Protection Effect and Mechanism of Huangshan Maofeng Tea Polyphenols Extracts on CCl₄-Induced Liver Injury

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Abstract. The purpose of this study is to observe whether Huangshan maofeng tea polyphenols extracts have protection effect for mice liver injuries caused by carbon tetrachloride (CCl₄) and its mechanism. Method: High (300 mg/kg), middle (150 mg/kg) and low (75 mg/kg) doses of Huangshan maofeng tea polyphenols were administered for 7 days. One hour after the last gavage, all groups (except the control group) will inject in peritoneal 0.10% CCl₄ olive oil solution. After 24 hours, blood from eyeball and liver tissue will be extracted. Changes in content of Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) will be detected; The expression of the mRNAs and proteins of the four liver enzymes were detected using Real-Time PCR and Western Blot. Result: Huangshan maofeng tea polyphenols low, medium, high dose groups can all decrease the activity of ALT and AST in serum of mice with acute liver injury; Huangshan maofeng tea polyphenols low, medium, high dose groups can all induce mice liver Cyp3a11 mRNA and protein expression. Conclusion: Huangshan maofeng tea polyphenols extracts can protect liver by inducing the expression of Cyp3a11.

Introduction

Chinese tea exists in various forms. Some of the major types include green tea, oolong tea and black tea. The subject of this experiment, Huangshan maofeng tea polyphenols, is a kind of green tea. Green tea is a kind of non-fermented tea which contains the natural characteristics of pure tea. The characteristic bioactive ingredient green tea contains is tea polyphenolic (TP) compounds. Green tea has the highest content of tea polyphenolic compounds, which is of the highest content along all soluble components in tea. It can be used as antioxidant and is able to prevent inflammation. It can also decrease the probability of cardiovascular diseases, prevent cancer, lower blood fat, lower body fat, prevent bacteria and change the environment of intestinal flora. Recent research shows that tea polyphenolic compounds can also protect or inhibit experimental liver injuries caused by carbon tetrachloride, acute cadmium poisoning and alcohol. Meanwhile, acute liver injury is now one of the most important causes of clinical death which may result in rapid increase of patients’ glutamic-pyruvic transaminase enzyme and glutamic-oxaloacetic aminotransferase.

Moreover, tea polyphenolic is mainly metabolized in liver by liver drug metabolizing enzyme (CYP450s) on the smooth endoplasmic reticulum of liver cells. CYP450s is a mixed-functional oxidase system responsible for the transformation of multiple types of endogenous and exogenous substance in human body. The three main gene families which are involved in most drug metabolism in human body are CYP1, CYP2 and CYP3. Main protein isoforms include CYP3A4, approximately occupying 30% of the oxidase content of CYP450s. Also, CYP3A4 mediate 50% of the metabolism process of clinical drugs. Since the drug metabolizing enzyme between mice and human are homologous, the expression Cyp3a11 in mice livers each corresponds to the expression of CYP3A4 in human body. This experiment focuses mainly on the protection tea polyphenolic provides for mice with acute liver injuries caused by CCL₄, and discuss the liver protect mechanism of tea polyphenolic through its influence upon Cyp3a11.
Experiment Material

Animals and Treatments

Female C57BL/6 mice were purchased from the Experimental Animal Center of Southern Medical University. The animals were fed rodent chow and accessed water freely in an environment of 25 ± 1 °C, 55 ± 5% relative humidity, and diurnallight supply. Mice were housed in groups of four in a cage andacclimatized to the animal facility for at least 1 week before the experiment. The animals were randomly divided into 8 groups with 6 mice per group: (1) blank control group; (2) CCl₄ model group; (3-5) treated group (low [75 mg/kg], middle [150 mg/kg], and high [300 mg/kg] doses of Huangshan maofeng tea polyphenols groups, respectively); (6) Bifendate group [200 mg/kg].

Main Reagent and Consumptive Material

Huangshan maofeng tea, purchased from yellow mountain, Anhui; Bifendate Pills, purchased from Beijing Union Pharm, batch number:14120103; ALT and AST kit, both purchased from Nanjing Jiancheng Bioengineering Institute. A total RNA column extraction kit, 1X TE buffer, and 5X sample loading buffer were purchased from Sangon Biotech (Shanghai, China). The primer was entrusted to and synthesized by Sangon Biotech. PrimeScript TMRT Master Mix (Perfect Real Time) and SYBR® Premix Ex TaqTM II (TliRNaseH Plus) were purchased from Takara (Takara Biotechnology Co., Ltd., Dalian, China). The primary antibodies (rabbit polyclonal antibodies against Cyp3a11) and horseradish peroxidase-labeled goat anti-rabbit IgG antibody were purchased from Abcam (Cambridge, MA, USA). ECL reaction buffer was purchased from BIO-RAD (Hercules, CA, USA).

