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Plasma metabolomics study of the hepatoprotective effect of glycyrrhetinic acid on *realgar*-induced sub-chronic hepatotoxicity in mice via ^{1}H NMR analysis

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ABSTRACT

Ethnopharmacological relevance: *Realgar*, a type of mineral drug that contains arsenic, is concurrently used with *Glycyrrhizae Radx et Rhizoma* to reduce its toxicity in many Chinese herbal formulations. Glycyrrhetinic acid (GA) is the bioactive ingredient in *Glycyrrhizae Radx et Rhizoma*. In this study, the protective effects of GA on *realgar*-induced hepatotoxicity was investigated using ^{1}H nuclear magnetic resonance (^{1}H NMR)-based metabolomic approaches.

Materials and methods: Mice were divided into control, GA, *realgar*, and GA and *realgar* co-administration groups. Their plasma samples were used for a metabolomics study.

Results: GA can protect the mice against *realgar*-induced hepatotoxicity to some extent by relieving alterations in the clinical biochemical parameters and the damage to *hepatocytes*. Metabolic profiling via principal components analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) indicated that the metabolic perturbation caused by *realgar* was reduced by GA. Six metabolites, including 3-hydroxybutyrate (3-HB), very low density/low density lipoprotein (VLDL/LDL), *N*-acetylglycoprotein (NAc), lactate, choline and D-glucose, were considered as potential biomarkers that are involved in the toxicity reduction effect of GA on *realgar*-induced hepatotoxicity. The correlation analysis showed that these potential biomarkers were all positively correlated with ALT and AST activities (correlation coefficient > 0.5). Lipid and energy metabolism pathways were found to be primarily associated with the hepatoprotective effect of GA.

Conclusions: GA has an effective protection function by regulating the lipid and energy metabolism to liver injuries that are induced by *realgar*.

1. Introduction

Realgar (Species: sulfide; 雄黄in Chinese; Xionghuang in pin yin; red orpiment in English) is a type of mineral drug that contains arsenic (Chinese Pharmacopeia Committee, 2010). It has been used as a traditional Chinese medicine (TCM) for more than 1500 years and has been mainly used for the treatment of carbuncles, boils, insect and snake bites, intestinal parasitosis, convulsive epilepsy and psoriasis (Nriagu, 2002). However, *realgar* has also been confirmed as one of the 28 types of toxic Chinese medicines in the Medical Treatment of Toxic Drugs Management Approach by the State Council due to its main component, arsenic.

The liver, which carries out many critical functions, is an important organ for metabolism and deposition of endogenous and exogenous substances. Previous studies have indicated that *realgar* has toxicological effects on the liver (Liang et al., 2011; Zhang et al., 2006; Huo et al., 2016). Therefore, there is a need to look for a potential protective agent against liver damage that is induced by *realgar*. Recently, herbal medicines have become popular for the treatment of hepatic disease and are attractive as putative hepatotherapeutics (Ferenci et al., 1989).

Compatible detoxification is an advantage of traditional Chinese medicines (TCMs). *Glycyrrhizae Radx et Rhizoma* (Species:

Abbreviations: ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GA, Glycyrrhetinic acid; CAT, catalase; CHE, choline esterase; CMC-Na, sodium carboxymethylcellulose; CPMG, Carr-Purcell-Meiboom-Gill; GSH, glutathione; GSH-Px, glutathione peroxidase; 3-HB, 3-hydroxybutyrate; ^{1}HNMR, ^{1}H nuclear magnetic resonance; LDL, low-density lipoprotein; NAc, *N*-acetylglycoprotein; PCA, principal component analysis; SOD, super-oxide dismutase; TC, total cholesterol; TCM, traditional Chinese medicine; TMAO, trimetylamine-N-oxide; TP, total protein; VLDL, low density lipoprotein

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Leguminsae; 甘草in Chinese; Gancao in pin yin, liquorice root in English) is a popular Chinese herbal medicine derived from the dried roots and rhizomes of *Glycyrrhiza uralensis* Fisch., *G. Glabra* L. and *G. Inflata* Bat. It is often used as an auxiliary medicine in a variety of Chinese patent medicines that contain harmful medicines because of its effects in regulating herbal properties and enabling detoxification. It has been reported that *Glycyrrhizae Radx et Rhizoma* in the *Niuhuang Jiedu* tablet can reduce the arsenic toxicity of *realgar* (Xu et al., 2013, 2014).

Glycyrrhetinic acid (GA, the chemical structure is shown in Supplementary Fig 1), the main active ingredient of *Glycyrrhizae Radx et Rhizoma*, is a major metabolite of glycyrrhizin (GL). Most GL is metabolized to GA in the intestine by bacteria. It has been confirmed to possess a variety of pharmacological effects, such as anti-inflammatory, antiallergic, anti-carcinogenic, anti-injury and antioxidant properties as well as liver protection, in numerous prior studies (Jin et al., 2009; Gong et al., 2008; Luo et al., 2008; Van Rossum et al., 1998). Recent studies have shown that GA can reduce the hepatotoxicity induced by retrorsine (Lin et al., 1999), titanium dioxide nanoparticles (Orazizadeh et al., 2014), cyclophosphamide (Mahmoud et al., 2015) and protect against carbon tetrachloride-induced liver injury (Jeong et al., 2002). The reported mechanism of the hepatoprotective effect of GA is mainly attributed to the induction of antioxidant defences, the suppression of inflammatory responses, and cytochrome P450 2E1 expression (Mahmoud et al., 2015; Jeong et al., 2002). Until now, whether GA can protect the hepatotoxicity induced by *realgar* and its concrete effects have not been reported. Therefore, studying the protective effect of GA on *realgar*-induced hepatotoxicity is important for clarifying the scientific idea of the compatibility of *realgar* and *Glycyrrhizae Radx et Rhizoma* and for providing experimental evidence for GA as a potential protective agent against *realgar*-induced liver damage.

