The effects of Tibetan medicine Zuotai and β-HgS on cytotoxicity and endoplasmic reticular stress-related genes expressions differentiate from HgCl₂ in PC-12 cells

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Background. Mercuric sulfide (HgS) has been used in traditional medicines for thousands of years, such as cinnabar, Zuotai and so on. Increasing research supports that HgS is different from mercury chloride (HgCl₂) and methylmercury (MeHg) in toxicity and pharmacology, due to the differences in chemical forms. However, the differences of the cytotoxicity and endoplasmic reticulum stress (ERs) among Zuotai, β-HgS and HgCl₂ is still unclear. Methods. This study was designed to investigate the differential effects of Zuotai (0.250 g/L, 0.116 g Hg/L), β-HgS (0.135 g/L, 0.116 g Hg/L) and HgCl₂ (0.0160 g/L, 0.0116 g Hg/L) on cell cytotoxicity and ERs. In this study, Cell viability, cellular morphology and the expressions of caspase family genes, ERs-related genes and metal sensitive genes were examined in PC-12 cells exposed with or without drugs. Results. Cell viability in Zuotai and β-HgS groups were higher than HgCl₂ group (p<0.05), and the cell viability were 94.79 ± 1.958%, 99.27 ± 1.328% and 65.82 ± 3.415% in Zuotai, β-HgS and HgCl₂ groups, respectively. Zuotai reduced the adhesion of cells slightly, β-HgS did not change cellular morphology, and HgCl₂ produced cell shrinkage and rounded up. Thus, Zuotai and β-HgS produced less cytotoxicity than HgCl₂. To examine the signaling pathways activated by Zuotai, β-HgS and HgCl₂, caspase family genes, ERs-related genes and metal sensitive genes expressions were examined. Zuotai decreased the expressions of Cas3, Cas8 and Cas9 and upregulated the expression of Cas12, while β-HgS decreased the caspase family genes (Cas3, Cas8, Cas9 and Cas12) expressions. Meanwhile, all three drugs upregulated the expressions of ERs-related genes (GRP78, Gadd45a and Gadd45b) and metal sensitive genes (MT-1 and HO-1). Conclusion. Zuotai and β-HgS had less cytotoxicity than HgCl₂. The cell viability of Zuotai and β-HgS were significantly higher than HgCl₂, and Zuotai and β-HgS inhibited caspase activity while HgCl₂ produced cell death through the caspase independent pathway. Zuotai, β-HgS and HgCl₂ triggered ERs and metal sensitive genes.
expressions. Taken together, Zuotai and β-HgS were different from HgCl₂ in cellular toxicity, and the effects of Zuotai and β-HgS at a high dose were both protective and destructive.
The effects of Tibetan medicine Zuotai and β-HgS on cytotoxicity and endoplasmic reticular stress-related genes expressions differentiate from HgCl\(_2\) in PC-12 cells

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Abstract:

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Methods. This study was designed to investigate the differential effects of Zuotai (0.250 g/L, 0.116 g Hg/L), β-HgS (0.135 g/L, 0.116 g Hg/L) and HgCl₂ (0.0160 g/L, 0.0116 g Hg/L) on cell cytotoxicity and ERs. In this study, Cell viability, cellular morphology and the expressions of caspase family genes, ERs-related genes and metal sensitive genes were examined in PC-12 cells exposed with or without drugs.

Results. Cell viability in Zuotai and β-HgS groups were higher than HgCl₂ group (p<0.05), and the cell viability were 94.79 ± 1.958%, 99.27 ± 1.328% and 65.82 ± 3.415% in Zuotai, β-HgS and HgCl₂ groups, respectively. Zuotai reduced the adhesion of cells slightly, β-HgS did not change cellular morphology, and HgCl₂ produced cell shrinkage and rounded up. Thus, Zuotai and β-HgS produced less cytotoxicity than HgCl₂. To examine the signaling pathways activated by Zuotai, β-HgS and HgCl₂, caspase family genes, ERs-related genes and metal sensitive genes expressions were examined. Zuotai decreased the expressions of Cas3, Cas8 and Cas9 and upregulated the expression of Cas12, while β-HgS decreased the caspase family genes (Cas3, Cas8 Cas9 and Cas12) expressions. Meanwhile, all three drugs upregulated the expressions of ERs-related genes (GRP78, Gadd45a and Gadd45b) and metal sensitive genes (MT-1 and HO-1).

