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Ameliorative effect of grape seed proanthocyanidin extract on thioacetamide-induced mouse hepatic fibrosis

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HIGHLIGHTS

- Administration of TAA for 9 weeks led to a serious liver necrosis and collagen deposition.
- GSPE diminishes TAA-induced mRNA expression of *TGF-β1*, *α-SMA* and *α1-(I)-collagen*.
- GSPE suppresses the collagen deposition and the activated HSCs induced by TAA.

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ABSTRACT

The present study was designed to examine the effect of the grape seed proanthocyanidin extract (GSPE) on developing hepatic fibrosis that was induced by thioacetamide (TAA) in mice. Administration of TAA for 9 weeks led to a serious necrosis and apoptosis of the parenchymal cells, which resulted in an accumulation of excessive collagen in the liver and an increase of transformed hepatic stellate cells (HSCs). In addition, the mRNA expression of transforming growth factor β1 (*TGF-β1*), α-smooth muscle actin (*α-SMA*), as the marker of the activated HSCs, and α1-(I)-collagen were all up-regulated significantly when compared with the control. However, combined oral administration of GSPE at 100 mg/kg suppressed the mRNA expression of *TGF-β1* and *α-SMA*, with decreased collagen accumulation as demonstrated by histomorphological evaluation and quantitative RT-PCR. The mRNA expression of the pro-inflammatory factors, including inducible nitric oxide synthase (*iNOS*) and cyclooxygenase-2 (*COX-2*), was remarkably enhanced by TAA treatment. However, their levels displayed a down-regulated trend beyond simultaneous GSPE treatment. Moreover, GSPE administration markedly suppressed lipid peroxidation. In conclusion, as a plant antioxidant, GSPE manifested effective hepatocellular protective action to ameliorate the developing liver fibrosis induced by chronic TAA administration in mice.

1. Introduction

Liver fibrosis is a wound-healing response to chronic noxious stimuli (Bedossa and Paradis, 2003). The vast majority of hepatocellular carcinomas and cases of hepatic cirrhosis develop in patients suffering from liver fibrosis (Kornek et al., 2006). Damage to the liver tissues can be brought about by long-term administration of various noxious substances which promote serious necrosis and apoptosis of parenchymal cells. When the liver's defensive capacities are exceeded and the loss of hepatocytes continues, a potent regenerative response is triggered. However, when hepatic injury persists further, the rebuilding process of damaged tissues will be disrupted and the extracellular matrix degradation process will be inhibited (Tipoe et al., 2010). This complex response process requires the activation of the hepatic stellate cells (HSCs). HSCs have been identified as the principal collagen producing cells in the liver, and are mediated by an intricate network of cytokines, mitogens, and growth factors (Friedman et al., 1985; Mann and Marra, 2010). At later stages, an excessive extracellular matrix is accumulated in liver tissues, which may lead to hepatic fibrosis or cirrhosis. As HSC plays a central role in the formation progress of liver fibrosis, reducing activated HSCs may represent an effective method of protecting liver against fibrogenesis.

Appropriate animal models are of great importance in the understanding of the mechanisms responsible for the acute or chronic liver injury process. Carbon tetrachloride (CCl_4) is the most common agent to cause liver damage and induce liver

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fibrosis. However, this is insoluble in water, and usually needs to be dissolved in vegetable or mineral oil and then administrated via repetitive intraperitoneal or subcutaneous injection (Erman et al., 2004; Miyazaki et al., 2005; Abdel Aziz et al., 2007). Its application to mice is inconvenient and associated with an intolerable high mortality rate (Kornek et al., 2006). The approaches of other chemicals to induce hepatic damage such as dimethylnitrosamine (DMN), thioacetamide (TAA), α-naphthyl-isothiocyanate, or 3,5-diethoxycarbonyl-1,4-dihydrocollidine has been well described in the literatures (for example Weiler-Normann et al., 2007).

