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^{1}HNMR-based metabonomic study of sub-chronic hepatotoxicity induced by realgar

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ABSTRACT

Ethnopharmacological relevance: Realgar has been used as a traditional Chinese medicine (TCM) for thousands of years. Recently, a number of realgar or realgar-containing medicines poisoning cases have been reported. However, the toxicological mechanism of realgar has not been clearly clarified. In present study, the subchronic hepatotoxicity of realgar on mice was investigated using ^{1}HNMR-based metabonomic approaches.

Materials and methods: Twenty-eight male mice were divided into control group and low (0.15 g/kg), middle (0.45 g/kg), high (1.35 g/kg) dosage realgar exposed groups. Their plasma and urine samples were used for NMR spectroscopic metabolic profiling. Principal component analysis (PCA) and pathway analysis were used to detect the hepatotoxic effects of realgar. Liver histopathological examination and plasma clinical chemistry analyses were also performed.

Results: Plasma clinical chemistry analyses showed increased levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total protein (TP), total cholesterol (TC) and choline esterase (CHE) in realgar-exposed mice indicating liver injury. The PCA score plots showed the metabolic profiles of realgar-exposed mice apparently separated from the controls. Obvious dose-dependent changes of metabolites in urine and plasma of realgar-exposed mice were observed. From the loading plots and boxplots results, the concentrations of VLDL/LDL, 3-HB, lactate, acetate, acetoacetate, creatine, glutamate, methionine, NAc, TMAO, alanine in plasma and pyruvate, succinate, 2-oxoglutarate, DMA, citrate, hippurate, glycine, taurine, phenylalanine, lactate in urine were significantly changed in realgar-exposed mice. The change trends of metabolites in urine and plasma from mice sub-chronic exposed to realgar are similar to those reported in rats acute exposed to realgar, which indicate the acute and sub-chronic toxic mechanism of realgar are same. The disturbed metabolic pathway include energy metabolism, amino acids metabolism and gut bacteria metabolism.

Conclusions: The present work illustrated the NMR-based metabonomic approach can capture and probe the metabolic alterations induced by traditional Chinese medicine in the toxicological effects.

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1. Introduction

Realgar, which has more than 90% arsenic disulfide (As_2S_2), has been used as a traditional Chinese medicine (TCM) for thousands of years. It has been used for the treatment of carbuncles, boils, insect- and snake-bites, intestinal parasitosis and convulsive epilepsy and psoriasis (Nriagu, 2002). Besides the major chemical composition of As_2S_2, realgar has a trace level of arsenic being present as non-sulfur bound species, such as arsenate (As^{V}) and arsenite (As^{III}). Arsenic is well-known for its acute and chronic toxicity (Zhang et al., 2011). In the past decades, a number of realgar or realgar-containing medicines poisoning cases have been reported due to overdose, long-term use and improper processing (Liu et al., 2008, 2011). The safety of realgar and realgar-containing TCMs is of concern.

Metabonomics is an emerging subject of the post-genome era. Metabonomics together with genomics, transcriptomics and proteomics, jointly constitutes the "Systems Biology" (Nicholson and Wilson, 2003). As a systemic approach, metabonomics adopts a "top-down" strategy to reflect the function of organisms from

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terminal symptoms of metabolic network and understand metabolic changes of a complete system caused by interventions in holistic context (Nicholson, 2006). This consists with the holistic thinking of TCMs and metabonomic techniques are extremely suitable for probing the mechanisms and evaluate the safety of TCMs (Zhang et al., 2010). NMR spectroscopy has already been one of the main analytical techniques for metabonomic research. It has the advantages of non-invasive and non-destructive to samples, not biased for any molecules, high throughput and require little sample preparation, and with good resolution and reproducibility. NMR-based metabonomics have been widely applied in TCMs toxicity research (Wang et al., 2015; Chang et al., 2015).

Recently, studies on the acute toxicological effects of realgar or realgar-containing medicines by ^{1}HNMR-based metabonomic approach have already been reported (Wei et al., 2009; Xu et al., 2013, 2014). However, in clinical, many realgar or realgar-containing medicines poisoning cases are caused by long-term or overdose use. Therefore, studying the chronic toxicity of realgar by metabonomic method is important for the further understanding of the toxic mechanism of realgar. In present work, we applied an ^{1}HNMR-based metabonomic approach to investigate the changes of metabolic profiles in urine and plasma of mice exposed to 0.15, 0.45 and 1.35 g/kg realgar for 8 weeks. We sought to describe the changes in urine and plasma metabonome of mice sub-chronic exposed to realgar as compared with controls and clarified the biochemical pathways affected by sub-chronic exposure to realgar. The study will promote realgar toxicity research and be beneficial to the safety assessment, rationale application and modernization of realgar.

