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Comparison of mercury sulfides with mercury chloride and methylmercury on hepatic P450, Phase-2 and transporter gene expression in mice.

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Running Title: HgS differs from HgCl₂ and MeHg in affecting drug processing genes

Keywords: Zuotai; mercury sulfide; mercury chloride; methylmercury; cytochrome P450, phase-2 genes; transporters.

Graphic abstract

Abstract

Zuotai (mainly β -HgS) and Zhusha (also called as cinnabar, mainly α -HgS) are used in traditional medicines in combination with herbs or even drugs in the treatment of various disorders, while mercury chloride (HgCl_2) and methylmercury (MeHg) do not have known medical values but are highly toxic. This study aimed to compare the effects of mercury sulfides with HgCl_2 and MeHg on hepatic drug processing gene expression. Mice were orally administrated with Zuotai (β -HgS, 30 mg/kg), α -HgS (HgS, 30 mg/kg), HgCl_2 (33.6 mg/kg), or MeHg (3.1 mg/kg) for 7 days, and the expression of genes related to phase-1 drug metabolism (P450), phase-2 conjugation, and phase-3 (transporters) genes were examined. The mercurials at the dose and duration used in the study did not have significant effects on the expression of cytochrome P450 1-4 family genes and the corresponding nuclear receptors, except for a slight increase in $\text{PPAR}\alpha$ and Cyp4a10 by HgCl_2 . The expressions of UDP-glucuronosyltransferase and sulfotransferase were increased by HgCl_2 and MeHg, but not by Zuotai and HgS. HgCl_2 decreased the expression of organic anion transporter (Oatp1a1), but increased Oatp1a4. Both HgCl_2 and MeHg increased the expression of multidrug resistance-associated protein genes (Mrp1, Mrp2, Mrp3, and Mrp4). Zuotai and HgS had little effects on these transporter genes. In conclusion, Zuotai and HgS are different from HgCl_2 and MeHg in hepatic drug processing gene expression; suggesting that chemical forms of mercury not only affect their disposition and toxicity, but also affect their effects on the expression of hepatic drug processing genes.

Keywords: Zuotai; mercury chloride; methylmercury; cytochrome P450, phase-2 genes; transporters

Abbreviations:

P450, Cytochrome P450 enzyme genes

Ugt1a1, UDP-glucuronosyltransferase family 1, subfamily A, polypeptide 1

Sult1a1, Sulfotransferase family 1, subfamily a, peptide1

Gst-mu. Glutathione S-transferase mu

Oatp1a1, Organic anion transporting polypeptide 1a1

Mrp2; Multidrug resistance associated protein 2

1. Introduction

Zuotai (mainly β -HgS) and Zhusha (also called as cinnabar, mainly α -HgS) are used in traditional Chinese medicines [1], Indian Ayurvedic medicines[2], and Tibetan medicines[3, 4] for thousands of years in the treatment of insomnia as a sedative agent, and also used for neuroinflammation, stroke, and brain trauma, and others. In contrast, HgCl_2 and MeHg are highly toxic, never used in traditional remedies. We have recently shown that the chemical forms of metals used in traditional medicines are a major determinant of their disposition, efficacy and toxicity [5]. For example, mercury sulfides are quite different from HgCl_2 and MeHg in producing liver toxicity and gene expression changes [6, 7].

It should be noted that mercury sulfides are not used alone, rather as the herb-metallic mixtures, or even with common drugs [2, 8]. Thus, the potential interactions of

mercury sulfides with herbs, drugs, and other dietary supplements could affect the efficacy and toxicity of traditional medicines, and are an important issue to be addressed [5].

Liver is the major organ of drug metabolism [9]. Herb-drug interactions involve the phase-1 and phase-2 drug metabolizing enzymes and transporters [10-12]. One of approaches to study the potential herb-drug interactions is to examine their effects on the expression of genes encoding liver phase-1 (cytochrome P450), phase-2 (conjugation reaction), and phase-3 (uptake and efflux transporters) [13, 14].

