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The Tibetan medicine Zuotai differs from $HgCl_2$ and MeHg in producing liver injury in mice

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ABSTRACT

Zuotai is composed mainly of β-HgS, while cinnabar mainly contains α-HgS. Both forms of HgS are used in traditional medicines and their safety is of concern. This study aimed to compare the hepatotoxicity potential of Zuotai and α-HgS with mercury chloride ($HgCl_2$) and methylmercury (MeHg) in mice. Mice were orally administrated with Zuotai (30 mg/kg), α-HgS (HgS, 30 mg/kg), $HgCl_2$ (33.6 mg/kg), or CH_3HgCl (3.1 mg/kg) for 7 days, and liver injury and gene expressions related to toxicity, inflammation and Nrf2 were examined. Animal body weights were decreased by $HgCl_2$ and to a less extent by MeHg. $HgCl_2$ and MeHg produced spotted hepatocyte swelling and inflammation, while such lesions are mild in Zuotai and HgS-treated mice. Liver Hg contents reached 45–70 ng/mg in $HgCl_2$ and MeHg groups; but only 1–2 ng/mg in Zuotai and HgS groups. $HgCl_2$ and MeHg increased the expression of liver injury biomarker genes metallothionein-1 (MT-1) and heme oxygenase-1 (HO-1); the inflammation biomarkers early growth response gene (Egr1), glutathione S-transferase (Gst-mu), chemokine (mKC) and microphage inflammatory protein (MIP-2), while these changes were insignificant in Zuotai and HgS groups. However, all mercury compounds were able to increase the Nrf2 pathway genes NAD(P)H:quinone oxidoreductase 1 (Nqo1) and Glutamate-cysteine ligase, catalytic subunit (Gclc). In conclusion, the Tibetan medicine Zuotai and HgS are less hepatotoxic than $HgCl_2$ and MeHg, and differ from $HgCl_2$ and MeHg in hepatic Hg accumulation and toxicological responses.

1. Introduction

In traditional Indian Ayurvedic medicines (Kamath et al., 2012), Chinese medicines (Pharmacopoeia of China (2015)), and Tibetan medicines (Chen et al., 2012; Kan, 2013; Li et al., 2015), mercury sulfides (α-HgS or β-HgS) are frequently included in the herbo-metallic preparations for the treatment of stroke, brain trauma, neuroinflammation, chronic ailments like syphilis, pneumonia, insomnia, and many other disorders. However, mercury is toxic heavy metal; the safety of these herbo-metallic preparations is of concern. In traditional Chinese medicines, the allowable amounts of cinnabar (96% α-HgS) were decreased from a daily allowable dose of 0.3–1.5 g in 1977 to 0.1–0.5 g in 2005) (Zhou et al., 2009). However, the total mercury content in these traditional medicines is still thousands of folds higher than environmental exposure levels. It was strongly recommended that well-designed studies are needed to address the true risk of the use of mercury-based traditional medicines (Kamath et al., 2012; Liu et al., 2008; Mao and Desai, 2009).

In discussing mercury toxicity, the chemical forms of mercury must be distinguished (Klaassen, 2006). Only mercury sulfides (α-HgS, β-HgS) were used in traditional remedies, while mercury chloride ($HgCl_2$) and methylmercury (MeHg) were never used in traditional medicines (Kamath et al., 2012; Pharmacopoeia of China (2015)).

Liver is the major target organ of mercury toxicity (Liu et al., 2008). We have previously shown that cinnabar (α-HgS) is

Abbreviations: MT-1, Metallothionein-1; Egr1, Early growth response protein gene; Ho-1, Heme oxygenase-1; Nrf2, Nuclear factor erythroid 2 [NF-E2]-related factor 2; Nqo1, NAD(P)H:quinone oxidoreductase 1; Gclc, Glutamate-cysteine ligase catalytic subunit; Gst-mu, Glutathione S-transferase-mu; mKC, Mouse chemokine; MIP-2, Microphage inflammatory protein 2.

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different from $HgCl_2$ and MeHg in producing liver injury in mice after acute exposure (Lu et al., 2011a) and chronic exposures (Lu et al., 2011b). However, whether the same scenario applies to β-HgS-containing Zuotai (Li et al., 2015) is not known. The goal of the current study was designed to compare the hepatoxicity potentials of Zuotai and α-HgS with $HgCl_2$ and MeHg in mice, focusing on liver Hg accumulation, pathology lesions, and gene expressions related to liver toxicity, inflammation, and the Nrf2 antioxidant pathways were examined.

2. Materials and methods

2.1. Chemicals and animals

Zuotai was provided by the Northwest Plateau Institute of biology of Chinese Academy of Sciences. The pure form of α-HgS (HgS), $HgCl_2$ and CH_3HgCl were purchased from Sigma Chemical Company (St. Louis, MO). Other reagents were of reagent grade.

Adult male Kunming mice, 25 ± 2 g, were purchased from the Laboratory Animals Center of the Third Military Medical University (Chongqing, China). Mice were maintained in animal facilities at 22 ± 2 °C with a 12 h light–dark cycle, and had free access to standard rodent chow and water. They were allowed to acclimate for at least 10 days prior to experiment. All the experiments were carried out in full compliance with the Chinese Guidance of Humane Care and Use of Laboratory Animals and approved by the Animal Use and Care Committee of Zunyi Medical College.

