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Olive oil combined with *Lycium barbarum* polysaccharides attenuates liver apoptosis and inflammation induced by carbon tetrachloride in rats

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ABSTRACT

Olive oil and *Lycium barbarum* polysaccharides were considered to be hepatoprotective for liver fibrosis. This study investigated anti-apoptotic, anti-inflammatory and anti-fibrotic effects of olive oil and/or *Lycium barbarum* polysaccharides (LBP) on CCl_4-induced liver fibrosis in rats. The results showed that CCl_4 caused liver fatty change, cell death, inflammation and collagen accumulation. The olive oil-treated groups reduced hepatic transforming growth factor (TGF)-β1 and tissue inhibitors of metalloproteinase (TIMP)-1 levels. The LBP-treated groups inhibited hepatic caspase-9 and caspase-3 activities, decreased hepatic tumor necrosis factor (TNF)-α levels, increased hepatic interleukin (IL)-10 levels and IL-10/TNF-α ratios, and reduced hepatic TGF-β1 and TIMP-1 levels. Olive oil combined with LBP suppressed liver apoptotic markers, inhibited liver inflammatory markers, and attenuated hepatic TGF-β1 levels. Therefore, LBP improves liver apoptotic, inflammatory and fibrotic markers, while olive oil combined with LBP has better effects on anti-apoptosis and anti-inflammation compared with olive oil treatment in rats with CCl_4-induced liver fibrosis.

1. Introduction

Liver disease is common all over the world, and the causes of liver disease involve virus infection, drug induction, alcohol abuse and metabolic diseases. While liver injury occurs, apoptosis and inflammation are induced. Then, hepatic stellate cells (HSC) transform into myofibroblasts followed by increasing the secretion and accumulation of extracellular matrix, and leads to liver fibrosis (Bataller & Brenner, 2005). Carbon tetrachloride (CCl_4) is hepatotoxic and commonly used in animal models to mimic human liver fibrosis. *In vivo*, CCl_4 can be metabolized to trichloromethyl radical ($CCl_3^{\cdot}$) and trichloromethyl peroxyl radical ($CCl_3OO^{\cdot}$) by liver cytochrome P450 enzymes which cause liver injury and liver fibrosis (Weber, Boll, & Stampfl, 2003).

Some dietary ingredients or Chinese medicine have been reported to show hepatoprotective activities. Olive oil is common dietary oil which is rich in monounsaturated fatty acids (MUFA), especially oleic acid (C18:1, n-9). A previous study found that thioacetamide-induced liver steatosis and fibrosis were alleviated in rats fed the diet rich in oleic acid (66% MUFA of fat) for 2 weeks (Fernandez, Torres, Gil, & Rios, 1997). Additionally, liver steatosis, inflammation and fibrosis induced by methionine- and choline-deficient diet were improved in mice given 0.5 mg/g bw oleic acid per day (Lee et al., 2011). *Lycium barbarum* polysaccharides (LBP) with molecular weight ranged from 24 kDa to 241 kDa are the most bioactive compound in wolfberry, and approximately 23% of dry weight in wolfberry (Cheng et al., 2015). The monosaccharides in LBP include xylose, mannose, arabinose, rhamnose, glucose, galactose and fucose (Cheng et al., 2015). The previous studies showed that LBP as an antioxidative substance increased antioxidant enzyme activities in rats with alcohol-induced liver injury (Cheng & Kong, 2011) and in high fat-fed rats (Wu et al., 2010), and decreased liver malondialdehyde (MDA) level in high fat-fed rats (Wu et al., 2010). The expression of cytochrome P450 2E1, the level of MDA and the expression of inflammatory markers in the liver were suppressed, but the activities of antioxidative enzymes were increased in mice fed LBP 2 h before acute liver injury induced by CCl_4 (Xiao et al., 2012).

Olive oil and LBP had potent hepatoprotective activities, but none of the studies had combined two for the treatment of liver fibrosis. Therefore, this study investigated whether the pre-treatment of olive oil combined with *Lycium barbarum* polysaccharides prevented the progression of liver injury induced by CCl_4 in rats via modulations of apoptosis, inflammation and fibrosis.