Conclusion. Zuotai and β-HgS had less cytotoxicity than HgCl₂. The cell viability of Zuotai and β-HgS were significantly higher than HgCl₂, and Zuotai and β-HgS inhibited caspase activity while HgCl₂ produced cell death through the caspase independent pathway. Zuotai, β-HgS and HgCl₂ triggered ERs and metal sensitive genes expressions. Taken together, Zuotai and β-HgS were different from HgCl₂ in cellular toxicity, and the effects of Zuotai and β-HgS at a high dose were both protective and destructive.
Key words: cytotoxicity, caspase family genes, endoplasmic reticular stress, Tibetan medicine
Zuotai, mercuric compounds.
1. Introduction

Mercurial sulfide (HgS) has been used in traditional Chinese medicine (Liu et al. 2008; Wang et al. 2007; Zhang et al. 2012), Tibetan medicine (Chen et al. 2012; Li et al. 2016) and Indian Ayurvedic medicine (Ernst 2002; Kamath et al. 2012) for thousands of years, and claimed to have therapeutic effects in these remedies. In Pharmacopeia of China (2015), there are 46 recipes (6.7%) containing HgS (cinnabar or Zuotai) (Chinese pharmacopoeia Commission 2015). An-Gong-Niu-Huang-Wan, Wan-Sheng-Hua-Feng-Dan, 70Wei Zhen-Zhu-Wan, Gui-Jiu-Wan, etc. were the famous and effective mercury-based medicines. HgS played an important role in the therapeutic effects of the mercury-based medicines, while removed or reduced HgS from medicines would reduce the therapeutic efficacy (Zhang et al. 2012; Zhu et al. 2007; Zhu et al. 2008).

Zuotai (about 54.5% β-HgS) (Wang et al. 2010; Zheng et al. 2015), a famous Tibetan medicine, was used in the majority of famous Tibetan medicines as a core component (Huang et al. 2013; Kan 2013; Li et al. 2014), and claimed to enhance the effects and decrease the toxicity of various prescriptions, including 70Wei Zhen-Zhu-Wan (for cardiovascular and cerebrovascular diseases), Ren-Qing-Chang-Jue (for intestines and stomach diseases), Er-Shi-Wu-Wei-Song-Shi-Wan (for liver disease), Gui-Jiu-Wan (for gynecological diseases), and so on. In addition, Zuotai was a nanoparticle drug, and its particle size was mainly in the range of 100-600 nm, even less than 100 nm, which was commonly further aggregated into 10 to 30 μm loose amorphous particles (Li et al. 2015). But the molecular mechanism of biology effect of Zuotai was little known.

HgS was structurally different from HgCl\textsubscript{2} and MeHg, and was the only chemical form of mercury used in oral traditional remedies. Increasing research supports that the chemical forms of mercury were the major determinant of mercury toxicity and therapeutic effects in traditional medicines (Liu et al. 2008; Wu et al. 2011). Compared to HgCl\textsubscript{2} or MeHg, HgS or HgS-containing traditional medicine (cinnabar, Zuotai, etc.) had lower toxic in acute toxicity (He et al. 2011; Li et al. 2014), chronic toxicity (Li et al. 2014; Zhu et al. 2013), developmental toxicity
(Dong et al. 2016; Huang et al. 2012), hepatotoxicity (Wu et al. 2016), nephrotoxicity (Lu et al. 2011; Shi et al. 2011), neurotoxicity (Chuu et al. 2001; Chuu et al. 2007), ototoxicity (Huang et al. 2008), and so on. The effects of HgS and HgCl$_2$ were also different in other aspects, such as in biochemistry and histological evaluation, mercury accumulation, mercury toxicity-related genes expressions et al. But whether the cascade reaction of caspases and endoplasmic reticulum stress (ERs) could trigger by mercuric or mercury based medicine was unclear.

Caspases were a family of genes and had important roles in maintaining homeostasis through regulating cell death and inflammation (McIlwain et al. 2013; Thornberry 1998). Accordingly, caspases had been broadly categorized by their known roles, such as apoptotic (Cas3, Cas6, Cas7, Cas8 and Cas9 in mammals), inflammatory (Cas1, Cas4, Cas5 and Cas12 in humans and Cas1, Cas11 and Cas12 in mice), and less easily classified roles (Cas2, Cas4 and Cas14). Briefly, Caspases involved in apoptosis had been sub-classified by their mechanism of action and were initiator caspases (Cas8 and Cas9) or executioner caspases (Cas3, Cas6 and Cas7). Cas12 was an ER resident caspase, the main caspase associated with the ER apoptosis pathway. Activation of apoptotic caspases results the generation of a cascade of signaling events permitting programmed cell death (Mehmet 2000; Nakagawa et al. 2000).