Metabolomics, which exhibit an impressive and ever increasing coverage of endogenous compounds, focus on the holistic investigation of multi-parametric metabolite responses in living systems (Lao et al., 2009). It offers an unbiased view of changes in the metabolism of entire metabolic pathways to characterize pathological states and reveals the subtle dynamic changes in response to toxicants (Nan et al., 2016). ^{1}H nuclear magnetic resonance (^{1}H NMR) spectroscopy provides a rapid, non-destructive and high-throughput method for the analysis of biofluids or tissues. Coupled with multivariate statistical chemometric methods, it offers a powerful approach for the assessment of metabolic structures.

Plasma is one of the commonly used biological samples in metabolomics studies, and it can reflect the changes in a variety of cells, tissues and organs. Many plasma/serum metabolomics studies on liver toxicity have been reported (Wang et al., 2016; Kim et al., 2013; Liang et al., 2016). Therefore, it is meaningful to conduct a plasma metabolomics study on *realgar*-induced hepatotoxicity and the hepatoprotective effect of GA.

We have reported a metabolomics study of sub-chronic hepatotoxicity induced by *realgar* in a previous paper (Huo et al., 2016). In this study, the effect of GA on the changes of plasma metabolic profiles induced by *realgar* was explored using an ^{1}H NMR-based metabolomic approach. We aimed to determine whether the metabolic profiles of mice sub-chronic exposed to counterbalanced *realgar* could be restored towards those of control mice, whether GA can alleviate the hepatotoxicity induced by *realgar*, and which biochemical pathways that are affected by *realgar* could be regulated by GA. Our research may facilitate understanding of the hepatoprotective mechanisms of GA. To our knowledge, this study is the first report to investigate the hepatoprotective effect of GA on *realgar*-induced liver toxicity via a metabolomics approach.

2. Experimental

2.1. Material and reagents

GA was obtained from Shanghai Yuanye Biotechnology (CAS#471-53-4; Lot: T09A7×1277; content: 97%, Shanghai, PR China; the chromatogram and MS/MS spectrum of GA for its purity test is shown in Supplementary Fig 1). *Realgar* was purchased from Shenyang Medical Materials Company (Shenyang, PR China; microscopic identification was used for the authentication of *realgar*, the microscopic characteristics of *realgar* is shown in Supplementary Fig 2), and the content of arsenic disulfide (As_2S_2) was in line with the standards of the Chinese Pharmacopoeia (2015). The assay kits for aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), albumin (ALB), total protein (TP), total cholesterol (TC) and choline esterase (CHE) were purchased from Dingguo Changsheng Biotechnology (Beijing, China). Kits for malonaldehyde (MDA), glutathione (GSH), glutathione peroxidase (GSH-Px), super-oxide dismutase (SOD) and catalase (CAT) were obtained from Jian Cheng Biological Engineering Institute (Nanjing, China). D_2O (deuterium oxide, 99.8% in D) and 3-(trimethylsilyl)-propionic-2, 2, 3,3-d_4 acid sodium salt (TSP-d_4) were obtained from Norell, Inc. (USA). All other chemicals were of analytical grade and commercially available.

2.2. Animal treatment

Male Institute of Cancer Research (ICR) mice (23–25 g, affiliated experimental animal centre of China Medical University) were used in this experiment. All mice were acclimatized for a week prior to group allocation and treatment. All mice were housed at 24 ± 1 °C and a relative humidity of 50 ± 5% under artificial lighting (12-h light/dark cycle) and allowed free access to food and water. The research was conducted in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals was adopted and promulgated by the China Medical University (Publication No. 85-23, revised 1985). All efforts were made to minimize the number and suffering of the animals.

The mice were divided into five groups randomly by weight, with seven mice in each group. Mice in group I (control group) were treated with 0.5% (*w*/*v*) sodium carboxymethylcellulose (CMC-Na); mice in group II (GA only group) were treated with 48 mg/kg GA; mice in group III (*realgar* only group) received *realgar* at a dose of 1.35 g/kg; mice in group IV and V were co-administered with GA and *realgar* (group IV, 16 mg/kg GA+1.35 g/kg *realgar*; group V, 48 mg/kg GA +1.35 g/kg *realgar*). All the mice were administered their treatments intragastrically for 8 consecutive weeks. Mice were sacrificed 12 h after final administration. Blood samples were collected from the eyeball vein plexus, and the livers were quickly excised from the mice. Plasma was obtained via centrifugation at 11,200 × *g* for 10 min at 4 °C and divided into two aliquots. One aliquot was used for biochemical assay, and the other was stored at −70 °C for NMR analysis. The excised livers were accurately weighted and stored for further analysis.

2.3. Biochemical assay and ultrastructure of hepatocyte

The activities of AST, ALT, ALP, ALB, TP, TC, and CHE in the plasma and GSH, MDA contents, GSH-Px, SOD, and CAT activities in the liver were determined using assay kits and operated in strict accordance with the instructions.

The fixed liver was made into 1-mm thick slices and postfixed in 1% OsO_4 containing 1.25% potassium ferrocyanide. The tissues were dehydrated in a graded series of acetone and embedded in spur resin. The blocks were stained with uranyl acetate and lead citrate and investigated for ultrastructural changes using a transmission electron microscope (JEM-1200EX, JEOL Corp., Japan) equipped with an ultrascan digital camera.