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2. Material and methods

2.1. Animals and treatments

Male Sprague-Dawley rats (250–300 g) were purchased from the BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). Rats were housed individually on a 12-h light/dark cycle at 22 ± 2 °C with a relative humidity of 65 ± 5%. After a one-week adaptation period, rats were randomly divided into 7 groups (n = 9–10 per group): normal group (4% soybean oil diet, N), soybean oil group (CCl_4 + 4% soybean oil diet, S), olive oil group (CCl_4 + 4% olive oil diet, O), mixed oil group (CCl_4 + 2% soybean oil/2% olive oil diet, M), soybean oil + LBP group (CCl_4 + 4% soybean oil diet + LBP, S + L), olive oil + LBP group (CCl_4 + 4% olive oil diet + LBP, O + L) and mixed oil + LBP group (CCl_4 + 2% soybean oil/2% olive oil diet + LBP, M + L).

Dietary intervention of oil and/or LBP was given orally from the beginning to the end of the experiment for 9 weeks. After a week of dietary pre-treatment (week 0), CCl_4 (Sigma-Aldrich Co. LLC, Germany) was administered intraperitoneally at a single dose of 1.0 mL/kg bw (40% in olive oil) once a week from week 1 to week 4 and twice a week from week 5 to week 8 to induce liver fibrosis. The normal group fed the modified AIN-93M diet containing 77.1% (w/w, 76.4% energy) carbohydrate from corn starch, dextrin, sucrose and cellulose, 14.0% (w/w, 14.4% energy) protein from casein and 4.0% (w/w, 9.2% energy) fat from soybean oil, and received the vehicle (olive oil) intraperitoneally in the same dose as CCl_4. The percentage of fat (w/w) was the same as the modified AIN-93M diet and the source of fat in the diet were 4% soybean oil as the modified AIN-93M diet, 4% olive oil to replace soybean oil completely or 2% soybean oil + 2% olive oil to replace half of soybean oil in the soybean oil groups, the olive oil groups or the mixed oil groups, respectively. According to the previous study by Cui et al. (2011), *Lycium barbarum* aqueous and ethanol extracts at a dose of 50 mg/kg bw/d given orally for 8 weeks significantly increased liver antioxidant capacity in rats with high fat diet-induced oxidative stress. In this study, the LBP groups were orally fed 50 mg LBP (GojiMax® 40%, Priority Healthfood Corporation, New Taipei City, Taiwan)/kg bw/d, which was mixed in the powdered feed, and the proportion of LBP in the feed was adjusted weekly according to body weight and food intake of rats. Food intake was monitored 3 times a week, and body weight was recorded once a week. Blood samples were drawn from the tail vein at weeks 0 and 4 after overnight fasting, and rats were sacrificed at week 8 after anesthetized by Zotile (0.5 mL/kg bw) and Rompum (0.5 mL/kg bw). Plasma and liver samples were collected and stored at −20 °C for further analyses. All animal protocols were approved and conducted under the guidelines of the Institutional Animal Care and Use Committee at Taipei Medical University.

2.2. Analysis of dietary oils and LBP

Fatty acid compositions in dietary oils were analyzed by gas chromatography and flame ionization detector. The total polysaccharides were 40% in LBP extracted by water using a spectrophotometer. Monosaccharide compositions in LBP were measured by Academia Sinica (Taipei, Taiwan) after hydrolysis by trifluoroacetic acid at 110 °C for 4 h using a high pH anion exchange chromatography-pulsed amperometric detection.

2.3. Histopathologic examination

Excised liver (1 cm × 1 cm) from 6 samples randomly selected in each group was fixed in 10% paraformaldehyde, and analyzed by the National Laboratory Animal Center (Taipei, Taiwan). Liver samples were then embedded in paraffin wax, and stained with hematoxylin and eosin (H&E) and Masson's trichrome. The pathological assessment of fat accumulation, necrosis and inflammation in liver was graded from 0 (none) to 5 (severe) by H&E stain (Shackelford, Long, Wolf, Okerberg, & Herbert, 2002). Liver fibrosis was graded from 0 (normal) to 4 (cirrhosis) by Masson's trichrome stain (Boigk et al., 1997).

