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Original Article

Serum metabolomics analysis reveals that obvious cardioprotective effects of low dose *Sini* decoction against isoproterenol-induced myocardial injury in rats

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ABSTRACT

Background: *Sini* decoction (SND) is used for cardiovascular disease over thousands of years in China. However, it is still lacking of dose-response relationship of SND in cardiovascular disease at the metabolic level.

Purpose: The present study is designed to explore the cardioprotective effects of different dosages of SND pretreatment on the isoproterenol (ISO)-induced myocardial injury and elucidate the mechanism underlying this protective effect.

Methods: The cardioprotective effects of different dosages of SND pretreatment on the isoproterenol-induced myocardial injury were compared through a serum metabolomics approach based on ultraperformance liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry (UPLC-Q-TOF-MS). In addition, the cardioprotective effects were evaluated by serum biochemical analysis and histopathological examination of myocardial tissue. Finally, in view of the fact that these perturbed bile acid and phospholipid metabolisms are connected with NF-κB signaling pathway, nuclear expression of NF-κB p65 and the activation of NF-κB were analyzed by immunohistochemistry, immunoblotting and electrophoretic mobility shift assay (EMSA), respectively.

Results: The cardioprotective effect was observed in SND pretreatment groups, especially in low dosage SND group. The results of serum enzyme activities and histopathology were consistent with the above effect. Meanwhile, fifteen latent biomarker candidates were identified involving glucose, phospholipid, bile acid and amino acid metabolisms. Among them, five bile acids including ursodeoxycholic acid, murideoxycholic acid, muricholic acid, hyodeoxycholic acid and cholic acid, were for the first time identified as latent pathological biomarkers related to ISO-induced myocardial injury. Further, different dose SND groups exerted different of inhibition degrees to the activation of NF-κB, which was obvious in the SND-L group.

Conclusion: The results revealed that *Sini* decoction protreatment protects myocardium better at a low dose level and one of possible cardioprotective mechanisms is modulating NF-κB signaling pathway against isoproterenol-induced myocardial injury through regulating phospholipid and bile acid metabolisms.

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Abbreviations: ANOVA, a one-way analysis of variance; BPI, base peak intensity; CK, creatine kinase; CK-MB, creatine kinase-MB; EMSA, electrophoretic mobility shift assay; ESI, electrospray ionization; FXR, farnesoid X receptor; H&E, hematoxylin and eosin; i.p., intraperitoneal injection; ISO, isoproterenol; LDH, lactate dehydrogenase; LSD, Least Significant Difference; LysoPC, lysophosphatidylcholine; MI, Myocardial infarction; MRM, multiple reaction monitoring; NF-κB, Nuclear factor kappa B; PC, phosphatidylcholine; PCA, Principal Component Analysis; PLS-DA, Partial Least Square Discriminant Analysis; PPAR, Peroxisome Proliferator –Activated Receptor; QC, quality control; RSDs, relative standard deviations; SND, *Sini* decoction; SND-H, High dosage SND pretreatment group; SND-M, Middle dosage SND pretreatment group; SND-L, Low dosage SND pretreatment group; UPLC-Q-TOF-MS, ultraperformance liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry.

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Introduction

Myocardial infarction (MI) is a common cardiovascular disease, resulting in myocardial injury and evolving into heart failure (Boon and Dimmeler, 2015). Isoproterenol (ISO) is used clinically for supporting heart rate and cardiac contractility (De Rasmo et al., 2011). However, excessive administration of isoproterenol may bring about complex biochemical and histological changes of myocardium which like those of human MI (Li et al., 2012). Therefore, many researchers investigated pathogenesis of cardiac dysfunctions and pharmacological effect of medicine in MI through establishing experimental rat model of ISO-induced myocardial injury (Liu et al., 2014a; Liu et al., 2013; Zhang et al., 2009).

Sini decoction (SND), described in a classic Chinese medicine literature of the Eastern Han Dynasty(*Shanghan Lun*), has been used as a Chinese Herbal Formulae to treat cardiovascular disease for many years(Tan et al., 2012) . SND is composed of three crude drugs: *Aconititum carmichaeli* Debeaux lateral root(Fuzi), *Glycyrrhizae uralensis* Fisch. root and rhizome, honeyed(Zhigancao) and *Zingiber officinale* Roscoe root(Ganjiang). *Aconititum carmichaeli* Debeaux lateral root, the main pharmacological component of SND, has narrow therapeutic indices ascribed to its strongest toxic ingredients- aconite alkaloids (Cai et al., 2013). At present, the main active compounds of SND were considered as Glycyrrhizinic acid and less toxic mono-esterified diterpenoid alkaloids, such as Benzoylaconitine, Benzoylhypacoitine and Benzoylmesaconitine (Peter et al., 2013; Zhang et al., 2015). But, a certain concentration of poisonous diester diterpene alkaloids seems to be necessary for clinical therapeutics (Peter et al., 2013). Hence, it is an urgent necessity to evaluate dose-response relationship of SND to provide guidelines for clinical therapy.