ERs had been implicated in several diseases, and multiple pathways linking ERs to cell death have been reported (Araki et al. 2003; Katayama et al. 2001; Ryu et al. 2002). The endoplasmic reticulum (ER) was an organelle that had essential roles in multiple cellular processes that were required for cell survival and normal cellular functions. ERs, represented a cellular stress induced by multiple stimuli and pathological conditions, triggered an evolutionarily conserved series of signal transduction events which could induce cell death when these events were severe or protracted. But whether ERs took a role in cell death induced by mercuric was not known.

This study investigated the effect of Zuotai (0.250 g/L), β-HgS (0.135 g/L) and HgCl$_2$ (0.0160 g/L) on cell death and ERs in PC-12 cells. Cell viability and cellular morphology was examined to value the cellular toxicity of Zuotai, β-HgS and HgCl$_2$. Caspase family genes,
caspase 3 (Cas3), caspase 8 (Cas8), caspase 9 (Cas9) and caspase 12 (Cas12), were tested to analyze the possible signaling pathways leading to cell death. The expressions of ERs-related genes, including glucose regulated protein 78 (GRP78), C/EBP homologous protein (CHOP, known as Gadd 153 and Ditt3) and growth arrest and DNA damage protein 45a and 45b (Gadd45a and Gadd45b), were examined to analyze whether Zuotai, β-HgS and HgCl₂ triggered ERs. And the expressions of metal sensitive genes (Metallothionein-1 (MT-1) and Heme oxygenase-1 (HO-1)) were tested to simply analyze the possible signaling induced by Zuotai, β-HgS and HgCl₂.

2. Materials and methods

2.1 Chemicals

Zuotai was provided by the Company of Tibetan Medicine in Tibetan Autonomous Region. β-HgS and HgCl₂ were from Sigma Chemical Company (St. Louis, MO). Other reagents were of reagent grade.

2.2 Cell cultures

PC-12 cells was provided by the Procell Life Science & Technology Co.,Ltd. (Wuhan, China). The cells were cultured in RPMI 1640 media (Gibco, USA) supplemented with 10% FBS (Gibco, USA) and 1% Penicillin-Streptomycin-Glutamine (100X, Gibco, USA) at 37 °C in a humidified 5% CO₂ incubator (Heal Force, China). In subsequent experiments, the cells were divided into four groups: normal control group, Zuotai group, β-HgS group, and HgCl₂ group. The normal control group was incubated with medium alone; the Zuotai group was incubated with Zuotai suspension (0.250 g/L, suspended in the medium, 0.116 g Hg/L); the β-HgS group was incubated with β-HgS suspension (0.135 g/L, suspended in the medium, equivalent Hg as Zuotai); the HgCl₂ group was incubated with medium containing HgCl₂ (0.0160 g/L, 1/10 Hg of Zuotai). And to avoid the influence of cell proliferation, two hours incubation period was adopted.
2.2 Cell viability

The cell viability was determined using sulforhodamine B (SRB) assay as described (Keepers et al. 1991; Papazisis et al. 1997). Briefly, PC-12 cells were seeded in 96-well plates at a density of $5 \times 10^5$ cells/well and cultured for 48 hours, followed by various treatments as described above. The cells were fixed with cold (4 °C) 10% trichloroacetic acid (TCA) solution. Plates were dried at room temperature after washed 5 times with ddH$_2$O, then 100μL 0.4% (w/v, in 1% acetic acid) SRB (Sigma, USA) solution were added to each well to stain for 20 min. Plates were dried again at room temperature after washed 5 times with 1% acetic acid. Bound SRB was solubilized with 200 μL 10 mM unbuffered Tris-base solution (Sigma, USA). Absorbance was read in a microplate reader (PerkinElmer, USA) at 560 nm. The cell viability was the product of the following equation: cell viability (%) = (OD$_{\text{drugs}}$/OD$_{\text{control}}$) × 100%.

2.3 Cell morphology observation

The haematoxylin and eosin (HE) stain assay kit (Beyotime, China) was used to observed the PC-12 cells after crawled on the slide. PC-12 cells were plated into a 6-well plate with prepared slides at a density of $1.5 \times 10^4$ cells/well and cultured for 48 hours. Then following treatments described in previous, the cells were washed in ice-cold phosphate buffered saline (PBS) and fixed with 95% ethanol. The PC-12 cells were then stained with haematoxylin and eosin to improve visualization and were observed under a microscopy (Olympus, Japan).

