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The Tibetan medicine Zuotai differs from HgCl₂ and MeHg in producing liver injury
in mice

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Running Title: HgS differs from HgCl₂ and MeHg in producing liver injury

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

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.

Keywords: Zuotai; cinnabar; mercury chloride; methylmercury; hepatotoxicity; electronmicroscopy; gene expression; Nrf2.

Introduction

In traditional Indian Ayurvedic medicines (Kamath et al., 2012), Chinese medicines (Pharmacopeia 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; Pharmacopeia 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 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

hepatotoxicity 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.

Materials and Methods

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 $22 \pm 2^\circ\text{C}$ 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.

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.

Histological Evaluation

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

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 (1x1x1 mm³) on ice pad. The liver sections were pre-fixed immediately in 3% glutaraldehyde for 36 hrs, 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.

Blood biochemistry

Blood was collected and allowed to clot at 4°C to separate serum by centrifugation at 3500 x 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).

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 hrs, 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).

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 seconds and denature at 95°C for 10 seconds. Dissociation curve was performed after finishing 40 cycles to verify the quality of primers and amplification. Relative expression of genes was calculated by the $2^{-\Delta\Delta C_t}$ -method and normalized to the house keeping gene β -actin.

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.

Results

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. 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, HgCl₂, and MeHg, respectively) were not significantly different among groups.

Liver Hg accumulation

To evaluate hepatic uptake and accumulation of mercury after orally administrated with Zuotai (30 mg/kg), HgS (30 mg/kg), HgCl₂ (33.6 mg/kg) and MeHg (3.1 mg/kg) for 7 days, the livers were removed, and a portion of the liver was digested in nitric acid and liver Hg contents were determined by Atomic Fluorescence Spectrometry as described in Methods. Hepatic Hg contents in controls was 0.22 ± 0.01 , in Zuotai groups was 1.15 ± 0.15 , in HgS group was 1.05 ± 0.13 ng/mg liver; while it reached 67.6 ± 6.11 in HgCl₂ group and 45.3 ± 4.0 ng/mg liver in MeHg group (Figure 2). The differences were dramatic.

Histopathological alterations

Representative H&E microphotos are showed in Figure 3. In livers of mice orally administrated with Zuotai (30 mg/kg) and α -HgS (30 mg/kg) for 7 days, the morphology of the liver was not different from controls; In comparison, in livers of mice orally dosed HgCl₂ (33.6 mg/kg) for 7 days, spotted hepatocyte degenerative lesions were observed; in livers of mice dosed with MeHg (3.1 mg/kg) for 7 days, the focal hepatocyte degeneration was observed. In general, at the dose of Hg compounds used in the present study, no apparent liver pathology lesions were evident.

3.4. Ultrastructural alterations

Representative electronic scanning images are showed in Figure 4. In livers of mice orally administrated with Zuotai (30 mg/kg) and α -HgS (30 mg/kg) for 7 days, the ultrastructure of the liver was not different from controls. In comparison, in livers of mice orally dosed HgCl₂ (33.6 mg/kg) or MeHg (3.1 mg/kg) for 7 days, more ultrastructural changes were observed, such as swollen of endoplasmic reticulum and lysosomes.

Expression of genes sensitive to toxic stimuli

To further examine the gene expression related to hepatic injury, the expression of metallothionein-1 (Lu et al., 2011a) and heme oxygenase-1 (Ho-1) (Lu et al., 2011b) was examined. Orally administration of Zuotai (30 mg/kg,) and HgS (30 mg/kg), did not show significant elevations in MT-1 (1.5-fold). In comparison, in livers of mice dosed with HgCl₂ (33.6 mg/kg) and MeHg (3.1 mg/kg) for 7 days, the expression of MT-1 was increased 12- and 5.5-fold, indicative of stress (Figure 5).

Ho-1 is a biomarker of oxidative liver injury (Lu et al., 2011b). The expression of Ho-1 was basically unchanged in livers of mice given orally Zuotai (30 mg/kg) and HgS (30 mg/kg). In comparison, in livers dosed with HgCl₂ (33.6 mg/kg) and MeHg (3.1 mg/kg) for 7 days, the expression of Ho-1 was increased 190% and 150% of controls, respectively, indicative of oxidative stress to the liver.

Expression of liver inflammatory genes

Expression of inflammatory marker genes Egr1, Gstm1, mKC, and MIP-2 are depicted in Figure 4. Zuotai (30 mg/kg) and HgS (30 mg/kg) had no apparent effects on the expression of these inflammatory genes, while HgCl₂ (33.6 mg/kg) and MeHg (3.1 mg/kg) increased the expression of Egr-1 by 5.8-fold and 3.3-fold, respectively; Gstm1 by 7.3-fold and 2.2-fold, respectively; mKC by 8-fold and 3.4-fold, respectively, and MIP-2 by 5-fold and 3-fold, respectively (Figure 6).