The hepatic toxic chemical TAA has been widely used in the study of the underlying mechanisms of hepatic fibrogenesis and the therapeutic effects of potential antifibrosis drugs. TAA is water soluble, and so can be easily administrated orally by being dissolved in drinking water (Salguero Palacios et al., 2008), or utilized in other approaches such as intraperitoneal injection (Baskaran et al., 2010; de David et al., 2011), or injections combined with 10% alcohol in drinking water (Kornek et al., 2006). TAA causes severe centrilobular necrosis and also induces apoptosis and periportal inflammatory cell infiltration in the liver. The initiation of the hepatotoxic effect of TAA requires metabolic activation (Wang et al., 2000; Ramaiah et al., 2001; Chilakapati et al., 2007).

In view of the widespread cases of liver fibrosis, there is a great demand to innovate or to find a potent antifibrotic agent for liver fibrosis patients. This aim so far, has remained elusive. Natural products extracted from plants or traditional Chinese medicine exhibit a variety of biological activities. Many of them can be options for the treatment of liver fibrosis. Among various natural plant extracts, grape seed proanthocyanidin extract (GSPE) is a powerful free radical scavenger which has been reported to possess a broad spectrum of biological, pharmacological and therapeutic effects. Proanthocyanidins are a class of phenolic compounds that take the forms of oligomers or polymers of polyhydroxy falvan-3-ol units, such as (+)-catechin, (−)-epicatechin (Yamakoshi et al., 2002). They accumulate predominantly in the lignified portions of grape clusters, especially in the seeds (Kovac et al., 1995). Currently commercial preparations of GSPE are marketed with over 95% standardized proanthocyanidins as dietary supplement due to its health benefits. Proanthocyanidins exhibit anti-inflammatory, antiallergic and antitumoral activities (Li et al., 2001; Singh et al., 2004; Mantena et al., 2006; Tang et al., 2012), beyond their free radical scavenging and antioxidant activity. Furthermore, they have been reported to modulate the activity of enzymes including phospholipase A_2, cyclooxygenase and lipoxygenase (Bagchi et al., 2000). As to fibrosis-related disease, GSPE was demonstrated to decrease the oxidative stress and reduce the fibrogenic effect of silica-induced pulmonary fibrosis (Hemmati et al., 2008). It also had the ability to reduce oxidative stress and fibrosis in experimental biliary obstruction (Dulundu et al., 2007) and exhibited hepatoprotective and anti-fibrogenic effects against DMN-induced liver injury *in vivo* (Shin et al., 2010). It also was demonstrated to inhibit the arsenic induced-rat liver injury through suppression of NADPH oxidase and TGF-β/Smad activation (Pan et al., 2011).

In light of the observations above, it seems feasible to expect that GSPE might be employed to reduce liver fibrosis and be an effective candidate for the desired anti-fibrotic drug innovation. The present study was designed to determine whether proanthocyanidins have an antifibrogenic effect on TAA-induced hepatic fibrosis in mice along with noting any possible changes in mRNA expression of the fibrosis markers of transforming growth factor β1 (TGF-β1), α-SMA and α1-(I)-collagen, the pro-inflammatory mediators of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). The results would provide a pivotal reference for treatment of liver fibrosis.

2. Material and methods

2.1. Animal model and treatments of chemicals

Female ICR mice weighing 18–22 g were obtained from the Laboratory Animal Center of Zhejiang University. Animals were kept at 22 ± 2 °C and relative humidity (60% ± 10%) under 12-h light/dark cycles. All experimental procedures were preformed in accordance with *the Guidelines for Care and Use of Laboratory Animals of Zhejiang University*.

We prepared the TAA-induced liver fibrosis as previously described (Teixeira-Clerc et al., 2006). The dosage of GSPE was based on several reported works (Saada et al., 2009; Pan et al., 2011; Tang et al., 2012). The animals were divided into four groups as follows: (1) control group with the administration of vehicles only. (2) GSPE control group. (3) TAA treatment group received 300 mg/L TAA (Aladdin reagent Co. Shanghai, China) in drinking water for 9 weeks. (4) GSPE + TAA group at a daily dose of 100 mg/kg GSPE by oral administration every day for the length of the study. The GSPE was generously provided by JF-NATURAL Co. (Tianjin, China). The total proanthocyanidins content of the GSPE is over 95% in this experiment.

