Realgar-induced neurotoxicity: Crosstalk between autophagic flux and the p62-NRF2 feedback loop mediates p62 accumulation to promote apoptosis

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Research Article

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Abstract

Realgar is a traditional Chinese medicine that contains arsenic. It has been reported that the abuse of medicine containing realgar has potential central nervous system toxicity, but the toxicity mechanism has not been elucidated. In this study, we established realgar exposure model in vivo and selected DMA, the end product of realgar metabolism, to treat SH-SY5Y cells in vitro. Many assays including behavioral, analytical chemistry, and molecular biology were used to elucidate the roles of autophagic flux and p62-NRF2 feedback loop in realgar-induced neurotoxicity. The results showed that arsenic could accumulate in the brain, causing cognitive impairment and anxiety-like behavior. Realgar impairs the ultrastructure of neurons, promotes apoptosis, perturbs autophagic flux homeostasis, amplifies the p62-NRF2 feedback loop, and leads to p62 accumulation. Further analysis showed that realgar activates JNK/c-Jun, promotes the formation of Beclin1/Vps34 complex, induces autophagy, and recruits p62. Meanwhile, realgar inhibits the activities of CTSB and CTSD, and changes the acidic of lysosomes, resulting in the inhibition of p62 degradation, and then accumulation. Moreover, the amplified p62-NRF2 feedback loop is also involved in the accumulation of p62. Its accumulation promotes neuronal apoptosis by up-regulating the expression levels of Bax and cleaved-caspase-9, resulting in neurotoxicity. Taken together, these data suggest that realgar can perturb the cross-talk between autophagic flux and the p62-NRF2 feedback loop to mediate p62 accumulation, promote apoptosis, and induce neurotoxicity.

1. Introduction

Realgar, a traditional Chinese medicine (TCM) whose key ingredient is arsenic sulfide (As$_2$S$_2$ or As$_4$S$_4$), has been used as a medicine for thousands of years in China and is widely used in the clinic [1, 2]. Some classical prescriptions, such as An-Gong-Niu-Huang Wan and Niu-Huang-Jie-Du Pian, contain 6–12% realgar [1]. However, under the influence of the concept of non-toxic side effects of traditional Chinese medicine, drug-induced arsenic poisoning events caused by long-term and excessive use of single realgar or its compound preparations have been reported [3]. Epidemiological and animal experiments have verified that long-term exposure to arsenic not only leads to skin damage, digestive system damage, and tumor occurrence but also causes central nervous system damage [4–8]. The effects of arsenic exposure and accumulation on human health caused by the medicinal use of realgar have aroused widespread public concern. The brain is one of the main target organs of arsenic toxicity [9]. Clarifying the neurotoxic effect of realgar and discovering the sensitive molecular targets of early changes in its toxicity has important theoretical and practical significance for early taking effective measures to prevent the occurrence of drug-induced arsenic poisoning and guiding clinical rational drug use.

Our previous study found that the multifunctional protein p62/SQSTM1, which is induced by stress and acts on selective autophagy, is continuously highly expressed in the cortex after realgar exposure[10]. But its molecular mechanism in realgar-induced central nervous system toxicity is not very clear. p62 is a ubiquitin-binding protein encoded by SQSTM1. Its amino acid sequence contains multiple functional domains, including PB1, TB, KIR, LIR, etc[11]. The LIR domain can interact with LC3 and then participate in the process of autophagy, also known as a marker protein for autophagy degradation [12]. In addition,
p62 competes with nuclear factor erythroid 2-related factor 2 (NRF2) to bind KEAP1 (Kelch-like ECH associated protein 1) through the KIR domain, promoting the dissociation of NRF2-KEAP1, and activates NRF2[13]. NRF2 is a key transcription factor involved in anti-apoptosis and antioxidant defense[14]. NRF2 activation can directly induce p62 transcription and promote p62 expression, thus forming a p62-NRF2 feedback loop[11, 15]. According to a previous study, p62 accumulation induces apoptosis by activating the caspase-8 inflammatory cascade and increasing caspase-9 cleavage[16]. However, the role of the p62-NRF2 feedback loop in realgar-induced neurotoxicity remains to be further explored.

Autophagy is an important regulatory mechanism for maintaining cellular homeostasis. In neural cells, disordered autophagy can accumulate protein aggregates, disrupt cellular homeostasis, produce neurotoxicity, and lead to neuronal apoptosis and cognitive dysfunction [17, 18]. Autophagic flux is a dynamic process, including autophagy induction, autophagosome formation, and autophagy degradation. Damage to any of these processes will disturb autophagic flux homeostasis[19]. Studies have shown that enhanced autophagy induction can promote the transport of p62 to lysosomes, thereby promoting p62 degradation [20]. The c-Jun N-terminal kinase (JNK) family, as one of the members of mitogen-activated protein kinase (MAPK), is involved in cell differentiation, apoptosis, and autophagy [21, 22]. Activated JNK/c-Jun, which is activated by cytokines, inflammatory factors, stress, and other factors, up-regulates the expression of autophagy-induced related protein Beclin1 and Vps34, and then the ECD of Beclin1 combines with the C2 of Vps34 to promote the formation of the Beclin1/Vps34 core complex and activate the autophagy induction[23]. LC3 is hydrolyzed to LC3I, and then LC3I is converted to LC3II, which recruits p62 through the LIR and binds together to the autophagosome membrane to participate in autophagosome formation, and is eventually degraded in the lysosome [24, 25]. However, if an external stimulus activates autophagy, the subsequent highly efficient degradation induced by autophagy is important for maintaining cellular homeostasis. Autophagy degradation is dependent on lysosomal hydrolysis enzymes and the acidic environment of the lysosome [26, 27]. When autophagy degradation is dysfunctional, the degradation of p62 is blocked and then accumulates. Excessive p62 further enhances the p62-NRF2 feedback loop, which promotes p62 accumulation and induces neuronal apoptosis in a crosstalk manner.