Expression of the Nrf2-related genes

Nrf2/ARE antioxidant pathways are a major defense mechanism against oxidative stress and toxicant could activate this pathway as an adaptive mechanism against oxidative liver injury (Liu et al., 2013). When cells receives toxic stimuli, Nrf2 is disassociate with its partner Keap1, and translocated into the nucleus to activate a battery of cell defense genes such as Nqo1 and Gclc, two major Nrf2 target genes. In the present study, all mercury compounds increased the expression of Nqo1 and Gclc 2-3 fold, with higher expression in HgCl₂-treated mice (Figure 7).

Discussion

The present study clearly demonstrated the hepatotoxicity potentials of Zuotai, and HgS differ from HgCl₂ (equal Hg as HgS) and MeHg (1/10 Hg as HgS). HgCl₂ and MeHg retarded animal body weight gain, while Zuotai and HgS did not affect the general health; HgCl₂ and MeHg produced more histopathological lesions in the liver than Zuotai and HgS; HgCl₂ and MeHg produced 45-70 ng Hg/mg liver accumulation, while Zuotai and HgS resulted in only 1-2 ng/mg; HgCl₂ and MeHg induced liver toxicity

gene MT-1 and Ho-1 expression, as well as increased expression of inflammatory genes, while Zuotai and HgS did not; Thus, chemical forms of mercurial compounds determine their tissue accumulation and toxicity in livers of mice.

Zuotai is one of the main raw materials of over 20 Tibetan medicines, and is thought to play important roles in the system of Tibetan medicine in the treatment of diseases (Huang et al., 2013; Kan, 2013; Li et al., 2014). Recent studies using advanced technology revealed that the main elements in Zuotai are Hg and S, with some other minor elements (Li et al., 2015) and the analysis also suggests that Zuotai is a kind of ancient micro-nano drug, with its particle size in the range of 100-600 nm and even less than 100 nm (Li et al., 2015). The mice could tolerant Zuotai at a single dose of 80 g/kg, the repeated subacute doses (13-2000 mg/kg), or the daily clinical dose (6.7 mg/kg) for 4.5 months, with only slight reversible kidney morphology alterations (Li et al., 2014). Rats received repeated β -HgS-containing Ayurvedic formulation (Sidh Makardhwaj) (10-100 mg/kg, po) for 28 days, and the hepatotoxicity potential was less than HgCl_2 (1 mg/kg) (Kumar et al., 2014). In the present study, we used 5-fold clinical dose (30 mg/kg) of Zuotai for 7 days, and no apparent toxic effects were observed, from the general health, liver Hg accumulation, and gene expressions related to liver toxicity, inflammation genes, fortifying the notion that β -HgS-containing Zuotai is relatively safe at clinical doses (Li et al., 2014).

HgS is more frequently used in Ayurvedic medicines (Ramath et al., 2012) and traditional Chinese medicines (Pharmacopia of China, 2015). Cinnabar is a naturally occurring mercuric sulfide containing more than 96% mercuric sulfide (α -HgS) and has been used in traditional Chinese medicine as a sedative and soporific agent for more

than 2000 years (Liu et al., 2008; Zhou et al., 2009). HgS is quite different from HgCl₂ and MeHg, in regard to its toxicokinetics and toxicological effects (Liu et al., 2008). We have previously shown that the hepatotoxicity of HgS is quite different from HgCl₂ and MeHg in mice after 44 days of administration (Lu et al., 2011b). Thus, it is not surprising that in the current study (30 mg/kg, po for 7 days), no overt toxicological effects and gene expressions related to hepatic toxicity and transporters were found, in agreement with the literature that the bioaccessibility of HgS is quite different from HgCl₂ (Koch et al., 2013; Wang et al., 2009; Tinggi et al., 2016; Zheng et al., 2015), resulting in much less toxicity potential (Lu et al., 2011b; Peng et al., 2012). It should be noted that the higher dose and longer-time administration of cinnabar (400-800 mg/kg for 90 days), liver toxicity did occur in rats (Liang et al., 2009). In another study, liver toxicity occurred after a large dose of cinnabar (1000 mg/kg) for 8-12 weeks (Wang et al., 2015). Thus, caution should be taken when taken cinnabar at the higher dose for the longer periods.

In contrast to Zuotai and HgS, HgCl₂ at the equivalent Hg content produced liver injury, as manifested by decreased body weight and histopathological findings. The expression of sensitive biomarkers for liver injury, such as MT-1 and Ho-1 (Lu et al., 2011a; 2011b), were increased. In addition, the increased inflammatory biomarkers such as Egr-1, Gsta1, mKC, and MIP-2 (Liu et al., 2013) were evident following HgCl₂ treatment. Exposure to non-toxic dose of HgCl₂ produces oxidative damage, leading to cell death (Patnaik et al., 2010). In HgCl₂-treated rats for 28 days, serum enzyme activities were significantly increased and serum levels of total protein, triglyceride, and low-density lipoprotein were significantly decreased (Uzunhisarcikli et al., 2015). Thus,

HgCl₂, even at the sub-lethal dose by mouth, could produce oxidative damage to the liver.

