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2 Mercury sulfides are much less nephrotoxic than mercury chloride and methylmercury in
3 mice

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13 Running Title: Nephrotoxicity of mercury sulfides differs from HgCl₂ and MeHg

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15 **Graphic abstract:**

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Abstract

Mercury sulfides (α -HgS, β -HgS) are frequently included in traditional medicines. Mercury is known for nephrotoxicity, their safety is of concern. To address this question, mice were orally administrated with Zuotai (mainly β -HgS, 30 mg/kg), α -HgS (HgS, 30 mg/kg), HgCl_2 (33.6 mg/kg), or MeHgCl (3.1 mg/kg) for 7 days, and nephrotoxicity was examined. Animal body weights were decreased by HgCl_2 and to a less extent by MeHg, but unaltered after Zuotai and HgS. HgCl_2 and MeHg produced renal tubular vacuolation, interstitial inflammation and cell degeneration with protein cysts in the tubular lumen, while these pathological lesions were mild in Zuotai and HgS-treated mice. Electron microscopy showed that HgCl_2 and MeHg produced spotted swelling endothelium reticulum, while these lesions were mild or absent in Zuotai and HgS-treated mice. Renal Hg contents reached 250-300 ng/mg kidney in HgCl_2 and MeHg groups as compared to 2-3 ng/mg in Zuotai and HgS groups. The expression of kidney injury biomarkers, kidney injury molecule-1 (Kim-1) and neutrophil gelatinase-associated lipocalin (Ngal), were increased after HgCl_2 and MeHg, but unaltered after Zuotai and HgS. The expression of renal influx transporters Oat3 and Oatp4c1 was decreased, while the expression of renal efflux transporter such as Mrp2, Mrp4, and Mate2 was increased following HgCl_2 and MeHg. These gene expressions were unchanged after Zuotai and HgS. In summary, both α -HgS and β -HgS are less nephrotoxic than HgCl_2 and MeHg, indicating that chemical forms of mercury are a major determinant of mercury disposition and toxicity.

47 **Keywords:** Mercury sulfides; Mercury chloride; Methylmercury; Nephrotoxicity;
48 Electromicroscopy; Renal Hg; Renal transporter; Kim-1; Oatp4c1; Mrp2.

49 **Abbreviations:**

50 Kim-1, Kidney injury molecule 1
51 Ngal, Neutrophil gelatinase-associated lipocalin
52 Oat1, Organic anion transporter 1
53 Oatp4c1, Organic transporting peptide 4c1
54 Mrp2, Multidrug resistance-associated protein 2
55 Mdr2, Multidrug resistance-associated protein 2
56 MeHg, Methylmercury

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Introduction

In traditional Indian Ayurvedic medicines, Chinese medicines and Tibetan medicines, mercury sulfides are frequently included in the herbo-metallic preparations in the treatment of various diseases for thousands of years. Nowadays, in Pharmacopeia of China (2015), over 30 recipes contain cinnabar (α -HgS); in Indian Ayurvedic medicine *Rasasindura* that is primarily composed of mercuric sulfide (α -HgS and metacinnabar, β -HgS), is included in over 20 recipes (Kamath et al., 2012); in Tibetan medicine, *Zuotai* that is mainly composed of β -HgS and is included in a dozen of Tibetan remedies (Kan, 2013; Li et al., 2015). Mercury sulfides-based traditional medicines are used for acute brain emergencies such as stroke, brain trauma, neuroinflammation, and high fever, but also used to treat chronic ailments like syphilis, pneumonia, insomnia, nervous disorders, paralysis of the tongue, and even used as a rejuvenator to improve strength, stamina and energy (Kamath et al., 2012; Kan, 2013; Li et al., 2015; Pharmacopeia of China, 2015). Mercury is a toxic heavy metal, and public concerns on the safety of these mercury-containing traditional medicines are increasing. For example, the allowable amounts of cinnabar in Chinese medicines have been dramatically decreased by as much as 65%, from a daily allowable dose of 0.3–1.5 g in 1977 to 0.1–0.5 g (Pharmacopoeia of China, 2015; Zhou et al., 2009). However, the total mercury contents in these traditional medicines are still thousands of folds higher than environmental exposure levels. An opposing opinion held that metal-containing traditional medicines, such as Ayurvedic medicines, are not necessarily toxic at safe therapeutic levels (Mao and Desai, 2009). It was strongly recommended that well-

designed studies are needed to address the true risk of the use of cinnabar-containing medicines (Kamath et al., 2012; Liu et al., 2008; Mao and Desai, 2009).