Therefore, our study will explore the molecular mechanism by which realgar promotes p62 accumulation by perturbing the cross-talk of autophagic flux and p62-NRF2 feedback loop in vitro and in vivo, which intensifies neuronal apoptosis and leads to central nervous system (CNS) toxicity. This will provide new ideas and basic experimental data for the study of the central nervous system toxicity of realgar.

2. Materials And Methods

2.1 Animals and reagents

Female Sprague–Dawley rats aged 3 weeks were purchased from the Laboratory Animal Center of China Medical University (Shenyang, China). Rats were fed in an environment on a 12 h light-dark cycle at 21–24°C. All animals received food and water ad libitum. All animal experiments were reviewed and approved
by the Institutional Animal Care and Use Committee of China Medical University (ethics committee approval numbers: CMU2019109 and CMU2021120). Realgar (> 90.0% As2S2) was purchased from Henan Sanmenxia Yuhuangshan Pharmaceutical Co., Ltd. (Henan, China; lot number: 180428). Information on all reagents is listed in Table S1.

2.2 Experimental protocol

We investigated realgar-induced neurotoxicity and disruptions in autophagy flux *in vivo* by arbitrarily dividing 3-week-old rats into 3 groups: (1) the control group of rats, (2) the 0.3 g/kg realgar group of rats, and (3) the 0.9 g/kg realgar group of rats. Animals in the control group received 0.5% (w/v) sodium carboxymethyl cellulose (CMC-Na) by oral administration once daily for 8 weeks, while those in the realgar groups received realgar suspensions at different dosages.

We investigated the effect of the JNK pathway on realgar-induced autophagy and neurotoxicity *in vivo* by randomly and equally dividing 3-week-old rats into four groups: (1) the saline + control group (n = 6 rats/group), (2) the saline + 0.9 g/kg realgar group (n = 6 rats/group), (3) the SP600125 (10 mg/kg) group (n = 6 rats/group) and (4) the SP600125 (10 mg/kg) + 0.9 g/kg realgar group (n = 6 rats/group). Rats in all SP600125 groups were injected with the same dose of SP600125.

2.3 The detection of metabolic products of realgar in rats

The contents of arsenic species were determined using a liquid chromatography-atomic fluorescence spectrometry (LC-AFS). Initially, urine was centrifuged at a 7500 g for 5 min at 4°C and diluted 30-fold before detection. Then, blood was digested with H₂SO₄ and diluted 30-fold before detection. Finally, a fifty-milligram cortex sample was homogenized in water, and the supernatant was digested in H₂SO₄ for detection. The LC-AFS6500 parameters were set as follows: the element of the detection tunnel was set to arsenic, the negative high voltage was 300 V, the total current was 90 mA, and the carrier gas flow rate was set to 5 ml/min.

2.4 Behavioral tests

In the novel object recognition test, the rats were first placed into the open field arena containing two identical objects (10 cm width ×10 cm length×10 cm tall) and allowed freely explore them for 3 min. After 24 h, one of the objects was replaced with a novel object (different shape and color than the old object) and the rats were allowed to explore for another 3 min. The time rats spent interacting with the novel and old objects was recorded. Calculate cognitive index: spend time in new or old object /Total time. The open-eld test was performed in a large acrylic cube measuring 50 cm tall and 100 cm wide with a black bottom. Briefly, rats were individually placed near the wall side and allowed to freely move for 5 min to assess locomotor’s activity. The time spent in the center zone (30×30 cm imaginary square), velocity and distance traveled were evaluated.

2.5 SH-SY5Y cell culture and treatment
As described previously, SH-SY5Y cells were cultured in Roswell Park Memorial Institute (RPMI 1640) medium supplemented with 12% fetal bovine serum (FBS) at 37°C in a humidified 5% CO₂ atmosphere. For the detection of autophagic flux, SP600125, Rapamycin, and Chloroquine were added to the medium before stimulation with DMA. The p62 siRNA or NRF2 siRNA was added to the medium 24 h before cell stimulation.

2.6 Electron microscopy

After gradient dehydration with ethanol solutions, the cerebral cortex was incubated with diluted acetone solutions, cleared with propylene oxide, and infiltrated overnight, followed by polymerization at 70°C overnight with a heating polymerization apparatus. Transverse cerebral cortical sections (50 nm) were acquired using an ultrathin slicing instrument. The tissue samples were stained with uranyl acetate and a 50% ethanol saturated solution for 15 min to 1 h. Finally, the samples were observed and photographed with a HITACHI H-7650 transmission electron microscope.

2.7 Western blot analysis

Cerebral cortical tissues or treated SH-SY5Y cells were homogenized in RIPA buffer containing protease inhibitors and phosphate inhibitors. The protein concentration was quantified with a BCA protein assay kit. The supematant was separated on a 10–12% SDS-polyacrylamide gel and then wet electrotransferred to a PVDF membrane. After blocking with 10% evaporated skim milk at room temperature for 1 h on a rocker, the PVDF membrane was incubated overnight at 4°C with corresponding primary antibodies. Blots were rinsed with TBST four times for 5 min each and incubated with the secondary antibody conjugated with horseradish peroxidase for 1 h. An ECL detection system was used to measure the levels of target proteins. The signal intensity was detected with Image J software.

2.8 Co-Immunoprecipitation assay

Cortex tissue weighing 100 mg or treated SH-SY5Y cells were homogenized, incubated for 30 min, and centrifuged at 4000 rpm for 10 min. The supematants were incubated with specific primary antibodies followed by the addition of 30 µL of PureProteome™ Protein A/G Mix Magnetic Beads and incubation with gentle rotation. The agarose beads were collected, washed four times with lysis buffer, and resuspended in loading buffer.

