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Zuotai and HgS differ from $HgCl_2$ and methyl mercury in Hg accumulation and toxicity in weanling and aged rats

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ABSTRACT

Mercury sulfides are used in Ayurvedic medicines, Tibetan medicines, and Chinese medicines for thousands of years and are still used today. Cinnabar (α-HgS) and metacinnabar (β-HgS) are different from mercury chloride ($HgCl_2$) and methylmercury (MeHg) in their disposition and toxicity. Whether such scenario applies to weanling and aged animals is not known. To address this question, weanling (21 d) and aged (450 d) rats were orally given Zuotai (54% β-HgS, 30 mg/kg), HgS (α-HgS, 30 mg/kg), $HgCl_2$ (34.6 mg/kg), or MeHg (MeHgCl, 3.2 mg/kg) for 7 days. Accumulation of Hg in kidney and liver, and the toxicity-sensitive gene expressions were examined. Animal body weight gain was decreased by $HgCl_2$ and to a lesser extent by MeHg, but unaltered after Zuotai and HgS. $HgCl_2$ and MeHg produced dramatic tissue Hg accumulation, increased kidney (kim-1 and Ngal) and liver (Ho-1) injury-sensitive gene expressions, but such changes are absent or mild after Zuotai and HgS. Aged rats were more susceptible than weanling rats to Hg toxicity. To examine roles of transporters in Hg accumulation, transporter gene expressions were examined. The expression of renal uptake transporters Oat1, Oct2, and Oatp4c1 and hepatic Oatp2 was decreased, while the expression of renal efflux transporter Mrp2, Mrp4 and Mdr1b was increased following $HgCl_2$ and MeHg, but unaffected by Zuotai and HgS. Thus, Zuotai and HgS differ from $HgCl_2$ and MeHg in producing tissue Hg accumulation and toxicity, and aged rats are more susceptible than weanling rats. Transporter expression could be adaptive means to reduce tissue Hg burden.

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1. Introduction

Metals (minerals) have been used in traditional medicines since ancient times, and are still in use today. In Ayurveda, 8% of the recipes contain 15 kinds of minerals (Joshi et al., 2017); In the 2015 Edition of Pharmacopoeia of China, 10 kinds of minerals are listed, including the most concerned cinnabar (HgS) and realgar (As_4S_4) (Pharmacopoeia of China, 2015). In Siddha Medicine used for diabetes, nearly half of the preparations contain inorganics, including cinnabar (Sathasivampillai et al., 2017). Metals in traditional medicine mixtures are of increasing public concern, and the existence of metals in traditional remedies is a reality challenging pharmacologists and toxicologists worldwide.

Mercury-based traditional medicines received the most concern. "Rasasindura" that is primarily composed of mercuric sulfide (HgS), has been used in Indian Ayurvedic medicines for treatment of chronic ailments like syphilis, high fever, pneumonia, insomnia, nervous disorders, and paralysis of the tongue (Kamath et al., 2012). In Tibetan and Mongolia medicines, Zuotai (mainly β-HgS) are frequently included in the herbo-metallic preparations for the treatment of stroke, brain trauma, neuroinflammation, and chronic ailments (Kan, 2013), and S-HgS is the basis of Mongolia traditional medicine Vermilion (Chen et al., 2012). Cinnabar (α-HgS) is a main "sedative drug" included in approximately 50 traditional Chinese medicine recipes (Pharmacopoeia of China, 2015).

Mercury sulfides present two isoforms: cinnabar is red in color (α-HgS), and is subjected to repeated grinding and washing (called Shui-Fei) for at least 3–4 times to remove impurities and this procedure is very important for the safe use of cinnabar in medication (Liu et al., 2008; Zhou et al., 2009; Pharmacopoeia of China, 2015); Zuotai is black in color (mainly β-HgS, also called metacinnabar) is also undergone the tedious processing procedures like Bhasmas in Ayurvedic medicine

Abbreviations: Mrp, Multidrug resistance-associated protein; Mdr1b, multidrug resistance gene 1b; OAT, organic anion transporter; OATP, organic anion-transporting polypeptides; OCT, organic cation transporters.

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(Kamath et al., 2012), which usually takes months of repeated incineration and treatment procedures using herbals and animal-based products (Chen et al., 2012; Kan, 2013). Both HgS and Zuotai are the major components in many famous Chinese medicines (Pharmacopoeia of China, 2015) and Tibetan medicines (Huang et al., 2013).

Cinnabar (α-HgS) and metacinnabar (β-HgS) are structurally different from mercury chloride ($HgCl_2$) and methylmercury (MeHg), and are the only chemical form of mercury used in oral traditional remedies. When discussing mercury toxicity, three mercury forms (elementary mercury, inorganic mercury, and organic mercury) must be distinguished, as chemical forms of mercury are a major determinant of their disposition and toxicity (Klaassen, 2006). We have recently shown that in young adult animals Zuotai and HgS are quite different from $HgCl_2$ and MeHg in Hg accumulation and toxicity in the liver (Wu et al., 2016) and kidneys (Liu et al., 2016). However, whether such scenario applies to weanling animals and aged animals is not known, and is the primary goal of the present study.