Mercury chloride and methylmercury have been reported to influence the expression and activities of cytochrome P450 [15-18], phase-2 enzymes such as UDP-glucuronosyltransferase (Ugt) [19], and transporters [20, 21]. However, little is known about the effects of mercury sulfides on these drug processing enzymes and their relative efficacy compared to HgCl_2 and MeHg . In discussing mercury toxicity, chemical forms of mercury should be distinguished [22]. The goal of the current study was designed to compare the effects of Zuotai and $\alpha\text{-HgS}$ with HgCl_2 and MeHg on Phase-1 P450, phase-2 Ugt and sulfotransferase (Sult) and Phase 3 (uptake and efflux transporters) gene expression in livers of mice to provide the potential metal-herb-drug interactions in traditional medicines, as the liver is the major target organ of Hg toxicity.

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, 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 a room 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 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*

Mice were divided randomly to five groups, 6-8 mice per group. Mice were orally given distilled water (Control), Zuotai (30 mg/kg, about 5-fold of clinical dose) [23], 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. The dose-selection was based on our recent publications[7].

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 the blood, Liver, and other tissues were collected for further analysis.

2.3. *Hepatotoxicity Evaluation*

Hepatotoxicity evaluation has been reported in our recent publication [7]. Briefly, hepatotoxicity was evaluated by serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), liver histopathology (H&E), liver mercury accumulation with Atomic Fluorescence Spectrometry, and by liver gene expression in response to Hg-induced stress and toxicity [7].

2.4. *RNA isolation and real-time PCR analysis*

Approximately 50-100 mg of liver tissue was homogenized in 1 ml TRIzol (Invitrogen, Carlsbad, CA), and total RNA was extracted according to manufacturer's instructions, followed by purification with RNeasy kits (Qiagen, Valencia, CA). The quality of RNA was determined by the 260/280 ratios. Purified RNA was reverse transcribed with Oligo-dT primers and MuLV reverse transcriptase. The Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) was used for real-time RT-PCR analysis. The primers were designed by Primer3 software and listed in Table 1. The expression of interested genes was first normalized with GAPDH of the same sample, and the relative transcript levels were calculated setting control as 100%.

Table 1: Primer sequences for RT-PCR analysis

Gene	GenBank Number	Forward	Reverse
AhR	NM_013464	ACCAGAACTGTGAGGGTTGG	CTCCCATCGTATAGGGAGCA
CAR	NM_009803	CTCAAGGAAAGCAGGGTCAG	AGTTCCTCGGCCCATATTCT
Cyp1a2	NM_009993	GACATGGCCTAACGTGCAG	GGTCAGAAAGCCGTGGTTG
Cyp2b10	AF128849	AAGGAGAAGTCCAACCAGCA	CTCTGCAACATGGGGGTACT
Cyp3a11	NM_007818	ACAAACAAGCAGGGATGGAC	GGTAGAGGAGCACCAAGCTG
Cyp4a10	NM_010011	CACACCCTGATCACCAACAG	TCCTTGATGCACATTGTGGT
GAPDH	M32599	AACTTTGGCATTGTGGAAGG	GGATGCAGGGATGATGTTCT
Gst-mu	NM_010358	CTCCCGACTTTGACAGAAGC	TTGCTCTGGGTGATCTTGTG
GST-pi	D30687	TGGGCATCTGAAGCCTTTTG	GATCTGGTCACCCACGATGAA
Mrp1	NM_008576	GCCCTCTTTGCAGTCATCTC	CAGTCTCTCCACTGCCACAA
Mrp2	NM_013806	TCCTAGACAGCGGCAAGATT	GCTAGAGCTCCGTGTGGTTC
Mrp3	NM_029600	TGGTCATGCTGTCAGCTTTC	AAGGACTGAGGGGAACGAAT
Mrp4	BC150822	GCAAAGCCCATGTACCATCT	ACCACGGCTAACAACCTACC
Oatp1a1	NM_013797	ATCCAGTGTGTGGGGACAAT	GCAGCTGCAATTTTGAAACA
Oatp1a4	NM_030687	GGAAGATTGGACACGCATCT	GGCATTGTGACTGAAGCAGA
Oatp1b2	NM_020495	CAAACCTCAGCATCCAAGCAA	GGCTGCCAAAAATATCCTGA
Oatp2b1	NM_020495	CTAGGCCAAATGCCAGAAAG	TTGCTTGGATGCTGAGTTTG
PPARa	NM_011144	GTCCTCAGTGCTTCCAGAGG	GGTCACCTACGAGTGGCATT
PXR	AF031814	CCCATCAACGTAGAGGAGGA	TCTGAAAACCCCTTGCATC
Sult1a1	NM_133670	GGATGTAGCTGAGGCAGAGG	CAGCTCCCAGTGGCATTAT
Sult1b1	NM_019878	GGTGGGAAAAGAGGGAAGAG	AAGGCCTCTTCATCCAAGGT

2.5. 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.