2.9 Analysis of mRNA levels

Total RNA was extracted from cerebral cortical tissues or SH-SY5Y cells with TRIZol reagent, and the RNA concentration was measured spectrophotometrically. The cDNA templates were synthesized using a cDNA Synthesis Kit according to the manufacturer's instructions. The analysis was performed using RT–PCR with gene-specific primers (Table S2) on a QuantStudio™6 Flex real-time fluorescence quantification system with TB Green Real-time PCR Master Mix. Amplification of target cDNA was normalized to GAPDH or ACTB expression. Relative levels of target mRNA expression were calculated using the 2^−ΔΔCt method.

2.10 Fluorescence and confocal microscopy analyses
Sections of the cortex were mounted on glass slides. Sections were washed with PBS and incubated with the following specific primary antibodies. SH-SY5Y cells were grown on glass coverslips. After presage processing, the cells were fixed with 4% PFA in PBS, followed by permeabilization with 0.2% Triton X-100 in PBS. Then, the cells were blocked with 5% BSA. The fixed cells were incubated with primary antibodies. The slides were then washed and incubated with a fluorescent secondary antibody. DAPI staining solution was used for nuclear counterstaining. The stained samples were examined using an Al + confocal microscope.

2.11 RFP-GFP-LC3B assay

Briefly, $1.5 \times 10^5$ SH-SY5Y cells were grown on glass-bottom dishes and infected with the adenovirus for 24 h. Then, the SH-SY5Y cells were treated with DMA for an additional 6 h. All samples were examined under an Al + confocal microscope equipped with a 60 × oil immersion objective.

2.12 LysoSensor Green DND-189 staining

The lysosomal pH was quantified using LysoSensor Green DND-189. Briefly, SH-SY5Y cells were loaded with LysoSensor Green DND-189 in a prewarmed basal medium for 30 min at 37°C. Then, the cells were washed three times with PBS. After treatment, the fluorescence intensity of the cells was quantified using an H1MD Multifunctional Microplate Reader with 443-nm excitation and 505-nm emission filters.

2.13 CTSD and CTSB activity assays

The Cathepsin D Activity Fluorometric Assay Kit and the Cathepsin B Activity Fluorometric Assay Kit were used to detect CTSD and CTSB catalytic activity. Briefly, the cortex was dissected from the brain and ground in CD/CB Cell Lysis Buffer with a Tissue Lyser II, followed by incubation on ice. After centrifugation, the supernatant was transferred to a 96-well plate, and a master assay mix containing CD/CB reaction buffer and CB substrate was added to each sample. The samples were incubated and were read with an HMID Multifunctional Microplate Reader with 328-nm/400-nm excitation and 460-nm/505-nm emission filters.

2.14 Proximity ligation assay

Protein interactions in SH-SY5Y cells were detected using the Duolink® PLA assay kit following the manufacturer’s protocol. After treatments, cells were fixed with 4% PFA and permeabilized with 0.3% Triton X-100. A blocking solution was added to the cells, which were incubated. Then, the slides were incubated with primary antibody and then with PLA probe solution. After being washed, the slides were incubated and then incubated with the amplification solution protected from light. Finally, cell nuclei were stained with DAPI, and the slides were imaged using Al + confocal microscope.

2.15 Synthesis of siRNAs
siRNAs targeting human p62/SQSTM1 or NRF2 were designed and synthesized by Guangzhou RIBOBIO Co., LTD.). The gene sequences were as follows: p62/SQSTM1 siRNA: 5'-GGAGTCGGATAACTGTTCA-3'; NRF2 siRNA: 5'-GCCCTCACCTGCTACTTTA-3'.

2.16 Statistical analysis

Data are presented as X ± SD. Statistical comparisons were performed using Student's t-tests or one-way analysis of variance (ANOVA), two-way ANOVA, and two-way repeated-measures ANOVA followed by Dennett's post hoc test. P < 0.05 was considered statistically significant. Origin 2018 and GraphPad Prism software version 8.00 were used for statistical analyses and graphic production.

3. Results

3.1. The content of arsenic species in cortex, blood and urine after realgar-exposed

First, we performed quantitative analyses of arsenite (As (III)), dimethylarsinic acid (DMA), monomethylarsonic acid (MMA) and arsenate (As (V)) in cortex, blood and urine after realgar-exposed by using LC-AFS. The results indicated that only DMA was detected in the cortex and blood, but As (III), MMA and As (V) were not detected (Fig. 1A). As (III), MMA and DMA were detected in urine, and As (V) was not detected (Fig. 1a). In the cortex, the content of DMA was significantly increased with a dose-dependent relationship in the 0.9 g/kg realgar group (P < 0.05), which was 4.6-fold higher than those in the 0.3 g/kg realgar group (Fig. 1b). In blood, compared with the 0.3 g/kg realgar group, the content of DMA in the 0.9 g/kg realgar group did not change significantly, suggesting that arsenic concentration reached a plateau value (Fig. 1c). In urine, the content of As (III), MMA, and DMA was significantly increased with a dose-dependent relationship in the 0.9 g/kg realgar group (Fig. 1d) (P < 0.05), which were 1.73-fold, 1.48-fold and 2.12-fold higher than those in the 0.3 g/kg realgar group, respectively. These data showed that the metabolites of arsenic in realgar can enter and accumulate in the brain, and are mainly dominated by DMA.

3.2. Effects of realgar on neurobehavior of rats

We evaluated the effect of realgar on rat neurobehavior by performing the novel object recognition test and open-field test. The novel object recognition test evaluates the cognitive function by recording the time spent exploring new and old objects and calculating the recognition index[28]. The rats were first placed into the open field containing two identical objects and allowed freely explore them for 3 min, called the training stage. After 24 h, one of the objects was replaced with a novel object and repeats the above steps, called the testing stage. The results indicated that there were no significant differences in the time spent exploring two old objects and their recognition index during the training stage, whether it was the control group, the 0.3 g/kg realgar group, or the 0.9 g/kg realgar group (Fig. 2a-b). In the testing stage, the control group spent more time exploring and in proximity to the new object, and its recognition
index was also increased \((P < 0.05)\), but the 0.3 g/kg or 0.9 g/kg realgar group showed no difference (Fig. 2c-d). It is suggested that realgar can impair cognitive ability.

