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Multitargeted inhibition of hepatic fibrosis in chronic iron-overloaded mice by *Salvia miltiorrhiza*

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ABSTRACT

Ethnopharmacological relevance: *Salvia miltiorrhiza* (SM, also known as Danshen) is a well-known Chinese medicinal herb, which has shown hepatoprotective effects with anti-fibrotic, anti-oxidative, anti-inflammatory and anti-apoptotic properties. To explore the effects and potential mechanism of SM against hepatic fibrosis induced by chronic iron overload in mice.

Materials and methods: Sixty male mice were randomized into five groups ($n=12$ in each group): control (saline), iron overload, iron overload with low-dose SM (3 g/kg/day), iron overload with high-dose SM (6 g/kg/day) and iron overload with deferoxamine (100 mg/kg/day) groups. The iron overload model was established by intraperitoneal injection with iron dextran at 50 mg/kg body weight/day, and the entire course lasted for 7 weeks. The major constituents of SM injection were quantified by high performance liquid chromatography. Changes of hepatic iron, hydroxyproline (Hyp), glutathione (GSH), superoxide dismutase (SOD) and malondialdehyde (MDA) were assayed by standard procedures. Protein expression levels of type I collagen, type III collagen, tumor necrosis factor-α (TNF-α) and interleukin-1α (IL-1α) were analyzed by immunohistochemistry, and mRNA levels of transforming growth factor-β (TGF-β), matrix metal proteinase-9 (MMP-9) and caspase-3 were detected by RT-PCR. Morphological changes were observed with Prussian blue, Masson's trichrome and hematoxylin–eosin staining.

Results: Treatment of chronic iron-overloaded mice with SM dose-dependently ameliorated changes in hepatic morphology and coefficient, reduced iron deposition and Hyp content, suppressed overexpression of type I collagen and type III collagen, downregulated expression of TGF-β mRNA, and upregulated expression of MMP-9 mRNA in the liver. Moreover, SM treatment contributed to decreased MDA content, increased SOD activity and GSH content, while it reduced expression of TNF-α, IL-1α and caspase-3.

Conclusions: SM displayed anti-fibrotic activity in the liver induced by chronic iron overload, which may be attributed to multitargeted inhibition of iron deposition and collagen accumulation, as well as oxidative stress, inflammation and apoptosis.

1. Introduction

Iron is an essential micronutrient for living organisms, but it is also potentially toxic (Hentze et al., 2010). The toxicity arises mainly due to iron-induced formation of reactive oxygen species (ROS), causing peroxidation of membrane lipids and oxidative damage to cellular proteins and DNA (Sochaski et al., 2002; Puntarulo, 2005). As a major site of iron storage (containing approximately one third of total body iron), the liver is more susceptible than other organs to the damaging effects of excess iron, which present in a wide variety of conditions, including hereditary (primary) hemochromatosis, hemosiderosis secondary to systemic disease (i.e., transfusions and hemolytic conditions) and cirrhosis-related hemosiderosis (i.e., hepatitis C and alcoholic liver disease) (Batts, 2007; Deugnier et al., 2008; Lee and Beutler, 2009). These patients with long-term iron-overload conditions are at risk for slowly developing chronic hepatopathy characterized by fibrosis and ultimately cirrhosis (Adams et al., 2006; Barton et al., 2012).

With the hemochromatosis epidemic, the prevalence of hepatic fibrosis is increasing worldwide (Adams et al., 2006). Hepatic fibrosis

Abbreviations: DFO, deferoxamine; DFP, deferiprone; ECM, extracellular matrix; GSH, glutathione; H&E, hematoxylin and eosin; HPLC–UV, high performance liquid chromatography–ultra violet; HSCs, hepatic stellate cells; Hyp, hydroxyproline; IL-1α, interleukin-1α; i.p., intraperitoneal; LVDCC, L-type voltage dependent calcium channel; MDA, malondialdehyde; MMP, matrix metal proteinase; ROS, reactive oxygen species; SM, *Salvia miltiorrhiza*; SOD, superoxide desmutase; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.

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is a reversible scarring response that occurs in almost all patients with chronic liver injury, and the unifying hallmark is excessive deposition of extracellular matrix (ECM) components including collagens (Friedman, 2008). In hemochromatosis patients, once the hepatic iron reaches the threshold concentration, it catalyzes the formation of ROS, promoting hepatic injury and activating hepatic stellate cells (HSCs) that initiate the deposition of ECM components and contribute to the development of fibrosis (Ramm and Ruddell, 2005; Lundvig et al., 2012). Moreover, the excess iron in some diseases may be responsible for inflammatory responses mediated by oxidative stress (Park et al., 2010). In a recent study on acute iron overload from our laboratory, intraperitoneal (i.p.) injections of an excess of iron also was shown to cause hepatocellular apoptosis (Gao et al., 2013).

In all iron-overload diseases, an effective life-saving strategy is iron removal by chelation with such agents as deferoxamine (DFO) and deferiprone (DFP) (Porter, 2001; Berdoukas et al., 2012). Although DFO reduces morbidity and mortality, the cumbersome administration schedule and parenteral infusions required on several days each week lead to poor compliance (Chaston and Richardson, 2003). DFP, with its oral route of administration and rapid access, can effectively chelate free iron, but the serious side effect of agranulocytosis may be life-threatening (Berdoukas et al., 2012). Although chelators are very effective, the recognized overwhelming barrier to their efficacy is long-term usage, and few patients with chronic iron-overload disorders obtain the maximum benefit (Galanello, 2001). Therefore, complications associated with long-term iron overload, such as fibrosis, are not timely and effectively resolved with chelation therapy.