2.4 Quantitative real time PCR (qRT-PCR)

The cells with and without drugs was homogenized in 1 mL TRIzol (Ambion, USA), and the total RNA was extracted according to the procedures of specification. 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 (Takara, China). The primers were designed by Sangon Biotech (Shanghai) Co., Ltd., and the detailed information was listed in Table 1. The 10 μL qRT-PCR reaction mix was prepared and operated according the guidelines of SYBR® Premix Ex Taq™ II (Takara, China). Relative expressions of target genes
were calculated by the $2^{\Delta \Delta Ct}$ method and normalized to the house keeping gene $\beta$-actin.

2.5 Statistical analysis

Statistical analysis was performed with the SPSS 21.0 (SPSS Inc., USA). Continuous variables were presented as mean ± standard deviation and 95 confidence intervals (95%CI). Continuous variables were examined for normality by Kolmogorov-Smirnov test. For normally distributed variables, differences between the groups were determined by analysis of variance (ANOVA), and Tukey test or Dunnett’s T3 test for post hoc comparisons. Kruskal-Wallis H test was used for abnormally distributed variables, followed by Mann Whitney U test for pairwise comparisons. Significance value (P-value) was set at 0.05.

3 Results

3.1 Cell viability

In the first series of experiments, HgCl$_2$ (0.0160 g/L, 1/10 Hg of Zuotai) induced significant loss of cell viability, compared with the control group (p<0.05), while the treatment of Zuotai (0.250 g/L) produced a 6% decrease in the cell viability (p<0.05). There was no cell loss happened in $\beta$-HgS group (0.135 g/L). (Fig.1)

3.2 Cell morphology

PC-12 cells were exposed to Zuotai, $\beta$-HgS and HgCl$_2$ for 2 hours and morphological changes were examined using HE stain assay kit. The cells in control group grew as a homogeneous monolayer, demonstrating a typical polygonal morphology (Fig. 2A). Zuotai caused the cytoplasm to become flocculent (Fig. 2B), and $\beta$-HgS did not change cellular morphology (Fig. 2C). HgCl$_2$ was more cytotoxic than did the Zuotai and $\beta$-HgS, even at 1/10 Hg dose. Significant morphological changes in PC-12 cells were observed after HgCl$_2$ exposure characterized by the features of apoptosis such as cell shrinkage and rounded up (Fig. 2 D). The black granules scattered in the slides of cells were Zuotai or $\beta$-HgS (Fig. 2B and Fig. 2C). The influence of HgCl$_2$ was most serious on cellular morphology, Zuotai had reduced the attachment.
of cells slightly, β-HgS produced no morphological change. The results agreed with cell viability.

3.3 Caspase family genes expressions

The expressions of \textit{Cas3, Cas8, Cas9} and \textit{Cas12} were quantified to determine that the cell death mechanisms triggered by Zuotai, β-HgS and HgCl\textsubscript{2} were caspase dependent apoptosis or caspase independent necrosis. Under the basal levels (control), the expression of \textit{Cas3} was 69.7%, 87.5% and 98.6%, the expression of \textit{Cas8} was 90.0%, 80.9% and 102.3%, the expression of \textit{Cas9} was 77.9%, 84.2%, and 102.9%, and the expression of \textit{Cas12} was 116.7%, 93.6% and 102.3% in Zuotai, β-HgS and HgCl\textsubscript{2} groups, respectively (Fig. 3). HgCl\textsubscript{2} was not statistically different in the expressions of \textit{Cas3, Cas8, Cas9} and \textit{Cas12} from control, and β-HgS decreased the expressions of \textit{Cas3, Cas8, Cas9} and \textit{Cas12} significantly, while Zuotai decreased \textit{Cas3, Cas8} and \textit{Cas9} expression but increased \textit{Cas12} expressions.