Experiment Method

The extraction of Huangshan Maofeng Tea Polyphenols Tea Polyphenolic

20g of precisely weighed Huangshan maofeng tea polyphenols tea powder will be added to a preheated 70% methanol with the proportion of 1:25(m/V). It will then be encased in a 1000ml round bottom flask and a glass rod will be used for stirring and madefaction. After that it will be immediately switched to a 70°C water bath and a condenser pipe will be installed. Reflux condensation should be extracted for 30min and cooled to room temperature after leaching. Next, the reflux condensation should be filtered and the filter residue will be poured back into the round bottom flask. The process of leaching needs to be repeated three times. All the filtrate will be combined and the methanol in the filtrate will be rotary evaporated. Then it will be extracted once using chloroform with three times volume. Then, it will be rotary evaporated again and the chloroform will also be evaporated. The rest will be dissolved into ultra pure water and centrifuged using high speed centrifuge (10000r/min, 15min). The supernatant will go through vacuum freeze-drying and will be kept away from light at normal temperature.

Animal Grouping and Disposition

36 grown male kunming mice in healthy condition (20±2g) randomly assigned into six groups. Each group contains 7 mice. Gavage will be performed on a successive 10 day period. One hour after the last gavage, all groups (except the normal group, which will be injected equal amount of olive oil in peritoneal) will inject in peritoneal 0.10% CCl₄ olive oil solution at the dose of 10ml/kg. The mice will be weighted after 24 hours and blood will be extracted to heparin sodium catheter. Whole blood will be placed in the refrigerated centrifuge for 10 min at the rate of 3000r/min after 30min. The upper plasma will be obtained and kept in 4°C refrigerator. Spine will suffer sudden death. Liver tissue will be extracted from mice and will be weighted after cleaning it in precooled normal saline. After that, it will be kept at -80°C after quick freezing by liquid nitrogen.

Detection of ALT

ALT will act on alanine and the substrate formed by α-Ketoglutaric acid to form pyruvate and
glutamate under the condition of 37°C and pH 7.4. 30 minutes after reaction (fixed time), add in 2,4-dinitrophenylhydrazine (DNPH) hydrochloride solution to terminate the reaction and at the same time DNPH will experience addition reaction with the carbonyl group in keto acid to form pyruvate phenylhydrazone. Phenylhydrazone will turn reddish brown in alkaline condition. Process will be conducted according to specification of the kit. The unit is expressed in Karmen unit. 1 Karmen unit=0.482 U/L, 25°C.

Detection of AST

AST α-Ketoglutaric acid. Ketasuccinic acid can self-decarboxylate during the reaction process to form pyruvate, which is able to form 2,4-dinitrophenylhydrazone by reacting with 2,4-dinitrophenylhydrazine (DNPH). Phenylhydrazone will turn reddish brown in alkaline condition. Process will be conducted according to specification of the kit. The unit is expressed in Karmen unit. 1 Karmen unit=0.482 U/L, 25°C.

RNA Extraction and Reverse Transcriptase-Real Time PCR

Total RNA was extracted with TRIzol reagent according to the manufacturer’s instructions. RNA concentration was determined spectrophotometrically by measuring the absorbance at 260 nm. Two micrograms of total RNA was used for cDNA synthesis with the High Capacity cDNA Archive kit. Real-time PCR was carried out with SYBR Green PCR Master Mix using ABI 7300 reverse transcription PCR equipment. The transcription level of the housekeeping gene, β-actin, was quantified for normalization. Analysis of real-time PCR was carried out by the ΔΔCt method. The expression level in control samples was arbitrarily set at 1.

Protein Extraction and Immunoblotting

Livers of female C57B/6J mice were harvested and rinsed with cold 1.15% potassium chloride. Hepatocytes were incubated on ice in PBS with 1 mM EDTA for at least 20 min before harvest and then collected by centrifugation at 1000g for 5 min. The livers or hepatocytes were then homogenized in homogenization buffer or sonicated in cell lysis buffer and then centrifuged at 11000g for 10 min. Anti-Cyp3a11 (1:2000) and GADPH (1:5000) antibodies were incubated overnight at 4°C, and then horseradish peroxidase-conjugated secondary antibody was incubated for 1 h at room temperature. Quantitative densitometric analyses of Western blot images were achieved using Image Lab software.

Statistical Analysis

Data are presented as mean±SD. Control and experimental groups were compared either by unpaired Student’s t test or one-way analysis of variance followed by Dunnett’s test. The criterion of significance was set at p<0.05. All statistical analysis was performed with SPSS software (SPSS Inc., Chicago, IL).

Result and Analysis

The Result and Analysis of ALT and AST Detection of Mice Serum

Table 1. Protective effect of Huangshan Maofeng Tea Polyphenols Extracts (TPE) on CCl₄ - Induced Liver Injury.