Salvia miltiorrhiza (SM, also known as Danshen), a member of the Labiatae family, is highly valued for its dried roots or rhizomes in traditional Chinese medicine. It was first indexed in Shennong's Classic of Materia Medica (206 B.C. to 8 A.D.), an early Chinese medicine monograph, as a tonic herb of the nontoxic superior class for promoting blood circulation and has been used in traditional Chinese therapies for more than 2000 years (Song et al., 2008). In recent decades, SM has been widely used in clinics in China, Korea, Japan and other Asian countries for treating various diseases involving microcirculatory disturbances, such as cardiovascular disease, liver injury and hepatic fibrosis (Wasser et al., 1998; Sugiyama et al., 2002; Hsu et al., 2005; Song et al., 2008; Park et al., 2009). China alone requires an estimated 80 million kilograms of SM annually (Hu et al., 2005). Clinically, SM is prescribed either in combination with other Chinese herbal medicines or individually, and many pharmaceutical dosage forms of SM are commercially available. SM injection, an aqueous extract of SM prepared based on the unified standard issued by the Ministry of Health of China, is believed to be one of the most highly recommended and widely accepted treatments for liver disease. As a well-known traditional medicine with hepatoprotective effects, SM has shown versatile anti-fibrotic, anti-oxidative, anti-inflammatory and anti-apoptotic effects (Liu et al., 1999; Nan et al., 2001; Kim et al., 2002; Oh et al., 2002; Tian et al., 2008; Yu et al., 2008; Cheung et al., 2013). The bioactive constituents of SM include lipid-soluble diterpenes and water-soluble phenolic compounds, the latter of which can reverse experimental hepatic fibrosis (Wasser et al., 1998). Some monomers, such as IH764-3, magnesium lithospermate B and Tanshinone II-A, also exert anti-fibrotic effects by regulation of collagen metabolism and HSCs (Lee et al., 2006; Fang et al., 2010; Paik et al., 2011; Liu et al., 2012). Recent studies have shown that dihydrotanshinone, a lipophilic component of SM, plays a cytoprotective role by blocking calcium channels, through which ferrous iron promiscuously enters cells (Oudit et al., 2003; Lam et al., 2008).

Based on the information above, we hypothesized that SM can inhibit the hepatic fibrosis in chronic iron-overload diseases. Despite numerous reports on the anti-fibrotic effects of SM, its exact role in hepatic fibrosis induced by iron overload is still largely unknown. In the present study, we investigated the anti-fibrotic effects of SM injection by assessing a series of changes in morphological and biochemical parameters in an iron overload mouse model established with long-term administration of an overdose of iron. We further explored the underlying mechanism of SM effects on hepatic fibrosis in this model by analyzing fibrosis-related molecules, oxidative stress markers, proinflammatory cytokines and apoptotic factor.

2. Materials and methods

2.1. Drugs and reagents

SM injection was purchased from Shenlong Pharmaceutical Co. Ltd. (Yancheng, China), and its standard was made according to vol. 20 of the traditional Chinese medicine prescription for preparation of medicines and chemical reagent standards issued by the Ministry of Health of China (executive standard number: WS_3-B-3766-98). SM injection was prepared with the dried roots of the plant at the concentration of 1.5 g aqueous extract per ml. The main active components of SM injection, as analyzed by high performance liquid chromatography with ultra violet (HPLC-UV) detection, included Danshensu, protocatechuic aldehyde and salvianolic acid B (Fig. 1). Identification of SM injection was confirmed by Jianping Zhang, Hebei Medical University (Shijiazhuang, Hebei, China). Voucher specimens (DBC20111028) were deposited in the Central Laboratory for Pharmacology of Hebei Medical University.

Iron dextran injection was supplied by Sunaccord Biological Technical Co. Ltd. (Hunan, China). DFO was purchased from Novartis Pharma AG (Basel, Switzerland). The assay kits for chemical analysis of hydroxyproline (Hyp), superoxide desmutase (SOD), glutathione (GSH) and malondialdehyde (MDA) were obtained from Jian Cheng Biological Engineering Institute (Nanjing, China). The SP-9002 Histostain™-Plus Kit purchased from ZYMED (Carlsbad, CA, USA) was used in immunohistochemistry stainings. Rabbit polyclonal antibodies of tumor necrosis factor-α (TNF-α), interleukin-1α (IL-1α), type I collagen and type III collagen were purchased from Boster Bio-engineering Co. Ltd. (Wuhan, China). The reverse transcription kit and multiplex PCR kit were obtained from TaKaRa Bio Inc. (Dalian, China). Trizol reagent was

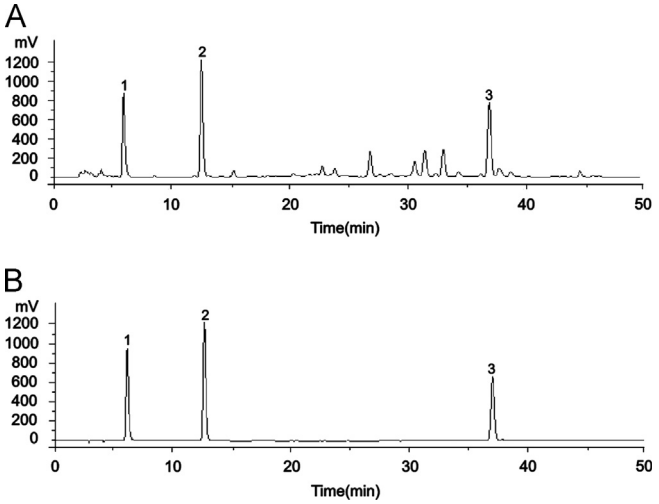

Fig. 1. HPLC–UV profiles of SM injection (A) and standards (B). Peaks represent: 1, Danshensu; 2, protocatechuic aldehyde; 3, salvianolic acid B.