The open-field test was used to analyze the changes in the emotions of rats (as reflected by the time spent in the center zone, the number entered the center zone, the distance and velocity traveled)[29]. The results indicated that the 0.3 g/kg realgar group had no significant difference in the time spent in the center zone compared with the control group, but the 0.9 g/kg realgar group significantly shortened the time spent in the center zone (Fig. 2e) \((P < 0.05)\). Meanwhile, we found that the 0.3 g/kg and 0.9 g/kg realgar groups exhibited significantly fewer entries into the center of the open field compared with the control group (Fig. 2e, g) \((P < 0.05)\). Moreover, we also found that the 0.9 g/kg realgar group significantly decreased the distance and velocity traveled compared with the control group and the 0.3 g/kg realgar group (Fig. 2h-i) \((P < 0.05)\). Based on these results, realgar can induce anxiety-like behavior in rats.

### 3.3. Effects of realgar on ultrastructure and apoptosis of cortex neurons

We first observed ultrastructural changes in neurons by using electron microscopy. The results indicated that in the control group, the nuclear membrane was smooth and complete, the nucleus was large and round, the chromatin was evenly distributed, the structure of the Golgi complex and endoplasmic reticulum in the cytoplasm was clear and neatly distributed, the mitochondria were round or oval, and obvious mitochondrial cristae were visible. In the 0.3 g/kg realgar group, neurons have large and rounded nuclei, but the cytoplasmic Golgi complex and endoplasmic reticulum are blurred, and mitochondrial cristae are broken. In the 0.9 g/kg realgar group, neurons exhibited an unclear boundary of the nuclear membrane and disorder of the Golgi complex and endoplasmic reticulum structure and distribution and expansion of mitochondria (Fig. 3a). It is suggested that realgar can damage the ultrastructure of cortex neurons.

Next, we explored the effect of realgar exposure on the apoptosis of cortex neurons. Compared with the control group, the percentage of apoptotic cells in the 0.3 g/kg and 0.9 g/kg realgar groups significantly increased in a dose-dependent manner, which was 17.02% and 34.22%, respectively (Fig. 3b) \((P < 0.05)\). Western blot showed that the Bax/Bcl-2 and cleaved-caspase-9/caspase-9 in the 0.3 g/kg realgar group did not change significantly, but their ratio was significantly increased in the 0.9 g/kg realgar group (Fig. 3c) \((P < 0.05)\), which were 1.4-fold and 1.3-fold that of the control group, respectively. It is indicated realgar can promote neuronal apoptosis. Taken together, these results suggest that realgar has central nervous system toxicity.

### 3.4. Realgar mediated p62 to promote neuronal apoptosis by perturbing autophagic flux homeostasis

Autophagy, an important self-regulation mechanism, eliminates aggregated and misfolded proteins and impaired organelles[30]. Autophagic flux homeostasis is one of the important ways to evaluate the autophagy function[31]. The results showed that the ratios of LC3II and LC3I in the 0.3 g/kg and 0.9 g/kg
realgar groups were significantly higher than those in the control group (Fig. 4a-b) \((P<0.05)\), which were 1.3-fold and 1.6-fold, respectively. In the 0.3 g/kg realgar group, the expression level of p62 protein was increased but there was no significant difference, and the p62 protein expression level in the 0.9 g/kg realgar group was significantly increased (Fig. 4a, c) \((P<0.05)\), which was 1.7 times that of the control group. Meanwhile, we also observed a significant increase in the number of intracellular autophagosomes under transmission electron microscopy after treating SH-SY5Y cells with DMA, the metabolic end product of realgar for 4 or 6 hours (Fig. 4d-e) \((P<0.01)\). It is suggested that realgar perturbs the cortex autophagic flux homeostasis, but the increase of p62 indicates that this may be related to the impaired autophagic degradation function.

p62 is not only a marker protein of autophagy degradation but also a key protein that is tightly linked to autophagy and apoptosis[16]. The results showed that cell viability decreased significantly after treating with different concentrations of DMA for 24 h, which was 74.4% at 5 mM (Fig. 4f) \((P<0.05)\) and 55.6% at 10 mM (Fig. 4f) \((P<0.01)\). In addition, compared with the control group, the p62 protein expression level did not change significantly when treated with 2.5 mM DMA. But the expression level of p62 was significantly increased when treated with 5 mM and 10 mM DMA (Fig. 4g-h) \((P<0.01)\), which were 1.6-fold and 1.9-fold, respectively. Moreover, the expression level of p62 was significantly decreased when SH-SY5Y cells were treated with DMA at 1 h \((P<0.01)\), but significantly increased at 12–24 h (Fig. 4i-j) \((P<0.01)\). It suggested that DMA could promote p62 protein expression level in SH-SY5Y cells. Next, we silenced p62, and the silent efficiency was identified as 72% (Fig. 4k) \((P<0.01)\). Compared with the DMA group, Bax/Bcl-2 and cleaved-caspase-9/caspase-9 were significantly decreased in the DMA+p62siRNA group (Fig. 4l-n) \((P<0.01)\), which was 63% and 85%, respectively. Taken together, these results suggested that p62 is involved in the DMA-induced apoptosis of SH-SY5Y cells.

3.5. Realgar activated autophagy through the JNK/Vps34 complex pathway to promote p62 aggregation

Under stress conditions, the expression of Beclin1 increases and forms a complex with Vps34[32]. Beclin1/Vps34 complex plays an important role in the induction of autophagy. To elucidate the molecular mechanism of autophagy activation by realgar, we first detected the expression of the Beclin1/Vps34 complex. Compared with the control group, the results showed that the expression levels of Beclin1 protein in the 0.3 g/kg and 0.9 g/kg realgar groups were significantly increased (Fig. 5a-b) \((P<0.05)\), which were 1.2-fold and 1.3-fold, respectively. And the expression level of Vps34 protein in the 0.3 g/kg realgar group was increased, but there was no significant difference, while the expression level of Vps34 protein in the 0.9 g/kg realgar group was significantly increased (Fig. 5a, c) \((P<0.05)\), which was 1.4-fold that of the control group. Moreover, CO-IP showed that the binding capacity of Beclin1/Vps34 complex in SH-SY5Y cells was enhanced after DMA treatment (Fig. 5d), suggesting that realgar exposure promoted the formation of Beclin1/Vps34 complex and activated the autophagy induction.