2.2. Experimental design

Adult mice were divided randomly to five groups, 6–8 mice per group. Mice were orally (po) given distilled water (Control), Zuotai (30 mg/kg, about 5-fold of clinical dose), α-HgS, (HgS, 30 mg/kg), $HgCl_2$ (33.6 mg/kg, equivalent Hg as α-HgS), and methylmercury chloride (MeHg, 3.1 mg/kg, 1/10 Hg of HgS), daily for consecutive 7 days. Animals were closely monitored throughout the entire experiment period and body weights were recorded every other day. At the end of experiment, animal body weights and liver weights were recorded and blood, Livers were collected for further analysis.

2.3. Histological evaluation

A portion of the liver was fixed in 10% neutral formalin for 48 h. The fixed tissues were paraffin embedded, sectioned at 6 μm and stained with hematoxylin and eosin (H&E) and examined with the light microscope.

2.4. Ultrastructural analysis

Mice were anesthetized with 10% Chloral hydrate, followed by perfusion through the heart with phosphate buffered saline and 4% paraformaldehyde. Livers were then quickly removed and cut in small pieces ($1 \times 1 \times 1$ mm^3) on ice pad. The liver sections were pre-fixed immediately in 3% glutaraldehyde for 36 h, and dipped in the fixative solution of 1% osmium tetroxide, and then processed with standard sample preparation for electromicroscopy (passed the graded alcohol for dehydration, embedded in spur epoxy resin, and cut thin slice of 70 nm). The section was stained with uranyl acetate and electronic lead nitrate for transmission electron microscope (Hitachi H-7650) examination.

2.5. Blood biochemistry

Blood was collected and allowed to clot at 4 °C to separate serum by centrifugation at 3500 × *g* for 10 min. The activities of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were quantified to evaluate the hepatotoxicity with the commercially available kits (Jiangcheng Co, Nanjing, China).

2.6. Determination of Hg in the liver

A portion (about 100 mg) of liver was digested in 5 ml 65% nitric acid at 163 °C for 2 h, and brought to 25 ml with distilled water. Aliquots of 5 ml were incubated 30 min with 5% sulfourea and ascorbic acid solution, then As and Hg contents were determined with Atomic Fluorescence Spectrometry (Kechuang Haiguan Instrument Co. Ltd, Beijing, China). These assays were performed by Guizhou Chemical Analysis Center of Chinese Academia of Sciences (Lu et al., 2011a, 2011b).

2.7. RNA isolation and real-time PCR analysis

Approximately 50–100 mg of livers was homogenized in 1 ml TRIzol (TakaRa Biotechnology, Dalian, China). The quality and quantity of RNA were determined by the 260/280 ratio and by gel-electrophoresis. Total RNA was reverse transcribed with the High Capacity Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA). The primers were designed with Primer3 software and listed in Supplemental Table 1. The 15 μl PCR reaction mix contained 3 μl of cDNA (10 ng/ μl), 7.5 μl of iQTM SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), 0.5 μl of primer mix (10 μM each), and 4 μl of ddH2O. After 5 min denature at 95 °C, 40 cycles will be performed: annealing and extension at 60 °C for 45 s and denature at 95 °C for 10 s. Dissociation curve was performed after finishing 40 cycles to verify the quality of primers and amplification. Relative expression of genes was calculated by the $2^{-\triangle\triangle}$Ct-method and normalized to the house keeping gene β-actin.

2.8. Statistical analysis

Data were expressed as mean and standard error. The SPSS 16 software was used for statistical analysis. For comparisons among three or more groups, data were analyzed using a one-way analysis of variance (ANOVA), followed by multiple range Duncan's test. P value < 0.05 was considered statistically significant. However, only the differences between control and treatment groups are labeled in the Figure.

3. Results

3.1. Animal body weight

Mice were orally administrated with Zuotai (30 mg/kg, 5-fold of clinical dose) daily for 7 days. Zuotai is mainly composed of β-HgS (Li et al., 2015). For comparison, α-HgS (HgS, 30 mg/kg), $HgCl_2$ (33.6 mg/kg, equivalent Hg content as HgS) and MeHg (3.1 mg/kg, 1/10 Hg of HgS) were also administered for 7 days, and animal body weights, activities and the general health were observed. The animal body weight gain in Zuotai and HgS groups were similar to controls, with normal activity and general health conditions. In comparison, $HgCl_2$ significantly retarded the animal net body weight gain, MeHg decreased animal body weights gain after 3 days of administration (Fig. 1). At necropsy, the liver weights were not significantly different among groups (Data not shown).

Blood biochemistry showed that the serum activities of alanine aminotransferase (23 ± 3, 20 ± 2, 21 ± 3, 28 ± 3 and 24 ± 3 for Control, Zuotai, HgS, $HgCl_2$, and MeHg, respectively) and aspartate aminotransferase (108 ± 13, 102 ± 12, 98 ± 13, 128 ± 15 and 119 ± 13 for Control, Zuotai, HgS, $HgCl_2$, and MeHg, respectively) were not