Children and elderly are sensitive to metal toxicology. During development, many physiological functions do not mature, and heavy metal poisoning, particular mercury, in children has become an increasingly serious health problem in the world (Daston et al., 1983, 1984, 1986; Rong et al., 2014). Aging often results in progressive losses of body defense mechanisms (Bridges et al., 2014). We have recently shown the age-related changes in 12 kidney transporters (Xu et al., 2017), and the major hepatic uptake transporters (Hou et al., 2014) and efflux transporters (Zhu et al., 2017), and whether age-related changes in transporters would be responsive for Hg toxicity is the second goal of this study.

The present study was designed to (1) compare the relative sensitivity of weanling rats and aged rats to Zuotai, HgS, $HgCl_2$ (at equivalent Hg amount as HgS), and MeHg (at 1/10 equivalent Hg amount as HgS) toxicity, focusing on toxicity-sensitive gene expressions; (2) to examine the major kidney and liver transporter gene expressions in response to the mercurial challenge.

2. Materials and methods

2.1. Chemicals

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

2.2. Animals

Adult Sprague Dawley (SD) rats (250–300 g) were purchased from the Experimental Animal Center of Third Military Medical University (Chongqing, China). Rats were maintained in SPF-grade animal facilities (Certificate No. SYXK 2011–004) at Zunyi Medical College, with controlled environment (22 ± 1 °C, 50 ± 2% humidity and a 12 h: 12 h light: dark cycle) and free access to purified water and standard laboratory chow. All animal care and experimental protocols are complied with the Animal Management Guidelines of the Chinese Ministry of Health and approve by Animal Use and Care Committee of Zunyi Medical College (2013–05). Rats were acclimatized for one week before timely mating overnight and a positive vaginal plug next morning was considered as Gestation Day 1. At the 21 days of age, rats were weaned, and divided into groups for the treatment (called weanling rats). Another set of 15-month old SD rats were used to assess effects of age on animal response to mercury (called aged rats).

2.3. Exposure of animals to Hg Compounds

The weanling rats (6/group) and the aged rats (5/group) were orally given distilled water (Control), Zuotai (30 mg/kg, about 5-fold of clinical dose, Li et al., 2014), the same dose was used for HgS (α-HgS, 30 mg/kg), $HgCl_2$ (34.6 mg/kg, equivalent Hg as HgS), and MeHg (MeHgCl, 3.2 mg/kg, 1/10 Hg of HgS), daily for consecutive 7 days and body weights were recorded daily. One hour after the last dose, rats were anesthetized with 7% chloral hydrate, and animal body weights and organ weights were recorded. Kidneys and liver samples were collected, snap frozen, and stored at −80 °C for future analysis.

2.4. Determination of Hg in the kidneys and liver

A portion of tissue, weighing 50–100 mg, was digested in 1 ml nitric acid at 60 °C for 72 h, and add distilled water to make dilution of 2–50 fold depending on Hg concentration. Hg contents were determined with cold-atom absorbed method using a F732-VJ cold vapor mercury detector (Huaguang Instrument Factory, Shanghai, China).

2.5. Real-time PCR

Approximately 50–100 mg of tissues was homogenized in 1 ml TRIzol (TakaRa Biotechnology, Dalian, China) and total RNA was extracted according to manufacturer's instructions. The quality and quantity of RNA were determined by the Nano Drop (Thermo Scientific, ND-2000, USA), with 260/280 ratio (>1.8). Total RNA was reverse transcribed with a High Capacity Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA, USA). The primers were designed with Primer3 software and listed (Supplemental Table 1). The 15 μl PCR reaction mix contained 3 μl of cDNA (10 ng/μl), 7.5 μl of iQ™ 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 $2^{-\Delta\Delta}$Ct-method and normalized to the house keeping gene β-actin and expressed as % of controls.

2.6. Statistical analysis

Data were expressed as mean and standard error. The SPSS 16 software was used for statistical analysis. Data were analyzed using a one-way analysis of variance (ANOVA), followed by Duncan's multiple range test. p value < 0.05 was considered statistically significant.

3. Result

3.1. Animal general health

Weanling (21d) and aged (450d) rats were orally given mercury compounds daily for 7 days. At the dose used in the present study, no mortality occurred. Tibetan medicine Zuotai and HgS did not affect the general health conditions, had no evident effects on weanling rat body weight gain, but slightly decreased aged rat body weight (~10 g of 650 rats), which was not significant. In contract, $HgCl_2$ at the equivalent Hg dose of HgS retarded the body weight gain in the weanling rats, and reduced the body weight of the aged rats for >6% (40 g of 650 g rats), and animal hair was not smooth. MeHg at 1/10 of the HgS dose also retarded the weanling rat growth, and reduced the body weight of aged rats by 2.5% (16 g of 650 g rats), but was not significantly different from controls (Fig. 1).

3.2. Accumulation of Hg in kidneys and liver

The accumulation of Hg in kidneys and liver of weanling and aged rats is shown in Fig. 2. There was no significant difference in Hg content between Zuotai, HgS and control groups. Zuotai- and HgS-treatment resulted in 2–3 fold increases in tissue Hg content over controls. In contract, Hg content in kidneys of $HgCl_2$ (250-fold in weanling rats, 285-

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