2. Experimental

2.1. Material and reagents

Realgar was purchased from Shenyang Medical Materials Company (Shenyang, PR China) and the content of As_2S_2 was in line with the standards of the Chinese Pharmacopoeia (2010). D_2O (deuterium oxide) and 3-(trimethylsilyl)-propionic-2, 2, 3, 3-d_4 acid sodium salt (TSP-d_4) were obtained from Norell Inc. (USA). Kits for plasma 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). All other chemicals were of analytical grade and commercially available.

2.2. Animal treatment

ICR male mice with initial body weights of 23–25 g were obtained from the Experimental Animal Center, China Medical University. All mice were acclimatized for seven days prior to group allocation and treatment. All mice were housed at 24 ± 1 °C with a relative humidity of 50 ± 5%, under artificial lighting (12 h light/dark cycle). The mice were randomly divided into four groups by weight with 7 mice in each group. The first group was designated the control group and was intragastrically (i.g.) treated with 0.5% (w/v) sodium carboxymethyl cellulose (CMC-Na); the second, third and fourth groups were exposed to realgar daily for 8 consecutive weeks at concentrations of 0.15 g/kg (low dosage), 0.45 g/kg (middle dosage) or 1.35 g/kg (high dosage) body weight suspended in CMC-Na. During the experiment, all mice were allowed free access to food and water and were observed for symptoms of toxicity every day. All animal treatments were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as 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.

2.3. Sample collection

Urine samples of each mouse were collected over a period of 24 h into ice-cooled tubes containing 50 μL sodium azide (1%, w/v) at the end of experimental period (day 56). Urine samples were subsequently stored at −70 °C prior to NMR analysis. All animals were sacrificed on day 57. Blood samples from sacrificed mice were centrifuged at $11{,}200 \times g$ for 10 min at 4 °C to obtain the resulted plasma samples. All plasma samples were divided into two aliquots. One aliquot was used for biochemical analysis, and the other was stored at −70 °C for NMR analysis. Livers were immediately removed from mice, accurately weighted and fixed with buffered 2.5% glutaraldehyde in 0.1 M PBS (pH 7.2–7.4) for ultrastructure examination.

2.4. Clinical biochemistry and histopathology

Standard spectrophotometric method was used to measure the following plasma parameters: AST, ALT, ALP, ALB, TP, TC and CHE.

The fixed liver was made into 1-mm thick slices. For electron microscopy, postfixation of the slice in 1% OsO_4 containing 1.25% potassium ferrocyanide was carried out. Afterwards, the tissues were dehydrated in a graded series of acetone and embedded in spur resin. Finally, the blocks were stained with uranyl acetate and lead citrate and photographed using a JEM-1200EX transmission electron microscope equipped with an ultrascan digital camera.

2.5. ^{1}HNMR analysis of urine profile

Frozen urine samples were thawed, and a total of 400 μL urine sample was mixed with 200 μL sodium phosphate buffer (pH 7.4; 0.2 M) to minimize variations in the pH of the urine samples. The resulting solution was left to stand for 5 min and centrifuged at $11{,}200 \times g$ for 5 min to remove any precipitates. 500 μL of the supernatant was transferred to 5 mm NMR tubes containing 40 μL TSP (1 mg/mL) and 20 μL D_2O. The TSP acted as a chemical shift reference (δ 0.0) and the D_2O provided a lock signal. NMR spectral measurements were acquired on a Bruker-AV 600 spectrometer at 298.2 K. Pulse program was 1D version of noesypr with presaturation during relaxation delay and mixing time. Typically, 64 free induction decays (FIDs) were collected into 64 k data points over a spectral width of 12,019.23 Hz with a relaxation delay of 3 s and an acquisition time of 2.73 s.

2.6. ^{1}HNMR analysis of plasma profile

Plasma samples were thawed and centrifuged at $11{,}200 \times g$ for 10 min at 4 °C. 50 μL of deuterium oxide (containing 1 mg/mL TSP) was added to a mixture containing 300 μL of plasma samples and 300 μL of sodium phosphate buffer (pH 7.4; 0.2 M). Then the mixture was centrifuged at $11{,}200 \times g$ for 10 min, and the supernatant of 600 μL was placed in a 5-mm o.d. NMR tube. NMR spectra of these samples were also recorded on a Bruker AV 600 spectrometer operating at 298.2 K. Water signals and broad protein resonances were suppressed by a combination of presaturation and the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence. ^{1}HNMR spectra were measured with 64 scans into 64 k data points over a spectral width of 12,019.23 Hz. The acquisition time was 2.73 s. All acquired NMR spectra were manually phased, baseline-corrected and chemical shift referenced to the methyl peak of TSP (CH_3, δ 0.0) within TOPSPIN (version 2.1, Bruker Spectrospin Ltd.).

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