatotoxin; thus, nonlethal doses of toxins/drugs for healthy people could potentially cause severe liver damage in NAFLD patients, and attention to this in a clinical setting is warranted.

Oxidative stress (OS) caused by reactive oxygen species (ROS) is involved in the mechanism of variant liver injury, including damage due to hepatotoxin and NAFLD. OS is one of the second hits, believed to be of major importance in mediating the progression of NAFLD to NASH. In simple hepatosteatosis, the hepatic anti-oxidant defense system is regulated to keep the balance with pro-oxidant factors. When the amount of ROS overwhelms the adaptive buffering capacity, DNA mutations, peroxidation of membranes, stellate cell activation, inflammation, apoptosis, and generation of additional free radicals may occur, leading to hepatic injury. MDA is one of the major aldehydic metabolites of lipid peroxidation and is widely utilized to reflect lipid peroxidation and oxidative stress level. SOD is a key anti-oxidative enzyme, and reduced GSH may reflect the anti-oxidative status. In our study, HF rats demonstrated a higher level of lipid peroxidation MDA compared with N rats, and lower levels of anti-oxidants reduced GSH and SOD activity. However, no apparent hepatocyte degeneration or apoptosis was observed, which suggests that the liver tissue of the HF group was adaptive of OS. No pathologic change occurs in normal liver tissue due to low dosage of CCl4 because of the liver function of detoxification. However, in NAFLD liver detoxification is associated with activated enzymes or molecules that may be almost exhausted; thus, CCl4 may be transformed, leading to the generation of more ROS and liver injury. Therefore, the livers of HF-CCl4 rats demonstrated an even higher content of MDA and lower level of GSH and SOD, with the appearance of dramatic hepatocyte ballooning degeneration and apoptotic bodies, as well as increased cleaved caspase-3 levels. CYP450 isoform 2E1 has been shown to be one of the most potent microsome cytochromes to generate ROS. Liver CYP2E1 is accepted as the hallmark of oxidative stress. In previous studies it has been shown to be invariably elevated in the livers of NASH patients.

In this study, we also observed elevated CYP2E1 expression in HF group and an even higher level in HF-CCl4 group.

To prevent or relieve severe liver injury caused by hepatotoxin in NAFLD patients, appropriate intervention should be taken to reduce hepatotoxic susceptibility. Herein, we studied the effect of liver-protective medicine Dangfei Liganning capsules and one of its components, Dangyao extract, as well as Diammonium Glycyrrhizinate as the positive therapeutic control. Our results demonstrated that, DF and G treatment of HF-CCl4 rats significantly improved liver function by decreasing the serum ALT and AST level, reducing liver TG accumulation, and lowering caspase-3 activation; the histopathology results exhibited less inflammation and loss of ballooning degeneration and apoptosis. This effect may be correlated with an adjustment of OS associated factors, as MDA content and CYP2E1 expression were markedly reduced, whereas GSH and SOD were elevated in the DF and G groups. D also exhibited a protective effect, however, it appeared to have a less efficient influence in reversing ALT, GSH and SOD levels; thus, in the livers of the D group ballooning hepatocytes still could be observed in proximity to the central vein. In addition, when compared with HF group, we demonstrated that DF also improved the pathogenic factors of NAFLD, including serum ALT, AST, liver TG, MDA, GSH, and CYP2E1 levels; moreover, the HE and oil red O staining results of the liver sections demonstrated fewer fatty droplets. These results suggest that DF not only extenuated the sensitivity of NAFLD to hepatotoxicity, but also improved the hepatosteatosis of NAFLD.

In summary, this study demonstrated that hepatosteatosis induced by a high fat diet significantly sensitizes liver tissue to hepatotoxic injury. One of the major underlying causes is the disrupted balance between pro-oxidants and anti-oxidants during NAFLD development as well as hepatotoxicity stimulation. Our findings also indicated that Dangfei Liganning capsules could attenuate this sensitivity and protect fatty liver tissue effectively from severe liver dysfunction, and that regulation for oxidative stress associated factors contributes to the therapeutic mechanism.
REFERENCES


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