3.4 ERs-related genes expressions

\textit{GRP, CHOP, Gadd45a} and \textit{Gadd45b} were tested to analyze whether HgS or mercuric triggered ERs. Zuotai and HgCl\textsubscript{2} increased \textit{GPR78} mRNA expression by 1.38-fold and 1.43-fold over control (p<0.05), respectively, while β-HgS was not statistically different from control. Zuotai and HgCl\textsubscript{2} significantly increased \textit{CHOP} mRNA expression by 8.82-fold and 8.29-fold respectively (p<0.05), whereas β-HgS decreased \textit{CHOP} mRNA expression to 82% (p<0.05). Zuotai, β-HgS and HgCl\textsubscript{2} increased \textit{Gadd45a} mRNA expression by 2.98-fold, 1.20-fold and 1.82-fold respectively (p<0.05), and upregulated the expression of \textit{Gadd45b} by 1.88-fold, 1.16-fold and 5.54-fold respectively (p<0.05) (Fig. 4). Zuotai and HgCl\textsubscript{2} upregulated the expressions of \textit{GRP78, CHOP, Gadd45a} and \textit{Gadd45b}, but β-HgS had reverse influence on the expression of \textit{CHOP} and increased the expressions of \textit{Gadd45a} and \textit{Gadd45b}.

3.5 Metal sensitive genes expressions

The expressions of \textit{MT-1} and \textit{HO-1} were determined as sensitive biomarker for Hg. \textit{MT-1} and \textit{HO-1} expressions were considered to be a good biomarker for metal exposure in aquatic,
and shown increase in a concentration dependent manner in the presence of heavy metal contaminants (Amiard et al. 2006; Chan 1995; Woo et al. 2006). The expressions of *MT-1* and *HO-1* were significantly increased after exposed to drugs. The expression of MT-1 was increased 2.67-fold, 1.30-fold and 4.66-fold after Zuotai, β-HgS and HgCl₂ respectively, and *HO-1* mRNA expression was increased 7.86-fold, 1.51-fold and 2.98-fold in Zuotai, β-HgS and HgCl₂ group respectively. (Fig. 5)

### 4. Discussions

To investigate the potential cytotoxicity and molecular base of Zuotai, the cell viability, cellular morphology and the expressions of caspase family genes, ERs-related genes and metal sensitive genes were tested. The results showed that: (1) Zuotai (0.250 g/L, 0.116 g Hg/L) did have sight cytotoxicity in PC-12 cells, but β-HgS (0.135 g/L, 0.116 g Hg/L) did not cause cytotoxicity, while HgCl₂ (0.0160 g/L, 0.0116 g Hg/L, 1/10 Hg of Zuotai) produced severe cytotoxicity; (2) the expressions of caspase family genes (*Cas3, Cas8* and *Cas9*) were significantly decreased by Zuotai and β-HgS, and the expression of *Cas12* was increased by Zuotai but decreased by β-HgS; (3) The expressions of ERs-related genes (*GRP78, CHOP, Gadd45a* and *Gadd45b*) were increased in Zuotai and HgCl₂ groups, while β-HgS increased *Gadd45a* and *Gadd45b* mRNA expressions and decreased the expression of *CHOP*; And (4) Zuotai, β-HgS and HgCl₂ were increased the expressions of metal sensitive genes (*MT-1* and *HO-1*). These results could add to the understanding of mercury in traditional medicine, and bring new insights of molecular base of Zuotai.

#### 4.1 Dose and cellular toxicity of Zuotai, β-HgS and HgCl₂

As a nanodrug, Zuotai was suspended in medium to investigate the cytotoxicity like other nanodrugs (AshaRani et al. 2009; Hussain et al. 2006; Patra et al. 2007). The dose of drugs used in vitro was not determined according to the dose of drugs used in clinical, because there was no dose conversion coefficient between cells and animals. The equivalent dose of Zuotai in mouse was 6.67 mg/kg/d, but no mouse was killed by toxicity of mercury at a dose of 40 g/kg/d for 7
days or 80 g/kg/d for 1 day (Li et al. 2014). The preliminary tests showed that Zuotai was well
tolerated in vivo.

To test the changes caused by the death of PC-12 cells, a very high dosage of Zuotai (0.250
g/L, 0.116 g Hg/L) was used in this study, more than 100 times as much as the accumulation of
Hg in vivo (Wu et al. 2016). The accumulation of mercury after orally administrated with Zuotai
(30 mg/kg/d, for 7 days) was 1.15 ng/mg kidney (Wu et al. 2016), which was the highest Hg
content reported in vivo (Li et al. 2014; Liu et al. 2016; Zheng et al. 2015). The equal Hg content
of Zuotai was used in β-HgS group (0.135 g/L, 0.116 g Hg/L), and 1/10 Hg content was used in
HgCl₂ group (0.0160 g/L, 0.0116 g Hg/L). 0.0160 g/L HgCl₂ contained 1.16 × 10⁴ ng Hg /mL,
less than the renal Hg (9.05 × 10⁴ ng/g) after orally administrated with HgCl₂ at dose of 20
mg/kg/d for 30 days (Shi et al. 2011). The cellular toxicity of Zuotai, β-HgS and HgCl₂ was
examined at the above mentioned dose.