3. Results

3.1. *Animal health*

Zuotai is mainly composed of β -HgS [4]. For comparison, mice were orally administrated with 5-fold of clinical dose (30 mg/kg) of Zuotai [23], similar amount of α -HgS (HgS, 30 mg/kg), equivalent Hg content of HgCl_2 (33.6 mg/kg, as HgS) and 1/10 Hg content of MeHg (3.1 mg/kg) for 7 days. 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 and MeHg retarded the animal net body weight gain [7]. However, serum activities of alanine aminotransferase and aspartate aminotransferase were within the normal range and were not significantly different among groups [7].

3.2. *Liver Hg accumulation and pathology*

Hepatic Hg contents reached 67.6 ng/mg in HgCl_2 group and 45.3 ng/mg liver in MeHg group as compared to 1.05-1.15 ng/mg liver in Zuotai and HgS groups, which were slightly higher than controls (0.22 ng/mg liver) [7]. These results are in the agreement of our prior publications studying the accumulation and toxicity of HgS, HgCl_2 and MeHg in livers of mice [6] and rats [26].

The morphology of the liver in Zuotai (30 mg/kg) and α -HgS (30 mg/kg) groups was not different from controls; In comparison, in livers of mice orally dosed HgCl_2 (33.6 mg/kg) or MeHg (3.1 mg/kg), the spotted pathological changes could be seen under the light microscopy and electromicroscope [7].

3.3. *Expression of cytochrome P450 genes*

Liver is the major organ of xenobiotic metabolism. Cytochrome P450 is the major phase-1 drug metabolism genes in the liver. Expression of hepatic cytochrome P450 1-4 family genes and the corresponding nuclear receptors [24] are shown in Fig. 1. AhR is the nuclear factor for the expression of cytochrome P450 1 family genes such as Cyp1a2. All mercury compounds did not show significant effects on the mRNA expression of AhR and Cyp1a2; CAR is the nuclear factor for the expression of cytochrome P450 2 family genes such as Cyp2b10, again, MeHg tended to increase the expression of Cyp2b10 (140% of control), but was not statistically significant. Other mercury compounds do not have apparent effects on mRNA expression of CAR and Cyp2b10. PXR is the nuclear receptor for Cyp 3 family genes such as Cyp3a11. Cyp3a11 is the major P450 gene responsible for most drug metabolism, and under the current experimental conditions, all mercury compounds did not show overt effects on the mRNA expression of PXR and Cyp3a11. PPAR α is the nuclear receptor for lipid metabolism genes such as Cyp4a10. The mRNA expression of PPAR α was increased 2-fold by HgCl₂, along with a slight increase (185% of control) in Cyp4a10, while no significant changes were evident in other groups (Figure 1).

Figure 1 The mRNA expression of cytochrome P450 genes and corresponding nuclear receptors 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 $p < 0.05$.

3.4. *Expression of Phase-2 conjugation enzyme genes*

Conjugation reactions are called phase-2 drug metabolism. In the liver, the important phase-2 conjugation enzymes include UDP-glucuronosyltransferase, sulfation transferase, and glutathione S-transferase [13, 24]. In the present study, HgCl_2 increased the expression of Ugt1a1 (195% of control) and Ugt1a6 (2-fold of control); MeHg increase the expression of Ugt1a9 (190% control) and Ugt2a3 (160% of control); while Zuotai and HgS do not affect the expression of these Ugts (Figure 2, top). For the sulfation enzyme genes, both HgCl_2 and MeHg increased the expression of Sult1a1 (160% of controls) and MeHg increased the expression of Sult1b1 (195% of controls). For the expression of glutathione S-transferase, both HgCl_2 (430% of controls) and MeHg (220% of controls) increased the expression of Gst-mu, and HgCl_2 also increased the expression of Gst-pi 2.5-fold. Zuotao and HgS did not have overt effects on Gst expressions (Figure 2, bottom).