Metabolomics is an approach to help understand the relationships and interactions between variations of 'endogenous' metabolic processes and environment triggers of disease or drug treatment. It provides a 'top-down', holistic perspective on the global metabolic state in complex biological organisms, which coincides with the holistic nature of Traditional Chinese Medicine (Ma et al., 2010; Nicholson and Lindon, 2008).Thus, it is applied widely in many fields of Traditional Chinese Medicine, such as, therapeutic effectiveness (Liu et al., 2014b), toxicological assessment (Cai et al., 2013) and the latent pharmacological mechanism (Tan et al., 2011).

Many studies on SND have employed metabolomics to understand pharmacological mechanisms and seek potential biomarkers of pharmacological effects (Tan et al., 2012; Tan et al., 2011; Wu et al., 2013). It is fortunate that many biomarker candidates have been successfully identified from cardiovascular disease. However, it is still lacking of the dose-response relationship of SND in cardiovascular diseases at the metabolic level. The present study aimed to observe the dose-response relationship through performing metabolomics to evaluate whether the cardioprotective effects are enhanced along with the increase of the dosage of SND.

Experimental

Reagents and materials

Isoproterenol hydrochloride, formic acid (LC-MS grade), chemical standards of cholic acid and glucose were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile, tetrahydrofuran and methanol were obtained from Merck (Darmstadt, Germany). 1-stearoyl-sn-glycero-3-phosphocholine (LysoPC(18:0)) and1-palmitoyl-sn-glycero-3-phosphocholine (LysoPC(16:0)) were obtained from Avanti Polar Lipids, Inc. (Alabster, AL, USA). The commercial assay kits used for creatine kinase-MB (CK-MB), creatine kinase (CK) and lactate dehydrogenase (LDH) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Chemical standards of benzoylaconitine, benzoylhypacoitine, benzoylmesaconitine, 6-gingerol and monoammonium glycyrrhizinate were purchased from National Institutes for Food and Drug Control(Beijing, China). Ultrapure water was produced by Direct Laboratory Water Purification system (Milli-Q, Bedford, MA, USA). All other chemical reagents were of analytical grade.

Aconititum carmichaeli Debeaux lateral root (batch number: 140101, collected in Sichuan, China), *Zingiber officinale* Roscoe root (batch number: 131101, collected in Sichuan, China) and *Glycyrrhizae uralensis* Fisch. root and rhizome, honeyed (batch number: 140101, collected in Xinjiang, China) were purchased from Zhejiang Chinese Medical University Medical Pieces Corp. (Hangzhou, China). These voucher specimens, which deposited in Traditional Chinese Medicine Herbarium of Zhejiang Chinese Medical University (ZZY20141107), were authenticated by Professor Fanzhu Li (School of Pharmacy, Zhejiang Chinese Medical University, Hangzhou, China) and meet their respective standards recorded in Chinese Pharmacopoeia 2010 edition.

Preparation of Sini decoction

Sini decoction was prepared through the method described in Chinese Pharmacopoeia 2010 edition with little modification as follow: The three sliced Chinese medicines were ground into powder (about 20 meshes) respectively. The powders of *Aconititum carmichaeli* Debeaux lateral root (300 g) and *Glycyrrhizae uralensis* Fisch. root and rhizome, honeyed (300 g) were soaked in 6 liters of deionized water and then boiled for two h using refluent water extraction method. The decoction was filtered with absorbent gauze. The residues was subsequently decocted with an eight-fold mass of deionized water (4.8 l) to reflux for additional 1.5 h, then filtered. The essential oil of *Zingiber officinale* Roscoe root powder (200 g) was extracted by conventional steam distillation for three h. The decoction from *Zingiber officinale* Roscoe root was filtered with the same method. All filtrates were merged and centrifuged at 3000 g for fifteen minutes. The supernatant was concentrated by decompressing at 55 °C–400 ml and then 3-fold volume of 95% ethanol (1200 ml) was added. The mixed solution was statically settled for 24 h and then centrifuged again. The supernatant was condensed under vacuum to about 200 ml. The concentrated solution and the aforementioned essential oil were blended into *Sini* decoction. The HPLC characteristic chromatographic profile of SND was shown in Fig. S1 (Supporting information). After measurement, the *Sini* decoction(5.0 g crude drug ml^{-1}) contains three active compounds (benzoylmesaconitine 0.672 mg ml^{-1}, glycyrrhizinic acid 3.812 mg $ml^{-1}$and 6-gingerol 1.313 mg ml^{-1}).The active compounds of benzoylaconitine, benzoylhypacoitine were not detected because of too low concentration. The *Sini* decoction was diluted with deionized water to attain a solution of 5.0 g crude drug ml^{-1}. And then, the solution was diluted with deionized water to the low, middle and high concentration of SND (1.0 g ml^{-1}, 2.0 g ml^{-1}, 4.0 g ml^{-1}, respectively).These *Sini* decoctions with different concentrations were stored at 4 °C before administration.

Animal experiments

Forty-five male Sprague-Dawley (SD) rats with specific pathogen free (SPF) (weight: 200 ± 20 g) were provided by the Experimental Animal Center, Zhejiang Chinese Medical University. The animals were raised in a standard animal room with twelve h light/dark cycle (temperature: 23 ± 2 °C, relative humidity: $50\% \pm 10\%$). All rats were randomly divided into five groups ($n = 9$) after acclimatization for seven days as follow: Control group, High dosage SND pretreatment (SND-H) group, Middle dosage

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