Cell viability and cellular morphology were the convenient and intuitive objects for
cytotoxicity. The cell viability of Zuotai, β-HgS and HgCl₂ were 94.79 ± 1.958%, 99.27 ± 1.328%
and 65.82 ± 3.415%, respectively. And Zuotai weakens the adhesion of cells slightly, β-HgS has
no change in cellular morphology, and HgCl₂ produces cell shrinkage and rounded up, like the
influence of HgCl₂ on opossum kidney cells (Carranza-Rosas et al. 2005). The order of cellular
toxicity was HgCl₂ >> Zuotai > β-HgS, even HgCl₂ was at 1/10 dose of Zuotai and β-HgS. The
results were consistent with the previous experimental studies on hepatotoxicity, nephrotoxicity,
development toxicity etc. (Dong et al. 2016; Liu et al. 2016; Wu et al. 2016).

4.2 Caspase family genes activated by Zuotai, β-HgS and HgCl₂

Caspases were central components of machinery responsible for apoptosis. The first
cysteine protease was discovered 8 years ago, called CED-3, which was a critical component
involved in apoptosis (Yuan et al., 1993). Since then, more and more evidences demonstrated
that a family of cysteine proteases named caspase was the executor of apoptosis, which was
evolutionarily conserved (Alnemri et al., 1996). At present, at least 14 distinct mammalian
caspases had been identified, and 8 of the 14 caspases played important roles during apoptosis
Cas3, Cas8, Cas9 and Cas12 played the important roles in apoptotic cascade, and could partly represent the signaling pathways of apoptosis. Cas3, the executioner of cascade reaction of caspase, was activated by limited proteolysis (Boatright et al. 2003). Cas8 was an initiator in death receptor mediated cell death (Juo et al. 1998). The trans-membrane death receptor trigged the extrinsic pathway which eliminated the unwanted cells during the normal physiological processes or functions. Cas9 was the apical caspase of the intrinsic pathway which was used to eliminate cells in response to external stimuli or pathological processes (Zou et al. 1999). Cas12 was an ER resident caspase, the main caspase associated with the ER apoptosis pathway, which upon activation by ERs and calcium release could promote Cas3 mediated apoptosis (Mehmet, 2000). The expressions of Cas3, Cas8, Cas9 and Cas12 were examined to value the possible pathways activated by Zuotai, β-HgS and HgCl₂ which leaded to apoptosis.

Zuotai increased Cas12 expression but decreased Cas3, Cas8 and Cas9 expressions, and β-HgS decreased the expressions of Cas3, Cas8, Cas9 and Cas12, while HgCl₂ was not statistically different in the expressions of Cas3, Cas8, Cas9 and Cas12 from control. The results reflected that Zuotai activated caspase related to ER and inhibited caspases in other pathways. So, Zuotai and β-HgS took effect on protecting by inhibiting caspases activity, while HgCl₂ produced cell death by caspase independent pathway.

4.3 ERs triggered by Zuotai, β-HgS and HgCl₂

Endoplasmic reticulum was an important organelle that had essential roles in multiple cellular processes that were required for cell survival and normal cellular functions. These processes included intracellular calcium homeostasis, protein secretion and lipid biosynthesis (Anelli & Sitia 2008; Ma & Hendershot 2004; Pizzo & Pozzan 2007). Multiple disturbances could trigger an evolutionarily conserved response termed ERs, such as oxidants, hypoxia, glucose deprivation, aberrations of calcium regulation, viral infection, high-fat diet and so on. And endoplasmic reticulum pathway regulated a wide array of cellular responses important in
physiological and pathological processes, for example, neurodegeneration (Katayama et al. 2001; Ryu et al. 2002), cardiac disease (Glembotski 2008; Okada et al. 2004), cancer (Romero-Ramirez et al. 2004; Shuda et al. 2003), diabetes (Araki et al. 2003; Yamada 2006), autoimmune disease (Blab et al. 2001; Corrigall et al. 2001) and other diseases.