The JNK/Vps34 complex pathway plays an important role in the induction of autophagy [33]. The results showed that compared with the control group, p-JNK/JNK and p-c-Jun/c-Jun in the 0.3 g/kg realgar
group increased, but there was no significant difference, p-JNK/JNK and p-c-Jun/c-Jun in 0.9 g/kg realgar group was significantly increased (Fig. 5e-g) \((P < 0.05)\), which were 1.3-fold and 2.0-fold that of the control group, respectively, suggesting that realgar activates JNK/c-Jun signaling pathway. Next, we used SP600125 (JNK-specific inhibitor) to intervene. Western blot results showed that compared with the 0.9 g/kg realgar group, it not only inhibited the expression level of p-c-Jun protein but also significantly decreased the protein expression levels of Beclin1 and Vps34. (Fig. 5h-i) \((P < 0.05)\), decreased by 23% and 20%, respectively. CO-IP also showed that the binding ability of Beclin1 to Vps34 was weakened after inhibiting the JNK pathway (Fig. 5j). PLA indicated that the formation of Beclin1/Vps34 complexes (red dots) was increased after DMA treatment in SH-SY5Y cells compared with the control group, and was decreased in the DMA + SP600125 group compared with the DMA group (Fig. 5k). These data suggest that realgar promotes Beclin1/Vps34 complex production by activating JNK/c-Jun to induce autophagy.

After autophagy induction, LC3 recruits a large amount of p62 to participate in autophagosome formation and promote autophagic flux [34]. We found that compared with the control group, the protein expression levels of LC3II/LC3I and p62 in the 0.9 g/kg realgar group were significantly increased (Fig. 5h-i) \((P < 0.05)\), indicating that realgar promotes the formation of autophagosomes, which is consistent with the above results (Fig. 3a). After using SP600125 to inhibit the induction of autophagy, the LC3II/LC3I in the realgar + SP group was significantly decreased compared with the 0.9 g/kg realgar group, (Fig. 5h-i) \((P < 0.05)\), which was 73% of it. However, although the expression level of p62 protein decreased, there was no significant difference (Fig. 5h-i). This data further suggests that autophagy degradation is impaired in the presence of autophagy-induced activation, which promotes p62 aggregation.

### 3.6. Realgar impairs autophagy degradation by inhibiting lysosomal hydrolase activity, not autophagosome-lysosomal fusion

After the formation of autophagosomes, a large amount of p62 accumulates in autophagosomes and is degraded by migrating lysosomes [35]. The degradation mainly depends on the fusion of autophagosomes and lysosomes and the activity of lysosomal hydrolase [36]. We employed mRFP-GFP-LC3, a tool for the detection of autophagosome and lysosome fusions, to trace autophagolysosome formation. In SH-SY5Y cells, LC3 (green dots) and autophagosome (yellow dots) were significantly increased in both the DMA group and the CQ group (Chloroquine, a specific inhibitor of autophagosome-lysosome fusion) compared with the control group by fluorescence microscopy (Fig. 6a-b) \((P < 0.05)\). However, compared with the CQ group, the number of autophagolysosome in the DMA group was significantly increased, and the number of autophagosomes was significantly decreased (Fig. 6a, c) \((P < 0.05)\). It suggested that DMA does not affect the fusion of autophagosome and lysosome.

The function of lysosomal hydrolases plays an important role in autophagy degradation. We found that the catalytic activities of CTSB and CTSD in the lysosome were decreased in the 0.3 g/kg realgar group compared with the control group, but not significantly different, and the activities of CTSB and CTSD were significantly decreased in the 0.9 g/kg realgar group (Fig. 6d–e) \((P < 0.05)\), 79% and 74% of the
control group, respectively. Next, we used LysoSensor Green DND-189 to detect the lysosomal acidic environment. The results showed that after DMA exposure, the green fluorescence intensity in SH-SY5Y cells was significantly reduced in a dose-dependent manner (Fig. 6f) \((P<0.05)\). These results suggested that DMA can alter the lysosomal acidic environment of SH-SY5Y cells. Therefore, it is speculated that realgar impairs autophagy degradation by inhibiting the activity of lysosomal hydrolase, resulting in the accumulation of p62.

3.7. Amplified p62-NRF2 feedback loop is involved in realgar-induced p62 accumulation

NRF2 is a key transcription factor in antioxidant defense[37]. Activation of the NRF2-ARE signaling pathway enhances p62 transcriptional activity and promotes its production[38].8-Hydroxydeoxyguanosine (8-OHdG) labeling of oxidized DNA can reflect ROS levels in cells[39]. In this study, 8-OHdG was colocalized with RBFOX3/NeuN antibody. We found that ROS levels were significantly increased in the 0.3 g/kg and 0.9 g/kg realgar groups (Fig. 7a-b) \((P<0.05)\), which were 2.7-fold and 6.8-fold that of the control group, respectively. Meanwhile, compared with the control group, the protein expression levels of NRF2 and p62 were increased in the 0.3 g/kg realgar group, but there was no significant difference and were significantly increased in the 0.9 g/kg realgar group (Fig. 7c and Fig. 3c) \((P<0.05)\). In addition, both ROS levels and NRF2 protein levels were dose-dependently increased after DMA treatment of SH-SY5Y cells (Fig. 7d-e) \((P<0.001)\). After silencing NRF2, the expression level of p62 protein was significantly decreased in the DMA + NRF2 siRNA group compared with the DMA group (Fig. 7f-g) \((P<0.01)\). It is suggested that DMA activates the NRF2 signaling pathway and promotes the expression of p62.