It should be noted that only mercury sulfides (α -HgS, β -HgS), but not mercury chloride (HgCl_2) and methylmercury (MeHg), that are included in traditional remedies, and their chemical structures are quite different (Figure 1). When discussing mercury toxicity, the chemical forms of mercury must be distinguished (Klaassen, 2006).

Figure 1. Mercury sulfides (cinnabar and metacinnabar) are structurally different from HgCl_2 and MeHg.

Kidney is the major target organ of mercury toxicity (Klaassen, 2006). We have previously shown that cinnabar (96% α -HgS) is different from HgCl_2 and MeHg in producing kidney injury in mice (Lu et al., 2011) and in rats (Shi et al., 2011). However, whether the same scenario applies to Zuotai that contains mainly β -HgS (Li et al., 2015) is not known. In addition, the differential effects of Zuotai and pure HgS from HgCl_2 , MeHg on renal transporters and renal Hg accumulation are not known. The goal of the current study was to compare the nephrotoxicity potentials of Zuotai (β -HgS) and pure form of mercury sulfide (α -HgS, HgS) with mercury chloride (HgCl_2) and organic

methylmercury (MeHg) in mice, focusing on renal mercury accumulation, ultrastructural changes, and gene expressions related to renal toxicity and transporters.

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 cinnabar (α -HgS), HgCl_2 and MeHgCl were from Sigma Chemical Company (St. Louis, MO). Other reagents were of reagent grade.

Adult Kunming outbred mice, 25 ± 2 g, male and female, 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 at least seven days prior to experiment. All the experiments were carried out in full compliance with the WHO Guidance of Humane Care and Use of Laboratory Animals, and approved by the Animal Care and Use 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 given Zuotai (mainly β -HgS, 30 mg/kg for 7days). A 5-fold of clinical dose of Zuotai ([Li et al., 2014](#)) was used to study nephrotoxicity. For comparison, α -HgS (HgS, 30 mg/kg), HgCl_2 (33.6 mg/kg, equivalent Hg as α -HgS), MeHg (3.1 mg/kg, 1/10 Hg of α -HgS), or distilled water (10 ml/kg) was gavaged daily for consecutive 7 days. Animals were closely monitored throughout the entire experiment period and body weights were

recorded daily. At the end of experiment, animal body weights and kidney weights were recorded and blood, kidneys were collected for further analysis.

2.3 Histological Evaluation

A portion of the kidney was placed in 10% neutral formalin. Fixed tissues were paraffin embedded, sectioned at 6 μm and stained with hematoxylin and eosin (H&E) and examined with Leica microscope. DP image software was used to capture images.

2.4. Ultrastructural analysis

The kidneys were quickly removed, and cut in small pieces (1x1x1 mm^3) on ice pad. The kidney tissues 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.

2.5 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 concentrations of blood urea nitrogen (BUN) and serum creatinine (CREA) were quantified to evaluate the potential nephrotoxicity with an Automatic Biochemical Analyzer at Zunyi Medical College Hospital as previously described ([Lu et al., 2011b](#)).

2.6 Determination of Hg in the kidney

A portion of kidney, weighing about 100 mg, 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, and then 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., 2011; Zhu et al., 2014; Sui et al., 2015).

2.7 RNA Isolation and Real-time PCR analysis

Approximately 50-100 mg of kidney 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 Supplemental Table 1. The expression of interested genes was first normalized with 18S of the same sample, and the relative transcript levels were calculated setting control as 100%.