The dysfunction of ER, such as the accumulation of protein unfolding or misfolding and aberrations of calcium regulation were mainly phenomena in ERs. Unfolded protein response (UPR) was a branch of ERs which was a well-studied pathway. And the consequences of triggering UPR in mammalian cells could be grouped into three types of effector functions: adaptation, alarm and apoptosis (Xu et al. 2005). The intent of the adaptive aspects of UPR was to reestablish homeostasis and normal ER function, and the UPR-induced alarm referred to signal transduction associated with cellular stress. The cell death was induced by severe and prolonged ERs which could proceed with or without caspase activity (Egger et al. 2003). Thus ERs had consequences for protective as well as destructive.

In this study, Zuotai and HgCl₂ significantly upregulated the expressions of ERs-related genes (GRP78, CHOP, Gadd45a and Gadd45b). But the sensitivity of genes was different between Zuotai and HgCl₂. β-HgS slightly increased the expressions of Gadd45a and Gadd45b but decreased the expression of CHOP. Summed up, Zuotai, β-HgS and HgCl₂ triggered ERs. But the consequences of ERs were protective and destructive. Therefore, the further study was needed to explain the biological effect of Zuotai in ER.

### 4.4 The metal sensitive genes induced by Zuotai, β-HgS and HgCl₂

MT-1 was an intracellular, low molecular, low molecular weight, cysteine-rich protein (Kramer et al. 1996). HO-1, also known as heat-shock protein 32 (HSP32), was the primary generator of carbon monoxide (CO) (Maines 1988). Both MT-1 and HO-1 were exquisitely sensitive, and could be rapidly induced by a wide range of metals, drugs, inflammation and so on. And the upregulation of MT-1 and HO-1 during stress was an adaptive mechanism to protect cells (Cha & Suh 2010; Klaassen et al. 1999). The expressions of MT-1 and HO-1 were also considered as good biomarkers for metal exposure in aquatic fish and shown to increase in a
concentration dependent manner in the presence of heavy metal contaminants (Amiard et al. 2006; Chan 1995; Woo et al. 2006). So MT-1 and HO-1 were determined as metal sensitive genes.

Zuotai, β-HgS and HgCl$_2$ significant increased the expressions of MT-1 and HO-1. And the sensitivity of genes was different between Zuotai and HgCl$_2$. β-HgS had mild effect on the expressions of MT-1 and HO-1. The expressions of MT-1 and HO-1 induced by mercuric and produced effect of protections, but the consequences were still unclear, for the mixed up the mercuric toxicity with adaptive protections. The further experiments would design to discussing this problem.

5. Conclusion

In summary, the present study has elucidated the influence of Zuotai, β-HgS and HgCl$_2$ on the cell viability, cell morphology and the expressions of caspase family genes, ERs-related genes and metal sensitive genes in PC-12 cells. In details, the results proved that Zuotai and β-HgS had far less cytotoxicity and triggered different signaling pathway leading to cell death compared with HgCl$_2$. As the results, HgCl$_2$ (0.0160 g/L, 1/10 Hg content of Zuotai) caused the lowest cell viability (65.82 ± 3.415%), produced significant morphological changes such as cell shrinkage and rounded up, triggered the ERs and metal toxicity reactions, and leaded to cell death through caspase independent pathway. β-HgS had no change in cell viability (99.27 ± 1.328%) and cellular morphology, mildly activated ERs and metal toxicity reactions, but limited caspase activity. Zuotai produced minor cellular toxicity (94.79 ± 1.958% in cell viability), reduced the adhesion of cells slightly, and produced ERs and metal toxicity reactions, but inhibited caspase activity.

These findings indicated that Zuotai and β-HgS were different from HgCl$_2$ in cellular toxicity, and the effects of Zuotai and β-HgS at a high dose were both protective and destructive. That might be a part of the foundation of drug action of HgS based medicines. But the toxicity and pharmacological basis of HgS based traditional medicines remains elusive, and further investigation was needed. This research provided new insights to understand the medicinal and
toxicological action of mercury contained medicines, and would bring some benefits to explore
the mechanism of action of mercury based traditional preparations.