MeHg mainly targets on the brain and kidney, but it can also target the liver (Liu et al., 2008; Lu et al., 2011b; Joshi et al., 2014), and affect liver lipid metabolism producing hypercholesterolemia (Moreira et al., 2012). MeHg is highly toxic among mercury compounds and only 1/10 of equivalent Hg dose of MeHg (3.1 mg/kg) was able to produce liver pathological lesions, and the liver injury was evident in nearly all parameters examined, just secondary to HgCl₂. In traditional medicines, no any record of using HgCl₂ and MeHg as therapeutics due to their severe toxicity, and HgCl₂ and MeHg should be distinguished from mercury sulfides.

One of the most interesting findings was the effects of mercurial on the Nrf2 antioxidant pathway gene Nqo1 and Gclc expression. Hg ions is known to increase Nqo1 (Wataha et al., 2008), and the induction of Nqo1 by Hg is mediated through the Nrf2 pathway activation (Amara and El-Kadi, 2011), probably as an adaptive mechanism in the body defense against toxic stimuli. The Nrf2 pathway gene induction could be beneficial as Berberine, a known Nrf2 inducer, is shown to protect against HgCl₂ hepatotoxicity (Othman et al., 2014). Activation of Nrf2 pathways protects against a panel of hepatotoxicants (Liu et al., 2013), and is a common mechanism for traditional remedies to exert hepatoprotective effects (Wu et al., 2014). Although the therapeutic basis of Zuotai and HgS remains an enigma, whether the induction of Nrf2 genes could be one of the mechanisms of their action require further investigation.

In summary, the present study demonstrated that Zuotai and HgS differ from HgCl₂ and MeHg in producing liver toxicity and this effect is related to differences in liver

Hg accumulation and toxicity-related gene expression. This study fortifies the notion that in the risk assessment of mercury-based traditional medicines, chemical forms matters.

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Figure legends:

Figure 1. Animal body weight gain in mice after orally administrated with Zuotai (30 mg/kg), HgS (30 mg/kg), HgCl₂ (33.6 mg/kg), and MeHg (3.1 mg/kg) for 7 days. Data are mean and SEM of 6-8 mice. *Significantly different from controls at $p < 0.05$.

Figure 2 Liver Hg contents in mice after orally administrated with Zuotai (30 mg/kg), HgS (30 mg/kg), HgCl₂ (33.6 mg/kg), and MeHg (3.1 mg/kg) for 7 days. Data are mean and SEM of 6-8 mice. *Significantly different from controls at $p < 0.05$.

Figure 3 Histopathological examinations with representative photos from livers of mice orally administrated with Zuotai (30 mg/kg), HgS (30 mg/kg), HgCl₂ (33.6 mg/kg), and MeHg (3.1 mg/kg) for 7 days. Magnitude 200X.

Figure 4 Electronic microscope examination of the liver with representative photos from livers of mice orally administrated with Zuotai (30 mg/kg), HgS (30 mg/kg), HgCl₂ (33.6 mg/kg), and MeHg (3.1 mg/kg) for 7 days. Magnitude 7000-10000X. Bar=2 or 5 μ m.

Figure 5 The mRNA expression of stress gene MT-1 and Ho-1 in the liver of mice orally administrated with Zuotai (30 mg/kg), HgS (30 mg/kg), HgCl₂ (33.6 mg/kg), and MeHg (3.1 mg/kg) for 7 days. . Data are mean and SEM of 6-8 mice. *Significantly different from controls at $p < 0.05$.

Figure 6 The mRNA expression of inflammatory genes Egr-1, Gst-mu, mKC and MIP-2 in the liver of mice orally administrated with Zuotai (30 mg/kg), HgS (30 mg/kg), HgCl₂ (33.6 mg/kg), and MeHg (3.1 mg/kg) for 7 days. . Data are mean and SEM of 6-8 mice. *Significantly different from controls at $p < 0.05$.

Figure 7 The mRNA expression of the Nrf2-targeted genes Nqo1 and Gclc in the liver of mice orally administrated with Zuotai (30 mg/kg), HgS (30 mg/kg), HgCl₂ (33.6 mg/kg,) and MeHg (3.1 mg/kg) for 7 days. . Data are mean and SEM of 6-8 mice.
*Significantly different from controls at $p < 0.05$.

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Figure 1

Figure 2

Figure 3

Control

Zuotai 30 mg/kg

HgS 30 mg/kg

HgCl₂ 33.6 mg/kg

MeHg 3.1 mg/kg

Figure 4

Control

Zuotai (30 mg/kg)

α-HgS (30 mg/kg)

HgCl₂ (33.6 mg/kg)

MeHg (3.1 mg/kg)

Figure 5

Figure 6

Figure 7

Mineral cinnabar
(96% α-HgS)

Mineral metacinnabar
(54% β-HgS)

Mercury sulfide
HgS

Mercuric chloride
HgCl₂

Methylmercury
CH₃Hg

Liver Hg

Liver H&E

Liver TEM

gene expression