Supplemental table 1. Primer sequence for real-time RT-PCR analysis

Gene	GenBank Number	Forward (5' - 3')	Reverse (5' - 3')
β -actin	M12481	GGCCAACCGTGAAAAGATGA	CAGCCTGGATGGCTACGTACA
Kim-1	AF035963	CACTCACTGCAGCGATTCTC	ATGCTCCCATGATTTTGCCC
Ngal	NM_008491	CTACAACCAAGTTCGCCATGG	ACA CTCA CCA CCA TTCA GT
Oat1	NM_008766	TGATGGCTGGGTCTATGACA	GCCAGGTAGCCAAACATCAT
Oat3	NM_031194	CCATTTCTTGAGAGCAAAGC	GCAACAATGCCTAGTGAGCA
Oatp4c1	NM_172658	TGTGCCCA CAC ACAA TCTT	TCCGA ACTTTGTCCCA TAGC
Mrp2	NM_013806	TCCTAGACAGCGGCAAGATT	GCTAGAGCTCCGTGTGGTTC
Mrp4	BC150822	GCAAAGCCCATGTACCATCT	ACCACGGCTAACAACTCACC
Mate2	NM_001033542	TGGCTGTGAGCTTGTCATTC	ACATGAGCTGCTCCTTTCGT

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.

3. Results

3.1 Animal body weight and kidney weight

Mice were orally administrated with Zuotai (30 mg/kg) daily for 7 days. Zuotai is mainly composed of β -HgS (Li et al., 2015) and is given at 5-fold of clinical dose (Li et al., 2014) in order to better study its nephrotoxic effects. 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. Fig. 2 top panel showed that the animal body weight

gain in Zuotai and HgS groups were similar to controls, with normal activity and general health conditions. In comparison, HgCl₂ significantly retarded the animal net body weight gain, and animal activity was apparently reduced, and the body weights were even lower than the beginning of the experiments. MeHg also decreased animal body weights gain, especially after 4 days of administration. Animal activity was also reduced, especially after 4 days.

Figure 2 Effect of Zuotai and mercury compounds on animal body weight and kidney index.

Mice were 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. Top: The net body weight gain; Bottom: the kidney index. Data are mean and SEM of 6-8 mice. *Significantly different from controls at $p < 0.05$.

At necropsy, the kidney weights in HgCl_2 and MeHg groups were increased, and when one normalized the kidney weight/body weights, the difference was even greater. The kidney weights in Zuotai and HgS groups were not different from controls (Fig. 2, bottom). Blood biochemistry showed that the concentrations of blood urea nitrogen (BUN, 6.2, 6.2, 6.3, 6.5 and 6.4 mmol/L for control, Zuotai, HgS, HgCl_2 , and MeHg, respectively) and serum creatinine (CREA, 10.2, 9.8, 9.9, 10.7, and 10.6 mmol/L for control, Zuotai, HgS, HgCl_2 , and MeHg, respectively) were not significantly different from controls.

3.2 Kidney Hg accumulation

To evaluate renal uptake and accumulation of mercury after orally administrated with Zuotai (30 mg/kg, α -HgS (30 mg/kg), HgCl_2 (33.6 mg/kg) and MeHg (3.1 mg/kg) for 7 days, the kidneys were removed, and a portion of kidneys was digested in nitric acid and kidney Hg contents were determined by Atomic Fluorescence Spectrometry as described in detail in Methods. Renal Hg contents in controls was 0.52 ± 0.06 , in Zuotai groups was 0.67 ± 0.08 , in HgS group was 1.08 ± 0.19 ng/mg kidney; while it reached 320 ± 25 in HgCl_2 group and 250 ± 14 ng/mg kidney in MeHg group. The differences were dramatic.

Figure 3 Renal 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 $p < 0.05$.