p62 can also compete with NRF2 for binding to KEAP1, forming a p62-NRF2 positive feedback loop[40]. We found by Co-IP results that DMA increased the formation of the KEAP1-p62 complex in SH-SY5Y cells (Fig. 7h) and decreased the formation of the NRF2-KEAP1 complex (Fig. 7i) compared with the control group. After silencing p62, the expression level of NRF2 protein in the DMA + p62siRNA group was significantly lower than that in the DMA group (Fig. 7j-k) \((P<0.05)\), suggesting that DMA activates NRF2 in a p62-dependent manner. Taken together, these data suggest that realgar promotes p62 accumulation by amplifying the p62-NRF2 feedback loop.

4. Discussion

Realgar, a mineral Chinese medicine containing arsenic, has a history of use of 2500 years in China [1]. The Chinese Pharmacopoeia shows that the main functions of realgar are detoxification, insecticide, anticonvulsant, and malaria treatment, and its compound preparations are used for encephalitis, cerebral palsy, heat-clearing, and detoxification. Some classic prescriptions, such as Angong Niuhuang Pill, a representative drug for the treatment of stroke, Niuhuang Jiedu Tablets, a representative drug for clearing heat and detoxification, and Xiaoerzhibaodan, a commonly used pediatric drug, all contain realgar. The researchers analyzed 191 Chinese patent medicines containing realgar and found that approximately
86% of them are designed for oral administration with a daily intake of arsenic greater than 10 mg/kg[41], which is much more than the 15 g/day PDE limit specified by ICH[42], suggesting that a potential health hazard exists if people use realgar-containing medicines. Clinical long-term abuse of realgar or realgar-containing compound preparations will cause side effects, manifested as multi-system damage, including skin damage, digestive system damage, nervous system damage, etc [1]. In a study, realgar was orally administered to mice for 8 weeks, and the dose-dependent increase in total arsenic levels in the brain resulted in potential central nervous system toxicity manifested as cognitive impairment[43]. Therefore, our study took the newly weaned rats as the subjects to explore the toxic mechanism of realgar on the central nervous system, and to provide theoretical basis and experimental data for clinical rational drug use and protection of people's health.

Studies have shown that after taking realgar or realgar-containing Chinese patent medicine, most realgar is excreted through feces, and only a trace amount of arsenic enters the body and accumulates in brain tissue through the blood-brain barrier, damaging the central nervous system of humans and animals [43, 44]. Arsenic is metabolized in the liver[45]. Briefly, iAs is methylated by arsenic-3-methyltransferase (AS3MT)[46] using S-adenosylmethionine (SAM) as the methyl donor to form monomethylarsonic acid (MMA)[47–49] and dimethylarsinic acid (DMA)[50]. DMA is an end product of metabolism[51]. In this study, the LC-AFS method was used to detect the levels of iAs (III), MMA, DMA, and iAs (V) in rat urine, blood, and cortex. iAs (III), MMA and DMA were detected in urine, and their contents had a dose-dependent relationship, and iAs (V) was not detected. Only DMA was detected in blood, and its contents were no significant difference in the 0.3 g/kg or 0.9 g/kg realgar group, indicating that the rate of metabolic production of DMA was consistent with its elimination rate. Only DMA also was detected in the cortex, and its content had a dose-dependent relationship. However, we have previously detected iAs and MMA in blood and MMA in brain using hydride generation-cold trapping-atomic fluorescence spectrometry (HG-CT-AAS). In this study, As (III), MMA and As (V) were not detected in the cortex and blood. Is the detection sensitivity of the LC-AFS low? Or do the pretreatment conditions need to be further optimized? The specific reasons for this need to be further explored. It is undeniable that the results of the two different instruments prove that arsenic in realgar can enter and accumulate in the brain, and it is mainly DMA. Therefore, we used DMA as a treatment factor to explore the effect of realgar on nerve cells in vitro.

The effect of realgar on central nervous system function was investigated. In this study, a novel object recognition test and open field test were used to observe the effect of realgar on the neurobehavior of rats. Based on our results, after realgar exposure, rats showed decreased ability to recognize new objects, produced anxiety-like behaviors, and decreased Spontaneous motor ability. Our results suggested that realgar caused a blurred neuronal membrane structure, reduced mitochondrial cristae, vacuolization and damage to the double membrane of some mitochondria, and disordered Golgi. In particular, mitochondrial damage is very serious. Meanwhile, after exposure to realgar, the number of TUNEL positive staining cells in neurons increased, and the apoptosis rate increased significantly. Apoptosis-related proteins Bax/Bcl-2 and cleaved-caspase-9/caspase-9 in 0.9 g/kg realgar group were significantly
increased. It shows that realgar promotes apoptosis of cortex nerve cells and has central nervous system toxicity, but its specific mechanism stills need to be further explored.

Previous studies have shown that realgar disrupts autophagy leading to central nervous system toxicity[10]. Autophagy is an important regulatory mechanism for maintaining cellular homeostasis [52] and central nervous system injury induced by an imbalance of autophagic flux homeostasis is related to neurodegenerative diseases[53]. In vivo, with the increase of realgar, the protein expression levels of LC3II/LC3I and p62, which are important indicators for monitoring the formation of autophagosomes, were significantly increased in the cortex. The accumulation of autophagosomes was observed in SH-SY5Y cells treated with DMA for 4 h and 6 h, indicating that realgar disturbed the autophagic flux homeostasis of the cortex. In vitro, When SH-SY5Y cells were treated with different concentrations of DMA, the cell viability decreased significantly. At 10 mM, the cell viability was 55.6%, and the expression level of p62 also increased in a dose-dependent manner. In the time analysis, results showed that the expression level of p62 significantly decreased after 1 h of DMA treatment, but increased significantly at 24 h, further confirming that realgar can promote the expression of p62 by disturbing the homeostasis of autophagic flux. Recent studies have shown that p62, a marker protein for autophagic degradation, can activate the apoptotic pathway through the caspase-8 cascade and increased cleaved-caspase-9 cleavage. It plays an important role in regulating the process of cell death or survival[54]. After silencing p62 in SH-SY5Y cells, DMA-induced Bax/Bcl-2 and cleaved-caspase-9/caspase-9 were significantly reduced, indicating that DMA can promote neuronal apoptosis by promoting p62 overexpression. Taken together, the results suggest that realgar can perturb autophagic flux homeostasis and mediate p62 overexpression to promote neuronal apoptosis.