<table>
<thead>
<tr>
<th>Groups</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.78±4.12</td>
<td>43.17±5.11</td>
</tr>
<tr>
<td>CCl₄ Group</td>
<td>227.80±6.12**</td>
<td>115.87±12.91**</td>
</tr>
<tr>
<td>TPE(75mg/kg)</td>
<td>178.53±17.20*</td>
<td>65.63±12.36##</td>
</tr>
<tr>
<td>TPE(150mg/kg)</td>
<td>145.25±10.65##</td>
<td>49.32±6.01**</td>
</tr>
<tr>
<td>TPE(300mg/kg)</td>
<td>201.83±9.43##</td>
<td>69.02±11.23##</td>
</tr>
<tr>
<td>Bifendate</td>
<td>93.53±15.49##</td>
<td>59.95±12.91##</td>
</tr>
</tbody>
</table>

Compared with Control group, **p<0.01; Compared with CCl₄ group, *p<0.05, ##p<0.01
The content of ALT and AST in mice serum was detected according to method 2.3 and 2.4 and
the results were shown in table 1. Compared to the normal group, the model group’s ALT and AST content in mice serum increased significantly (P<0.01), implying the success of modeling. Compared to the model group, the Huangshan maofeng tea polyphenols groups’ ALT and AST content in mice serum decreased significantly, especially the medium dose group. There was a very significant decrease in ALT content (p<0.01).

The Result and Analysis of Mice Liver Cyp3a11 mRNA and Protein Expression

The result of RT-PCR was shown in Figure 1. Compared to the blank control group, Huangshan maofeng tea polyphenols groups can all evidently induce the expression of Cyp3a11 mRNA in mice liver tissue (p<0.01). The effect was most obvious for low dose group. Huangshan maofeng tea polyphenols extracts display the tendency of opposite dose-dependent manner on the expression of mice liver Cyp3a11 mRNA.

The result of Western Blotting experiment was shown in Figure 2. Compared to the blank control group, Huangshan maofeng tea polyphenols groups can all evidently induce the expression of CYP3A11 protein in mice liver tissue (p<0.01), implying that Huangshan maofeng tea polyphenols extracts may increase the amount of expression of protein Cyp3a11 through inducing the expression of Cyp3a11 mRNA. Huangshan maofeng tea polyphenols extracts display the tendency of dose-dependent manner on the expression of mice liver Cyp3a11.

Discussion

Carbon tetrachloride is a kind of widely used liver injury initiator. After carbon tetrachloride enters the body, it will be metabolized by liver cytochrome enzyme P450 to form trichloromethyl free radical. Free radical can attack cell membrane and other membrane-like substances to initiate lipid peroxidation which results in damage of cell membrane structure. ALT is mainly distributed in
liver cytoplasm. AST is mainly distributed in liver cytoplasm and mitochondria. As a kind of liver cell endoenzyme, it plays an important role in the synthesis and catabolism of amino acid. Under normal circumstances, only a very small amount of ALT and AST would enter the blood. When liver tissue are acutely damaged or permeability of cell membrane increase, a lot of the two kinds of enzyme would enter the blood to cause a significant increase in the activity of serum enzyme. The increase amplitude of serum transaminase can reflect the necrosis extent of liver cell.

During the process of experiment, after the mice in model group were injected with CCl\textsubscript{4}, the liver injury caused the content of ALT and AST in serum increased faster compared to the normal group. On the other hand, the content of ALT and AST of those that had already been given Huangshan maofeng tea polyphenols extracts showed a significant decrease compared to the model group. This implies that Huangshan maofeng tea polyphenols extracts can effectively inhibit the oxidative damage of mice liver. Compared to the model group, according to Huangshan maofeng tea polyphenols's influence upon ALT and AST in mice serum, all dose groups showed a significant decrease in enzyme activity (p<0.05), especially the medium dose group (p<0.01). This gives a hint that Huangshan maofeng tea polyphenols extracts can most effectively inhibit acute mice liver injury caused by carbon tetrachloride at 150mg/kg.

Cyp3a11 is one of the most important members of P450 enzyme system. It approximately makes up 30% of liver P450 content and is capable of metabolizing more than 50% of clinical drug with different structure and property. In the experiment result, compared to the blank control group, all Huangshan maofeng tea polyphenols groups showed significant induction of mice liver Cyp3a11 mRNA and protein (p<0.01), implying that Huangshan maofeng tea polyphenols extracts can effectively induce the content of Cyp3a11 in mice liver to some extent.

In conclusion, Huangshan maofeng tea polyphenols extracts can serve as a good protection for acute mice liver injury caused by CCl\textsubscript{4} and can prominently induce the expression of Cyp3a11 mRNA and protein in mice liver tissue, implying that Huangshan maofeng tea polyphenols extracts may achieve the goal of liver protection through inducing Cyp3a11 and promoting the metabolism and excretion of CCl\textsubscript{4}. Its detailed mechanism needs further investigation.

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References