3.3. Histopathological alterations

Representative H&E microphotos are showed in Figure 4. In kidneys of mice orally administrated with Zuotai (30 mg/kg) and α -HgS (30 mg/kg) for 7 days, the morphology of the kidneys was not different from controls; only spotted cell swellings can be seen. In comparison, in kidneys of mice orally dosed HgCl₂ (33.6 mg/kg) for 7 days tubular cellular vacuolation and degeneration lesions were evident, with inflammatory cell infiltration. Similarly, in kidneys of mice dosed with MeHg (3.1 mg/kg) for 7 days, the renal tubular cell degeneration, apoptosis and spot necrosis were evident. Cell degeneration with protein cysts in the tubular lumen was evident (thick arrows) and spotted inflammation (thin arrows) could be seen.

Figure 2 Histopathological examinations with representative photos from kidneys 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. Arrows indicate tubular degeneration, and arrowheads indicate tubular cell death. Magnitude 200X.

3.4. Ultrastructural alterations

Representative electronic scanning images are showed in Figure 3. In livers of mice orally administrated with Zuotai (30 mg/kg) and a-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) for 7 days, spotted swelling endothelium reticulum were observed. Similarly, in livers of mice dosed with MeHg (4.6 mg/kg) for 7 days, the disturbance of ultrastructure can be seen.

Figure 5 Ultrastructural examinations kidneys 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. Pathological lesions were more evident in HgCl₂ and MeHg-treated mice. Magnitude 10000X.

3.5 Expression of genes sensitive to toxicity

To further examine the gene expression related to renal injury, the expression of the biomarker gene Kim-1 (Kidney injury molecule-1) ([Shi et al., 2011](#)) and Ngai (neutrophil gelatinase-associated lipocalin) ([Bridges et al., 2014](#)) was examined. Orally administration of Zuotai (30 mg/kg) and HgS (30 mg/kg) did not show significant elevations in Kim-1 (0.555% of β -actin for control, 0.567% of β -actin for Zuotai and 0.831% of β -actin for HgS). In comparison, in kidneys dosed with HgCl₂ (33.6 mg/kg)

238 and MeHg (3.1 mg/kg) for 7 days, the expression of Kim-1 was increased about 10-fold,
 239 indicative of renal injury.

240
 241 **Figure 6** Expression of Kidney injury molecule-1 (Kim1, top) and Neutrophil gelatinase-
 242 associated lipocalin (Ngal, bottom) in kidneys of mice orally administrated with Zuotai (30
 243 mg/kg), HgS (30 mg/kg), HgCl₂ (33.6 mg/kg), and MeHg (3.1 mg/kg) for 7 days. Data are mean
 244 and SEM of 6-8 mice. *Significantly different from controls at p < 0.05.

245 Ngal is a novel biomarker of kidney injury ([Bridges et al., 2014](#)). The expression of the
 246 biomarker gene Ngal was basically unchanged in kidneys of mice give orally Zuotai (30
 247 mg/kg,) and HgS (30 mg/kg) (0.113% β -actin for control, 0.121% for Zuotai and 0.183%
 248 for HgS). In comparison, in kidneys dosed with HgCl₂ (33.6 mg/kg) and MeHg (3.1

249 mg/kg) for 7 days, the expression of Ngai was increased 8- and 5-fold, respectively,
250 indicative of renal injury, and more increases were evident in HgCl₂ group.

251 **3.6 Expression of renal uptake transporters**

252 Kidney Oat1 and Oat3 are important for renal uptake of drugs and toxicants ([Nigam et](#)
253 [al., 2015](#)), especially for HgCl₂ ([DiGiusto et al., 2009](#)). Expression of kidney uptake
254 transporters Organic anion transporters Oat1 and Oat3, and organic transporting
255 peptide Oatp4c1 are shown in Figure 7. Zuotai (30 mg/kg) and HgS (30 mg/kg) had no
256 apparent effects on the expression of these transporter genes, while HgCl₂ (33.6 mg/kg)
257 and MeHg (3.1 mg/kg) decreased the expression of Oatp4c1 by 50%, and MeHg also
258 decreased the expression of Oat3 by 50%. HgCl₂ and MeHg under the current
259 experimental conditions did not show significant suppression on Oat1, even though the
260 expression of Oat1 was reduced 25-35%.