Autophagy is a highly dynamic process that includes autophagy induction, autophagosome formation, the autophagosome fusion with the lysosome, and the degradation stage in the lysosome [55, 56]. Autophagy induction is often controlled by the mTOR-dependent PI3K/Akt pathway [57] or mTOR-independent JNK/Vps34 pathway [58, 59]. Li found that realgar induces oxidative stress in liver tissue, activates the JNK/c-Jun signaling pathway, and then regulates the release of inflammatory factors [2]. Our results found that with the increase of realgar, the protein expression levels of p-JNK, p-c-Jun, Beclin1, and Vps34 in the cortex were significantly increased. The binding capacity of the Beclin1/Vps34 complex was enhanced in DMA-treated SH-SY5Y cells at 24 h. These results suggest that realgar exposure activates the JNK/c-Jun signaling pathway and up-regulates the Beclin1/Vps34 complex. After the intervention of JNK specific inhibitor SP600125, the protein expression levels of p-c-Jun, Beclin1, Vps34, and LC3II induced by realgar were significantly decreased and the interaction ability of Beclin1/Vps34 complexes was significantly weakened in vivo, and the formation of Beclin1/Vps34 complexes was significantly reduced after DMA treatment for 24 h in vitro. It indicates that realgar activates autophagy induction by activating the JNK/Vps34 complex pathway. However, after SP600125 intervention, the expression level of p62 induced by realgar decreased with no significant difference. This further suggests that in the case of realgar-activated autophagy induction, autophagic degradation is impaired, thereby perturbing autophagic flux homeostasis and promoting p62 aggregation.
Efficient autophagy degradation is critical for maintaining autophagic flux homeostasis in the presence of enhanced autophagy induction. Two types of deficits in autophagy-mediated degradation have been identified: (i) inhibition of autophagosome and lysosome fusion\[60\] and (ii) the disruption of lysosomal degradation function\[61\]. mRFP-GFP-LC3B is a tool for detecting autophagosome and lysosome fusion. CQ is a specific inhibitor that inhibits autophagosome and lysosome fusion. We transfected the GFP-mCherry-LC3 and used CQ as a positive control group. The results showed that CQ significantly reduced the number of autophagolysosome, while DMA significantly increased the number of autophagolysosome, indicating that DMA did not affect the fusion of autophagosomes and lysosomes.

Lysosomes contain a variety of acid hydrolases, which can degrade a variety of endogenous and exogenous macromolecules and can directly respond dynamically to autophagy\[62\]. The function of the lysosome is mainly determined by the activity of lysosomal hydrolase and the acidic environment of the lysosome\[63\]. CTSB and CTSD play important roles in the Cathepsin family \[64, 65\]. Lysosomal dysfunction due to lysosomal hydrolase dysfunction is strongly linked to neuropathology\[27\]. With the increase of realgar, the activity of CTSB and CTSD in the cortex decreased significantly in vivo, and the lysosomal acidic environment was also significantly altered. Taken together, these results suggest that realgar impairs autophagic degradation by inhibiting lysosomal hydrolase activity rather than autophagosome-lysosome fusion leading to p62 accumulation.

p62 has multiple functional domains and can interact with other proteins to mediate a variety of cellular functions. In addition to autophagy, p62 also plays an important role in antioxidant responses \[40, 66\]. The KIR domain of p62 interacts with Keap1 to regulate the expression of NRF2, a key antioxidant factor. Activated NRF2 up-regulates the level of the p62 mRNA and promotes the synthesis of the p62 protein to form a p62-NRF2 feedback loop\[40\]. Our results showed that with increasing doses of realgar exposure, both ROS levels and NRF2 protein expression levels in the cortex were significantly increased and with increasing DMA concentrations, their levels in SH-SY5Y cells also increased significantly increased.

Meanwhile, the expression level of p62 was significantly reduced after silencing NRF2 in SH-SY5Y cells. It indicates that realgar induces NRF2 activation and promotes p62 production. In addition, the binding of the p62-KEAP1 complex was enhanced and the binding of the NRF2-KEAP1 complex was weakened after DMA treatment of SH-SY5Y cells. The expression level of NRF2 was significantly reduced after silencing p62 in SH-SY5Y cells. These results suggest that NRF2 is activated in a p62-dependent manner and that NRF2 activation indirectly increases p62 transcriptional activity, activating the p62-NRF2 feedback loop, which in turn promotes p62 accumulation in DMA-exposed SH-SY5Y cells. However, the increase in ROS level may be directly induced by realgar exposure, or it may be closely related to realgar-induced protein accumulation, autophagosome accumulation, or mitochondrial damage, which still needs to be further explored.

In summary, our results suggest that realgar mediates p62-promoted neuronal apoptosis by perturbing cross-talk of autophagic flux and p62-NRF2 feedback loop, which produces CNS toxicity. On the one hand, realgar induces autophagy by activating the JNK/Vps34 complex pathway and recruits a large number of p62 aggregation; on the other hand, realgar impairs autophagic degradation by reducing the
activity of lysosomal hydrolase and changing its acidic environment, reducing the degradation of p62, leading to excessive accumulation of p62. Moreover, the amplified p62-NRF2 feedback loop also promotes the accumulation of p62 in this process. Accumulation of p62 eventually induces neuronal apoptosis. These results provide a new perspective and experimental data for the study of the mechanism of realgar-induced central nervous system toxicity and are also of great significance for clinical guidance of the rational use of realgar.