2.2. Sample preparation

At the end of the treatment period the mice were sacrificed after anesthesia with sodium pentobarbital. Blood and liver samples were then collected for further analysis. The liver tissue blocks were washed with ice-cold saline, and either frozen in liquid nitrogen and kept at −80 °C for biochemical analyses, or fixed in 4% paraformaldehyde for histological studies. Serum was obtained by centrifugation of blood at 13,000 rpm for 15 min at 4 °C and stored at −80 °C for analysis.

2.3. Histological and immunohistochemical staining

For histomorphological evaluation, a portion of fixed liver tissue was dehydrated, embedded in paraffin and sectioned at 5 μm. The sections were stained with hematoxylin and eosin and Masson's trichrome stain. The morphological changes were examined under a microscope (Eclipse 80i, Nikon, Japan), and the pictures were captured with a digital camera (DS-Fi1, Nikon, Japan). Grading and staging of hepatic histopathological changes was performed by using a previous reported system (Ishak et al., 1995).

The immunohistochemical stains were carried out by using anti-α-smooth muscle actin (α-SMA) as previously described (Guesdon et al., 1979). Briefly, five-micrometer liver sections were deparaffinized, rehydrated, and dipped in 3% H_2O_2 for 30 min to quench endogenous peroxidase activity. Antigen retrieval was carried out in a citrate buffer (pH. 6.0) at 95 °C for 60 min. 5% bovine serum albumin was used to block the nonspecific staining. The histological sections were then incubated with the monoclonal antibody for α-SMA (Boster Bioengineering Co., Wuhan, China) at a dilution of 1:200 overnight at 4 °C. After washing with PBS, sections were incubated with the biotinylated secondary antibodies. Then the immunoreaction was amplified with streptavidin–avidin–peroxidase complex (SABC), and the sections were visualized by using 3,30-diaminobenzidine tetrahydrochloride (DAB) and lightly counter-stained with hematoxylin.

2.4. RNA extraction, cDNA synthesis, and quantitative real-time PCR

The extraction of total RNA, preparation of cDNA and the amplification of target genes by PCR were carried out according to the manufacturer's instructions. Briefly, liver tissues were homogenized in Trizol reagent (Invitrogen, Carlsbad, CA, USA) to extract total RNA. The RNA purity and concentration were determined spectrophotometrically at 260/280 nm in the range of 1.8–2.0 by NanoDrop 2000 (Thermo Scientific, Waltham City, MA, USA). Total RNA (3 μg) was reverse transcribed by Fermentas One step RT-PCR kit (MBI Fermentas, Burlington, ON, Canada) and amplified by PCR. The sequences of the primers are listed in Table 1.

The quantitative real-time PCR was carried out on the ABI 7300 HT real time PCR machine (Applied Biosystems, Foster City, CA, USA) with the reaction volume of 20 μl consisting of a 2 or 4 μl cDNA template, 0.4 μl of each of the gene-specific forward and reverse primers and a 10 μl SYBR® Premix Ex Taq™ (TaKaRa Bio Inc. Co., Japan). Experiments were repeated twice. All samples were normalized against 18S rRNA using the comparative CT method (ΔΔCT) (Livak and Schmittgen, 2001).

2.5. Biochemical analysis of malondialdehyde and serum aminotransferases

As one endpoint of lipid peroxidation, malondialdehyde (MDA) indirectly reflects the level of hepatocellular oxidative injury. MDA concentrations were calculated by detecting the absorbance of thiobarbituric acid reactive substances at 532 nm and were expressed in nmol/mg protein (Agostinho et al., 1997). Frozen liver tissue was homogenized with ice-cold saline. The homogenate was centrifuged for 15 min at 1700 × *g* and the supernatant was used for further measurements (Bu et al., 2011). The serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined with a biochemical analyzer (7180, HITACHI, Japan).