Figure 7 Expression of Kidney uptake transporters Mrp2, Oat1, and Oatp4c1 in kidneys 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.

3.7 Expression of renal efflux transporters

The renal efflux transporters are important for renal elimination of toxicants (Klaassen and Aleksunes, 2010). Expression of kidney efflux transporters multidrug resistance-

associated protein Mrp2, Mrp4 and Multidrug resistance-associated protein 2 (Mate2) are shown in Figure 8. Zuotai (30 mg/kg) and HgS (30 mg/kg) had no apparent effects on the expression of these transporter genes, while HgCl₂ (33.6 mg/kg) and MeHg (3.1 mg/kg) increased the expression of Mrp2 (4-fold), Mrp4 (2-fold), and Mate2 (2-3 fold), indicating an adaptive mechanism to eliminate Hg from the kidneys.

Figure 8. Expression of Kidney efflux transporters Mrp2, Mrp4, and Mate2 in kidneys of mice are shown in Figure 8. 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 $p < 0.05$.

Discussion

The present study clearly demonstrated the nephrotoxicity produced by mercury sulfides is different from HgCl_2 (equal Hg as HgS) and MeHg (1/10 Hg as HgS). HgCl_2 and MeHg retarded animal body weight gain and increased the kidney weights, while Zuotai and HgS did not affect the general health; HgCl_2 and MeHg produced histopathological changes in the kidney, while these lesions were mild or absent in Zuotai and HgS group; HgCl_2 and MeHg produced over 100-fold Hg accumulation in the kidney, while Zuotai and HgS only increased renal Hg content 2-3 fold; HgCl_2 and MeHg induced renal toxicity gene Kim-1 and Ngal expression, while Zuotai and HgS did not; HgCl_2 and MeHg decreased the expression of renal uptake transporter Oat3 and Oatp4c1 and increased the efflux transporter Mrp2. Mrp4 and Mate2, while these transporters were largely unaltered following Zuotai and HgS treatment at equivalent Hg doses. Thus, chemical forms of mercurial compounds determine their tissue accumulation and toxicity in kidneys 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 ([Kan, 2013](#); [Huang et al., 2013](#)). Recent studies using energy dispersive spectrometry of X-ray (EDX), X-ray fluorescence spectroscopy (XRF), synchrotron radiation X-ray absorption fine structure (SR-XAFS), X-ray diffraction (XRD), scanning

electron microscope (SEM) and atomic force microscope (AFM) revealed that the main elements in Zuotai are Hg and S. XRD assay found that β -HgS are the main phase compositions in Zuotai (Li et al., 2015). Zuotai is also 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). Higher mercury dissolutions of Zuotai were observed in artificial gastrointestinal fluid, followed by β -HgS, cinnabar and α -HgS (Zheng et al., 2015). The mice could tolerant Zuotai at a single dose of 80 g/kg, after the repeated subacute doses (13-2000 mg/kg), or the daily clinical dose (6.7 mg/kg) for 4.5 months, with only mild reversible kidney morphology alterations (Li et al., 2014). In another study, rats were orally given Zuotai at daily doses of 17-67 mg/kg for 6 months, and kidney Kim-1 and metallothionein-1 (MT-1) were increased, but returned to normal levels 30 days after discontinuation of Zuotai, suggesting that Zuotai at the high dose (10 times the clinical dose) for 6 months could produce reversible kidney injury (Xiang et al., 2014). Our recent study also demonstrated that Zuotai and HgS are much less toxic to the liver as compared to HgCl_2 and MeHg in mice (Wu et al., 2016). In the patients taken Zuotai-containing Tibetan medicine Danzuo for one month, no apparent abnormality of serology was evident (Li 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, renal Hg accumulation, and gene expressions related to renal toxicity and transporters. Zuotai and HgS had little effects on phase-1, phase-2, and transporter genes in the livers of mice (Xu et al., 2015). Taken together, these results fortify the notion that β -HgS-containing Zuotai is relatively safe at clinical doses.

