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Protective effects of *Salvia miltiorrhiza* on adenine-induced chronic renal failure by regulating the metabolic profiling and modulating the NADPH oxidase/ROS/ERK and TGF-β/Smad signaling pathways

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ABSTRACT

Ethnopharmacological relevance: Chronic renal failure (CRF) is defined as a progressive and irreversible loss of renal function and associated with inflammation and oxidative stress. *Salvia miltiorrhiza* (SM) is an important Chinese herb used in traditional Chinese medicine for treating cardiovascular diseases. The previous studies showed the SM exhibited significant protective effects on CRF. In this present study, the metabolic profiling changes and action mechanism of SM on CRF were explored.

Aims of the study: The aims of this study were to illustrate the metabolic profiling changes of adenine induced CRF and analyze the protective effects and action mechanisms of SM ethanol extract (SMEE) and water extract (SMWE).

Materials and methods: The animals were divided into normal group, CRF model group, Huangkui capsule-treated group, SMEE-treated group and SMWE-treated group. The UPLC−QTOFMS coupled with multivariate statistical methods were used to explore the changes of metabolic profile in plasma, urine and renal tissue from CRF rats simultaneously after treatment with SMEE and SMWE. Hematoxylin eosin (HE) staining and Masson staining were applied to observe pathological changes in renal tissue. Biochemical indicators including serum urea nitrogen (BUN), urine protein (UP) and serum creatinine (Scr) were measured according to the manufacturer's instructions of kits. Furthermore, HK-2 cell damaged model induced by ISF was established to access the protective effects and action mechanism. The dichlorodihydrofluorescein diacetate (DCFH-DA) assay was used to determine the reactive oxygen species (ROS) and Western blot was applied to analyze the expression of pathogenesis-related proteins in different groups.

Results: The results showed that the ethanol extract (SMEE) and water extract (SMWE) of SM significantly inhibited the elevation of serum creatinine (Scr), blood urea nitrogen (BUN), urine protein (UP) and indoxyl sulfate (ISF) in adenine-induced CRF rats, especially SMEE exhibited more significant effects. Moreover, SM extracts obviously improved the symptoms of glomerular and tubular atrophy, focal calcium deposits, interstitial fibrosis, interstitial inflammation, and renal tissues. By metabolomics analysis, fifty-nine metabolites (thirteen in plasma, twenty-seven in urine and nineteen in kidney tissue) were up-regulated or down-regulated and contributed to CRF progress. After treatment of SM extracts, the altered metabolites were restored back to normal level. These potential biomarkers underpinning the metabolic pathways are including phenylalanine metabolism, pyrimidine metabolism, purine metabolism and tryptophan metabolism. Furthermore, SM extracts prevent epithelial-mesenchymal transition (EMT) of human renal tubular epithelial (HK-2) cell by inhibiting NADPH

Abbreviations: CRF, Chronic renal failure; SM, Salvia Miltiorrhizae Radix et Rhizoma; SMWE, Saliva Miltiorrhizae Radix et Rhizoma ethanol extract; SMWE, Salvia Miltiorrhizae Radix et Rhizoma water extract; EMT, epithelial-mesenchymal transition; PCA, Principal components analysis; PLS-DA, Partial least squares discriminate analysis; OPLS-DA, Orthogonal partial least squares discriminate analysis; UPLC-QTOF/MS, Ultra performance liquid chromatography combined with time-of-flight mass spectrometry; UPLC-MS/MS, Ultra performance liquid chromatography-mass spectrometry; VIP, Variable importance in the projection; Scr, serum creatinine; BUN, blood urea nitrogen; UP, urine protein; ISF, Indoxyl sulfate

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oxidase/ROS/ERK and TGF-β/Smad signaling pathways.
Conclusions: SMEE and SMWE can significantly alleviate adenine-induced CRF via regulation of the metabolic profiling and modulation of NADPH oxidase/ROS/ERK and TGF-β/Smad signaling pathways, which provided important supports for the development of protective agent of SM for CRF.

1. Introduction

Chronic renal failure (CRF) is a chronic disease characterized by renal function loss, and it's morbidity and mortality have increased year by year. In China, it has emerged about 119.5 million patients with CRF, with an overall prevalence of 10.8% in 2012 (Liu, 2013; Zhang et al., 2012). And the prevalence rate was from 13.1% at 2004 up to 14.4% at 2010 in the USA (Bethesda, 2010; Beger et al., 2010). The basic clinical manifestation forms of chronic renal diseases including proteinuria, hematuria, hypertension, edema, leading to various attack patterns and degrees of renal dysfunction and eventually developing CRF of a group of glomerular disease (Bethesda, 2010). The therapeutic drugs for CRF in clinic were using diuretics to reduce proteinuria and edema, such as furosemide and bumetanide. And renin-angiotensin system antagonists to relieve hypertension were also applied, such as fluvastatin and nitrendipine (Coresh et al., 2007). Nevertheless, confronted with the multiple pathogenic factors of CRF, the hitting-one-target therapeutic strategy was poor efficiency. What's more, the application compatibility to different drugs always has the effect to induce much syndromes, such as hepatitis, hypotension, high drug-resistant, anaphylaxis (Zhu et al., 2007). And some studies showed drug factors have become a leading cause of acute renal failure (Nangaku and Fujita, 2008; Liu and Li, 2008). So, more and more attention has been paid to the discovery of new and effective preparations with little side effects. Tubulointerstitial fibrosis is a major character and factor of renal dysfunction. Previous studies have reported that epithelial to mesenchymal transition (EMT) in human renal epithelial (HK-2) cells is possibly involved in the irreversible progression of tubulointerstitial fibrosis (Carew et al., 2012). While intracellular reactive oxygen species (ROS) plays a critical role in the induction of tubular EMT and subsequent renal fibrosis (Chen et al., 2013). What's more, the production of intracellular ROS was associated with NADPH oxidase in HK-2 cells (Li and Shah, 2003). α-SMA, fibronectin and E-cadherin were the critical markers of EMT (Wang et al., 2006). In addition, TGF-β1 and its receptors (TGF-βRI and TGF-βRII) play a key role in EMT and renal fibrosis (Bottinger, 2007). The downstream signaling molecules, containing smad2, smad3 and smad7, were involved in TGF-β-induced EMT, and Smad7 can block the function of smad3.