**Ethics approval**

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of China Medical University (ethics committee approval numbers: CMU2019109 and CMU2021120).

**Declarations**

**Ethics approval**

All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of China Medical University (ethics committee approval numbers: CMU2019109 and CMU2021120).

**Consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Data Availability**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing Interests**

The authors have no conflicts of interest to declare.

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**Author Contributions**
All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Rui Feng, Jieyu Liu and Zhao Yang. The first draft of the manuscript was written by Rui Feng and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Authors' information


Disclosure of potential conflicts of interest

Not applicable.

Research involving Human Participants and/or Animals

Not applicable.

Informed consent

Not applicable.

References


Figures
Figure 1

The content of arsenic species in cortex, blood and urine after realgar-exposed

a Chromatograms of different forms of arsenic in rat cortex, blood, and urine. b Content of different forms of arsenic in the cortex (n=10). c Content of different forms of arsenic in the blood (n=10). d Content of different forms of arsenic in urine (n=10). -: not detected. compared with the control group, *P<0.05.

Figure 2

Effects of realgar on neurobehavior of rats
a The trajectory diagram of the training stage in the novel object recognition test. b Recognition index of rats during the training stage (n=10). c The trajectory diagram of the testing stage in the novel object recognition test. d Recognition index of rats during testing stage (n=10). e Trajectory diagram of the open field test. f The time spent in the center zone (n=10). g The number of times to enter the center zone (n=10). h The travel distance of the rat (n=10). i The travel velocity of the rat (n=10). compared with the control group, *P<0.05. compared with the 0.3 g/kg realgar group, #P<0.05.

Figure 3

Effects of realgar on ultrastructure and apoptosis of cortex neurons

a Electron microscopy of ultrastructure of rat cortex neurons. b Fluorescence images of rat cortex neurons detected by TUNEL staining (n=3). c Apoptosis rate of rat cortex neurons (n=3). d Protein expression image of Bax, Bcl-2, caspase-9 and cleaved-caspase-9. e The ratio of Bax and Bcl-2 (n=6). f The ratio of cleaved-caspase-9 and caspase-9 (n=6). compared with the control group, *P<0.05.
Figure 4

Realgar mediated p62 to promote neuronal apoptosis by perturbing autophagic flux homeostasis

a Protein expression image of LC3I, LC3II, and p62. b The ratio of LC3II and LC3I (n=6). c Relative expression levels of p62 protein (n=6). d Observation of autophagosomes under an electron microscope. e Statistics of the number of autophagosomes (n=4). f SH-SY5Y cell viability (n=5). g Protein expression
image of p62 in SH-SY5Y cells. h Relative expression levels of p62 protein (n=3). i Protein expression image of p62 in SH-SY5Y cells after DMA treatment for different times. j Relative expression levels of p62 protein (n=3). k After silencing p62, the relative expression levels of p62 mRNA (n=3). l After silencing p62, protein expression image of Bax, Bcl-2, caspase-9, and cleaved-caspase-9. m The ratio of Bax and Bcl-2 (n=3). n The ratio of cleaved-caspase-9 and caspase-9 (n=3). compared with control group, *P<0.05, **P<0.01, compared with DMA group, ##P<0.01.

Figure 5
Realgar activated autophagy through the JNK/Vps34 complex to promote p62 aggregation

a Protein expression image of Beclin1 and Vps34. b Relative expression level of Beclin1 protein (n=6) c Relative expression level of Vps34 protein (n=6) d The binding of Beclin1-Vps34 complex was detected by CO-IP. e Protein expression image of p-JNK,JNK,p-c-Jun and c-Jun f The ratio of p-JNK and JNK (n=6). g The ratio of p-c-Jun and c-Jun (n=6). h Protein expression image of p-c-Jun,Beclin1,Vps34,p62 and LC3II/LC3I. i Relative expression level of p-c-Jun,Beclin1,Vps34,p62 and LC3II/LC3I(n=6). j The binding of Beclin1-Vps34 complex was detected by CO-IP after SP600125 intervention. k Detection of Beclin1-Vps34 complex formation by PLA. compared with the control group, *P<0.05. compared with the 0.9 g/kg realgar group, #P<0.05.

Figure 6
Realgar impairs autophagy degradation by inhibiting lysosomal hydrolase activity

a Fluorescence images of autophagosome and autolysosome after DMA and CQ treatment. b-c After DMA and CQ treatment, the statistics of the number of autophagosomes and autolysosome (10 cells per group). d Statistical chart of CTSB activity detection (n=10) e Statistical chart of CTSD activity detection (n=10). f LysoSensor Green DND-189 fluorescence image (n=3). compared with control group, *P<0.05, **P<0.01, ***P<0.001. compared with DMA group, #P<0.05.

Figure 7
Amplified p62-NRF2 feedback loop is involved in realgar-induced p62 accumulation

a ROS expression and co-localization fluorescence image of cortex neurons, the bar is 200 μm (n=3). b Relative expression level of NRF2 protein (n=6). c ROS detection flow graph. d Statistical analysis chart of ROS level (n=3). e After DMA treatment, the expression of NRF2 protein in SH-SY5Y cells and its relative
expression level (n=3) f Protein expression image of p62 after silencing NRF2. g Relative expression level of p62 protein after silencing NRF2 (n=3). h The binding of the p62-NRF2 complex was detected by CO-IP. i The binding of NRF2-KEAP1 complex was detected by CO-IP. j Protein expression image of NRF2 after silencing p62. k Relative expression level of NRF2 protein after silencing p62 (n=3). compared with control group, *P<0.05, **P<0.01, ***P<0.001. compared with DMA group, #P<0.05, ##P<0.05.

Supplementary Files

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