Cinnabar, mainly composed of α -HgS, is included in Ayurvedic medicines (Ramath et al., 2012) and traditional Chinese medicines (Pharmacopia of China, 2015). Cinnabar has been used in traditional Chinese medicine as a sedative and soporific agent for more than 2000 years (Chen et al., 2012; Zhou et al., 2009). HgS is quite different from HgCl_2 and MeHg, in regard to its toxicokinetics and toxicological effects (Liu et al., 2008). We have previously shown that the kidney toxicity of HgS is much less than environmental mercurials in mice (300 mg/kg, po for 6 weeks) (Lu et al., 2011, and in rats (200 mg/kg, po for 60 days) (Shi et al., 2011). 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 renal toxicity and transporters were found, in agreement with the literature about the bioaccessibility of HgS (Koch et al., 2013; Wang et al., 2013; Tinggi et al., 2016) and our recent studies on cinnabar-containing traditional medicines on renal transporters (Zhu et al., 2014; Sui et al., 2015). However, it should be noted that the higher dose and longer-time of cinnabar administration, renal toxicity did occur (400-800 mg/kg, po for 90 days in rats) (Liang et al., 2009). In another study, kidney toxicity is evident after cinnabar at a dose of 1000 mg/kg, po for 8-12 weeks (Wang et al., 2015), and at the higher dose of cinnabar, renal efflux transporter Oat1 and Oat3 could be decreased (Yu et al., 2015). Thus, caution should be taken when taken cinnabar at the higher dose for the long-term.

HgCl_2 is a potent nephrotoxicant and a very concerned environmental pollutant (Liu et al., 2008). In contrast to Zuotai and HgS, HgCl_2 is highly nephrotoxic at the equivalent Hg content in the present study, as manifested by decreased body weight, histopathological findings, and gene expressions. The expression of sensitive

biomarkers for kidney injury, such as Kim-1 and Ngal ([Bridges et al., 2014](#)) was significantly increased, despite no overt elevations in serum biomarkers BUN and creatinine. In addition to toxicity-related gene expressions, the alteration of renal transporters could be another sensitive biomarker for kidney injury, such as the decreased expression of Oat3 and Oatp4c1 and increased expression of Mrp2, Mrp4, and Mate2 (Fig. 7 and 8), and the effects of HgCl₂ on these transporters such as Oat1, Oat3 ([Lash et al., 2005](#); [Di Giusto et al., 2009](#); [Zhu et al., 2014](#); [Sui et al., 2015](#)) and Mrp2 ([Bridges et al., 2013](#); [Zalpus et al., 2014](#)) are also in agreement with the literature. This is consistent with the mercury bioaccumulation in mice following different mercurials, in that HgCl₂ was the highest, while β-HgS, α-HgS, and cinnabar were very low ([Zheng et al., 2015](#)). Thus, the differential mercury disposition could affect their tissue accumulation and toxicity.

MeHg mainly targets on the brain, but it can also reach the kidney, portion of the administered MeHg can be converted to HgCl₂ in the gut, and then go to kidney to produce nephrotoxicity ([Klaassen, 2006](#); [Liu et al., 2008](#)). MeHg is highly toxic among mercury compounds and only 1/10 of equivalent Hg dose of MeHg (3.1 mg/kg) was used in the present study, and the renal injury was evident in nearly all parameters examined. In MeHg-treated animals, severe renal glomeruli and tubule damages and interstitial inflammation are evident, consistent with the literature ([Eto et al., 1997](#)). Another novel findings of the present study are the ultrastructural changes after MeHg at this dose, and mitochondria swollen can be seen in MeHg group only, together with endoplasmic reticulum swollen, which can also be seen with HgCl₂, but to a lesser extent with HgS and Zuotai. In our previous study, MeHg was even more nephrotoxic

during chronic exposures in mice (Lu et al., 2011, 1.4 mg/kg, 1/100 Hg of cinnabar) and in rats (Shi et al., 2011, 1 mg/kg), producing even pronounced renal injury. Compared to HgCl₂, MeHg-induced quite different gene expression profiled in C-elegans (McElwee et al., 2013), but little is known about MeHg-induced renal gene expression changes related to transporters and renal toxicity, the current study demonstrated the suppression of MeHg on renal uptake transporters and upregulation of efflux transporters, adding to our understanding of molecular effects of MeHg.