Danshen, a traditional herbal medicine, derived from the root of *Salvia miltiorrhiza* Bunge. belonging to *Salvia* genus of family of Labiatae, has been used widely in clinic for treatment of coronary heart diseases, particularly angina pectoris and myocardial infarction (Tanonaka et al., 1990; Cheng, 2006). The previous researches demonstrated that Danshen had various pharmacological effects on improving cerebral ischemia reperfusion injury, blood rheology, platelet function, anti-hypertensive, anti-inflammatory and protecting the cardiovascular system (Li et al., 2015; Kang et al., 2002; Gao et al., 2013; Sun et al., 2005; Zhang et al., 2014), which relied on its multiple bioactive components mainly including salvianolic acids and tanshinones. While the injection of Danshen preparation can significantly reduce serum urea nitrogen and serum creatinine and improve renal function (Yin et al., 2014; Lu et al., 2015; You et al., 2012). The main bioactive component of salvianolic acid B exhibited obviously effect on renal ischemia reperfusion injury in rats (Kang et al., 2004), and tanshinone IIA significantly attenuating renal fibrosis in 5/6 nephrectomized rats (Wang et al., 2015). However, the action mechanisms of SM on CRF have not yet been revealed completely and the studies about metabolic profiling changes, metabolites and metabolic pathway analysis on CRF rats were quite rare (Zhao et al., 2013).

Metabolomics, a subdiscipline of biological metabolome technology, provides a comprehensive and simultaneous analysis about profile of metabolic changes occurring in living creature in response to pathophysiological stimuli or genetic modification, which was first propounded by Nicholson in 1999 (Nicholson et al., 1999). The means has become a highly ingenious and forceful tool for screening potential biomarkers from the metabolic pathways (Nicholson and Wilson, 2003) and further elucidate the potential action mechanisms, which has been widely used in various fields, such as disease, drug development, toxicology and mechanisms study (Marco et al., 2011; Justyna et al., 2009; Nicholson et al., 2002; Su et al., 2013; Yin et al., 2008), and so on. Metabolomics relies principally on NMR and MS combined with LC or GC with precolumn derivatization as a supplementary means to separate the metabolites (Theodoridis et al., 2012; Timothy et al., 2015; Van der Kloet et al., 2012). Especially, Ultra-performance liquid chromatography-quadrupole time-of-fight mass spectrometry (UPLC-QTOF/MS) has been proven to be an effective technique for metabolites identifications and quantifications due to its excellent resolution and sensitivity (Xie et al., 2008; Zhou et al., 2014).

The aim of the study is to explore the protective effects and action mechanisms of SM ethanol extract (SMEE) and SM water extract (SMWE) on adenine-induced CRF rats. The potential low molecular weight biomarkers and metabolic profiling based on plasma, urine and renal tissue metabolomics using UPLC-QTOF/MS were identified and constructed the metabolic pathways and explored the action mechanisms comprehensively. In addition, the ISF induced EMT in HK-2 cells model *in vitro* was established to evaluate the curative effect of SM on renal fibrosis by determination of ROS and related proteins expression levels.

2. Materials and methods

2.1. Instruments and chemicals

Waters Acquity™ Ultra Performance LC system (Waters, USA) equipped with a Quattro Micro MS spectrometer and a Waters Xevo™ G2 Qtof MS (Waters MS Technologies, Manchester, UK). Ultrapure water meter (Nanjing Pu Yi Yida Science and Technology Development Co., Ltd.). MassLynx v4.1 and MassLynx XS v4.1 workstation was adopted to analyze the data. Milli-Q system was from Millipore Bedford, MA, USA, Vacuum freeze-drying equipment (Labconco, UK) and Ultra-high speed centrifuge at low temperature (Thermo, UK) were used.

Huangkui capsule was obtained from SZYY Group Pharmaceutical Limited (15101211, Jiangyan, China). *Salvia Miltiorrhizae* Radix et Rhizoma was bought from Bozhou city traditional Chinese medicinal materials market (140922, Bozhou, China) which was identified by professor Duan Jinao from Nanjing University of Chinese Medicine (Nanjing, China), and the producing area was Shandong province. UPLC-grade acetonitrile and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Adenine (S18009, 98%) was bought from shanghai inke biological technology co., LTD (Shanghai, China). Serum urea nitrogen (BUN) reagent kit, serum creatinine (Scr) reagent kit, urine protein (UP) reagent kit, and Indoxyl sulfate (ISF) standard substance (98%) were bought from Nanjing jiancheng Bioengineering