Figure 2 The mRNA expression of phase-2 conjugation enzyme genes (sulfate transferase, UDP-glucuronotransferase and glutathione S-transferase) genes 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.

3.5. *Expression of the transporter genes*

Drug transporters are also called the phase-3 metabolism. In the liver, Oatp transporters are major uptake transporters for taking xenobiotics into the liver, while Mrp transporters are major efflux transporters pumping xenobiotic metabolites into the bile or back to the blood stream [9]. For uptake transporters, HgCl₂ decreased the expression of Oatp1a1 (50% of control) but increased the expression of Oatp1a4 (280% of control); MeHg also increased the expression of Oatp1a4 (290% of control), while Zuotai and HgS did not affect liver uptake transporters (Figure 3, left panel). For efflux transporters, MeHg increased the expression of Mrp1 (180% of controls), and both HgCl₂ (210% of control) and MeHg (240% of control) increased the expression of Mrp2, which is important for biliary excretion of xenobiotic conjugates [9]. Mrp3 and Mrp4 are transporters to transporting xenobiotics back to blood. HgCl₂ (235% of control) seemed to have more effects on Mrp3 than MeHg (170% of control), but both had similar effects on Mrp4 (225% of controls). HgS tended to increase the expression of Mrp3 (140% of control), but was not statistically significant; In general, Zuotai and HgS did not have significant effects on these efflux transporters (Figure 3, right panel).

Figure 3 The mRNA expression of Oatp uptake transporters and Mrp efflux transporters 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 $p < 0.05$.

4. Discussion

To examine the effects of xenobiotics on drug processing genes is an important strategy to predict metal-herb-drug interactions [24, 13, 14]. The present study compared the effects of mercury sulfides with HgCl_2 (equivalent Hg) and MeHg (1/10 of Hg) in drug metabolizing enzyme gene expression in livers of mice. Under the experimental conditions of the present study, Zuotai and HgS did not show significant effects on the expression of cytochrome P450 1-4 family genes, phase-2 conjugation enzyme genes, and transporters genes; HgCl_2 slightly increased $\text{PPAR}\alpha$ and Cyp4a10 , while MeHg increased Cyp2b10 . For phase-2 enzyme genes, HgCl_2 increased Ugt1a1 , Ugt1a6 , Gst-mu and Gst-pi , while MeHg increased Ugt1a9 , Ugt2a3 , Sult1b1 , and Gst-mu . For uptake transporters, HgCl_2 decreased oatp1a1 , but increased Oatp1a ; while both HgCl_2 and MeHg increased the efflux transporter Mrp1 , Mrp2 , Mrp3 , and Mrp4 . Thus, Zuotai and HgS differ from HgCl_2 and MeHg in affecting the expression of drug metabolism genes in livers of mice.

Cytochrome P450 is the major Phase-1 metabolic enzymes converting xenobiotics to more soluble forms for Phase-2 conjugation. AhR, CAR, PXR, and $\text{PPAR}\alpha$ are nuclear receptors regulating Cyp1a1 , Cyp2b10 , Cyp3a11 , and Cyp4a10 in mouse livers, respectively [24]. Parenteral administration of HgCl_2 is known to induce Cyp1a enzyme gene expression [16, 25], and in our prior studies, oral prolonged (1-2 months) administration of HgCl_2 can also induce Cyp gene expression in the liver [6, 26]. At the repeated higher doses, HgCl_2 could inhibit Cyp2e1 but had little effects on Cyp3a1 -mediated metabolism [16]. MeHg could also induce P450 genes [6]. Although both HgCl_2 and MeHg did not produce overt effects on hepatic cytochrome P450 gene

expression under current experimental conditions, this does not exclude the possibility that MeHg could affect P450 (either increase or decrease) at higher dose and longer time administration. Zuotai and HgS at the clinical dose seemed to have little influence on P450 expression.