In summary, the present study demonstrated the differential toxicity of Zuotai, HgS, HgCl₂, and MeHg in producing kidney toxicity and related gene expressions. Zuotai and cinnabar used in traditional medicines have much lower toxicity potential as compared to HgCl₂ and MeHg, producing much less renal mercury accumulation and alterations in Hg handling transporter expressions. As a result, Zuotai and cinnabar at clinical doses produce mild and reversible pathological changes and renal toxicity-related gene expression. The current study adds to our knowledge on the risk assessment of mercury-based traditional medicines, chemical form matters.

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Conflict of interest

The authors do not have conflict of interest.

Figure legends:

Figure 1. Zuotai and cinnabar are structurally different from HgCl_2 and MeHg.

Figure 2 Effect of Zuotai and mercury compounds on animal body weight and kidney weight. Mice were orally administrated with Zuotai (30 mg/kg), HgS (30 mg/kg), HgCl_2 (33.6 mg/kg), and MeHg (3.1 mg/kg) for 7 days. Top: The net body weight gain; the bottom the kidney weights. Data are mean and SEM of 6-8 mice. *Significantly different from controls at $p < 0.05$.

Figure 3 Renal Hg contents in mice after orally administrated with Zuotai (30 mg/kg), HgS (30 mg/kg), HgCl_2 (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 4 Histopathological examinations with representative photos from kidneys of mice orally administrated with Zuotai (30 mg/kg), HgS (30 mg/kg), HgCl_2 (33.6 mg/kg), and MeHg (3.1 mg/kg) for 7 days. Arrows indicate tubular degeneration, and arrowheads indicate tubular cell death. Magnitude 200X.

Figure 5 Ultrastructural examinations kidneys of mice orally administrated with Zuotai (30 mg/kg), HgS (30 mg/kg), HgCl_2 (33.6 mg/kg), and MeHg (3.1 mg/kg) for 7 days. Mitochondria and Endoplasmic reticulum swollen can be seen in MeHg group, followed by HgCl_2 . These alterations are mild in HgS and Zuotai groups. Magnitude 10000X.

Figure 6 Expression of Kidney injury molecule-1 (Kim1, top) and Neutrophil gelatinase-associated lipocalin (Ngal, bottom) in kidneys of mice orally administrated with Zuotai

409 (30 mg/kg), HgS (30 mg/kg), HgCl₂ (33.6 mg/kg), and MeHg (3.1 mg/kg) for 7 days. .
410 Data are mean and SEM of 6-8 mice. *Significantly different from controls at p < 0.05.

411 **Figure 7** Expression of Kidney uptake transporters Mrp2, Oat1, and Oatp4c1 in
412 kidneys of mice orally administrated with Zuotai (30 mg/kg), HgS (30 mg/kg), HgCl₂
413 (33.6 mg/kg), and MeHg (3.1 mg/kg) for 7 days. . Data are mean and SEM of 6-8 mice.
414 *Significantly different from controls at p < 0.05.

415 **Figure 8.** Expression of Kidney efflux transporters Mrp2, Mrp4, and Mate2 in kidneys of
416 mice are shown in Figure 8. Orally administrated with Zuotai (30 mg/kg), HgS (30
417 mg/kg), HgCl₂ (33.6 mg/kg), and MeHg (3.1 mg/kg) for 7 days. . Data are mean and
418 SEM of 6-8 mice. *Significantly different from controls at p < 0.05.

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