2.4. Biochemical analyses

Plasma aspartate aminotransferase (AST) activity, alanine aminotransferase (ALT) activity, triglycerides (TG) and total cholesterol (TC) concentrations at week 0, 4 and 8 were measured by Lezen Reference Lab (Taipei, Taiwan) using a reflection photometer (Vitros 950 analyzer, Ortho Clinical Diagnostics Inc., Raritan, NJ, USA). Plasma high-density lipoprotein-cholesterol (HDL-C) level was assessed at 500 nm by a spectrophotometer using the kit from Randox Laboratories Ltd. (Antrim, UK). Low-density lipoprotein-cholesterol (LDL-C) level was calculated by Friedewald's formula: LDL-C (mg/dL) = TC-(HDL-C + TG/5). Hepatic TG and TC were extracted by chloroform/methanol (2/1, v/v) and chloroform/methanol/water (3/48/47, v/v/v), and measured at 500 nm by a spectrophotometer using the kits from Randox Laboratories Ltd. (Antrim, UK).

Liver apoptotic markers were analyzed spectrophotometrically, and liver inflammatory and fibrotic markers were measured quantitatively by enzyme-linked immunosorbent assay (ELISA) rather than semi-quantitatively by Western blot analysis. Homogenized liver supernatant in radioimmunoprecipitation assay (RIPA) buffer was added to reaction buffer and a chromogenic substrate (Leu-Glu-His-Asp-p nitroanilide for caspase 9 or Asp-Glu-Val-Asp-p nitroanilide for caspase 3) at 37 °C for 1 h, and hepatic caspase-9 and caspase-3 activities were determined colorimetrically at 400 nm by a spectrophotometer using the kits from BioVision, Inc. (Milpitas, CA, USA). Liver homogenate was incubated with anti-tumor necrosis factor (TNF)-α or anti-interleukin (IL)-10 antibody for 2 h at room temperature, mixed with biotinylated secondary antibody for 2 h, reacted with streptavidin-horseradish peroxidase conjugate for 20 min, and samples were washed 3 times by wash buffer (Tween 20 in phosphate buffered saline) in between each incubation step. The substrates (H_2O_2 and tetramethylbenzidine) were added for 20 min at room temperature, and the reaction was terminated by adding stop solution (H_2SO_4). Liver TNF-α and IL-10 levels were measured colorimetrically at 450 nm and 540 nm for correction by an ELISA reader using the kits from R&D Systems Inc. (Minneapolis, MN, USA). Liver homogenate was added to 96-well plate for 90 min at 37 °C, incubated with biotinylated anti-transforming growth factor (TGF)-β1 or anti-metalloproteinase (MMP)-2 antibody for 60 min at 37 °C, mixed with avidin-biotin-peroxidase complex for 30 min at 37 °C, and samples were washed 5 times by phosphate buffered saline (PBS) in between each incubation step. The substrates (H_2O_2 and tetramethylbenzidine) were added for 25 min at 37 °C, and the reaction was terminated by adding stop solution (H_2SO_4). Liver TGF-β1 and MMP-2 levels were analyzed colorimetrically at 450 nm by an ELISA reader using the kits from Boster Biological Technology Co., Ltd. (Pleasanton, CA, USA). Liver homogenate was added to 96-well plate for 2 h at 37 °C, incubated with biotinylated tissue inhibitors of metalloproteinase (TIMP)-1 or TIMP-2 antibody for 1 h at 37 °C, mixed with avidin conjugated horseradish peroxidase for 1 h at 37 °C, and samples were washed 5 times by PBS in between each incubation step. The substrates (H_2O_2 and tetramethylbenzidine) were added for 15 min at 37 °C, and the reaction was terminated by adding stop solution (H_2SO_4). Liver TIMP-1 and TIMP-2 levels were determined colorimetrically at 450 nm by an ELISA reader using the kits from Cusabio Biotech Co., Ltd. (College Park, MD, USA). Liver homogenate was added to the coated 96-well plate with anti-hydroxyproline antibody, mixed with horseradish peroxidase conjugate for 40 min at 37 °C, and samples were washed 5 times by PBS. The substrates (H_2O_2 and tetramethylbenzidine) were added for 20 min at 37 °C, and the reaction was terminated by adding stop solution (H_2SO_4). Liver hydroxyproline levels were measured colorimetrically at 450 nm by an ELISA reader using the kit from Cusabio Biotech Co., Ltd. (College Park, MD, USA).