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**Table 1** (on next page)

Primer sequences for qRT-PCR
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’ to 3’)</th>
<th>Reverse (5’ to 3’)</th>
<th>Access</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>TGTCACCAACTGGGACGATA</td>
<td>GGGGTGTTGAAGGTCTCAAA</td>
<td>B661201 (Sangon, China)</td>
</tr>
<tr>
<td>Cas 12</td>
<td>TTACACCATCTTCTGCATCA</td>
<td>CCTGAGGAACTGTAAGCATT</td>
<td>NM_130422.1</td>
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<tr>
<td>Cas 3</td>
<td>ACAGAGCTGGACTGCGTAT</td>
<td>AATAGTAACCGGTTGGGTA</td>
<td>NM_012922.2</td>
</tr>
<tr>
<td>Cas 8</td>
<td>TCCTTCAGTGATTCACAGC</td>
<td>CTTTGTCATCCATGGATAGG</td>
<td>NM_022277.1</td>
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<tr>
<td>Cas 9</td>
<td>CTTCTCTGCTTCATCTCCTG</td>
<td>GGGGTGTAACAGGCTTCTGGA</td>
<td>NM_031632.1</td>
</tr>
<tr>
<td>CHOP</td>
<td>TATCTCTATCCCAGGAAACG</td>
<td>CTTTGAGGTTTGCTTGTGAC</td>
<td>NM_001109986.1</td>
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<tr>
<td>Gadd 45a</td>
<td>TCTGTTCGGAGGAACGACATC</td>
<td>CAGTGTAAGCCGGGCTCTG</td>
<td>NM_024127.2</td>
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<tr>
<td>Gadd 45b</td>
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<td>GCCTCGTTTGTGCCTAGAT</td>
<td>NM_001008321.1</td>
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<tr>
<td>GRP 78</td>
<td>AAGAGAAGCTTGGAGGTAAG</td>
<td>GTCTTCAATGTCTGCATCCT</td>
<td>NM_013083.2</td>
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<tr>
<td>HO-1</td>
<td>GGTGACAGAAGAGGCTAAG</td>
<td>TCTGGCGAAGAAACTCTG</td>
<td>NM_012580.2</td>
</tr>
<tr>
<td>MT-1</td>
<td>CGGCTGCAAGAACTGCAAT</td>
<td>ACTTGTCGGAGGCACCTTTG</td>
<td>NM_138826.4</td>
</tr>
</tbody>
</table>
Figure 1 (on next page)

The effect of Zuotai, β-HgS and HgCl₂ on cell viability. PC-12 cells were treated with Zuotai (0.250 g/L), β-HgS (0.135 g/L) and HgCl₂ (0.0160 g/L, 1/10 Hg of Zuotai) for 2 hours. Data were the mean and SEM of 10 repeats. *Significantly
The graph shows the viability of PC12 cells treated with different compounds: control, Zuotai, β-HgS, and HgCl₂. The x-axis represents the treatments, and the y-axis represents the viabilities in percentage. The data indicates that β-HgS and HgCl₂ have significantly lower viabilities compared to the control and Zuotai treatments.
**Figure 2** (on next page)

HE staining of the slides of cells with and without drugs exposed for 2 hours. A: morphological characterization of PC-12 cells in control; B: Zuotai 0.250 g/L, C: β-HgS 0.135 g/L, D: HgCl₂ 0.0160 g/L. Magnitude 400 ×.
Figure 3 (on next page)

Effect of Zuotai, β-HgS and HgCl₂ on the expressions of caspase family genes. PC-12 cells were given Zuotai (0.250 g/L), β-HgS (0.135 g/L) and HgCl₂ (0.0160 g/L) for 2 hours. Data were the mean and SEM of 6 repeats. *Significantly different from...
Caspase family mRNA expression (β-actin)

- Cas3
- Cas8
- Cas9
- Cas12

control  Zuotai  β-HgS  HgCl2

* Indicates statistically significant differences from the control group.
Figure 4 (on next page)

Effect of Zuotai, β-HgS and HgCl₂ on the expressions of ERs-related genes. PC-12 cells were given Zuotai (0.250 g/L), β-HgS (0.135 g/L) and HgCl₂ (0.0160 g/L) for 2 hours. Data were the mean and SEM of 6 repeats. *Significantly different
**Figure 5** (on next page)

Effect of Zuotai, β-HgS and HgCl$_2$ on the expressions of metal sensitive genes. PC-12 cells were given Zuotai (0.250 g/L), β-HgS (0.135 g/L) and HgCl$_2$ (0.0160 g/L) for 2 hours. Data were the mean and SEM of 6 repeats. *Significantly dif
The diagram shows the mRNA expression levels of MT-1 and HO-1 under control conditions, treatment with Zuotai, β-HgS, and HgCl₂. The expression levels are normalized to β-actin. The data for MT-1 and HO-1 are presented separately, with asterisks indicating statistically significant differences compared to the control group.