Phase-2 conjugation is an important step after Phase-1 to conjugate xenobiotics with glucuronic acid, 3'-phosphoadenosine 5'-phosphosulfate (PAPS), or glutathione to facilitate their biliary excretion [24]. Little is known about the effects of mercury on Phase-2 conjugation metabolism. Consistent with induction of UDP-glucuronosyltransferase by HgCl₂ [16], we also observed effects of HgCl₂ on increasing Ugt1a1 and Ugt1a6 in the present study (Fig. 2). MeHg can also increase Ugt1a9 and Ugt2a3. Both HgCl₂ and MeHg increase the expression of Gst-mu and Gst-pi, possibly as a result of acute oxidative stress. The increase of the GST genes would facilitate biliary excretion of mercury-glutathione conjugates [27], probably as adaptive responses to eliminate mercury from hepatocytes. Zuotai and HgS at the dose investigated had no apparent effects on these Phase-2 enzyme gene expressions.

Transporters are important in producing herb–drug interactions [11, 28]. In the liver, the major uptake transporters are Oatp1a1, Oatp1a4, Oatp1b2, and Oatp2b1, and the major efflux transporters are Mrp1, Mrp2, Mrp3, Mrp4, and Bcrp [9, 29]. HgCl₂ inhibited Oatp1a1 but increased Oatp1a4. This is similar to the kidney, in which HgCl₂ decreased the renal uptake transporter Oat1 and Oat3, probably as an adaptive mechanism to reduce Hg²⁺ to get into the cells [30,31], and deletion of the uptake transporter could protect against HgCl₂ toxicity [32]. HgCl₂ and MeHg are known to affect Mrp2 and Bcrp transporters in the kidney [20, 21]. Mrp1 is also involved in efflux

of mercury from term placenta [33]. In our prior studies, HgCl_2 and MeHg increased Mrp2 or Mrp4 expression in the kidney [30, 31], while HgS had no effects. In agreement of prior observations on kidney transporters [30, 31], HgS had little effects on liver transporters in the present study. In general, HgCl_2 and MeHg could inhibit uptake transporters while increase efflux transporters as an adaptive responses to reduce cellular Hg burden, while HgS had little effects on transporters.

Zuotai is mainly composed of β -HgS, and is thought to play important roles in Tibetan medicine in the treatment of various diseases such as neuroinflammation, brain stroke, and diabetes [3, 23]. Mice were tolerant Zuotai at the clinical dose (6.7 mg/kg) daily for 4.5 months, with slight reversible kidney morphology alterations [23]. In rats given Zuotai at the dose of 3.8 – 12 mg/kg for 12 days, the mRNA expression of Cyp1a2 and NT2 was decreased [34]. However, in the present study in mice, 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 and P450 genes, fortifying the notion that β -HgS-containing Zuotai is relatively safe at clinical doses [7].

HgS more frequently used in Ayurvedic medicines [2] and traditional Chinese medicines [1]. Cinnabar is a naturally occurring mercuric sulfide and 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 [35]. HgS is quite different from HgCl_2 and MeHg, in regard to its toxicokinetics and toxicological effects [6, 8, 26]. We have previously shown that the hepatotoxicity of HgS is quite different from HgCl_2 and MeHg in mice after 44 days of administration [6]. 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 liver toxicity and drug processing genes were found, in agreement with the literature that the bioaccessibility of HgS is quite different from HgCl₂ [36, 37], resulting in much less toxicity potential [26, 38]. Compared to prior studies on the induction of Cyp1a, 3a, and 4a genes [26, 38], no apparent effects of cinnabar on cytochrome P450 is evident under the present experimental conditions (lower dose and shorter time). Indeed, when cinnabar is administered at the higher dose and longer-time period, liver toxicity does occur in rats (400-800 mg/kg for up to 90 days, 8-16-fold clinical dose) [39]. In another study in rats, liver toxicity occurred after given a large dose of cinnabar (1000 mg/kg) for 8-12 weeks [40]. Thus, caution should be taken when taken cinnabar at the higher dose for the longer time periods.

In summary, the present study demonstrated that Zuotai and HgS differ from HgCl₂ and MeHg in producing liver toxicity and the alterations on Phase-1, Phase-2, and Phase-3 drug processing genes. This study fortifies the notion that in the risk assessment of mercury-based traditional medicines, chemical forms matters.

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