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Short communication

Alteration of amino acid neurotransmitters in brain tissues of immature rats treated with realgar

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ABSTRACT

Realgar is a traditional Chinese medicine, which has been used for thousands of years and are claimed to have therapeutic effects. The toxicity from realgar or realgar-containing traditional medicines has raised public concern. However, the neurotoxicity induced by realgar is less reported. Amino acid neurotransmitters are closely linked to the vulnerability of the immature brain to neuronal injury. The investigation of amino acid neurotransmitters is important to understand the evolution of developmental brain damage. An improved HPLC–UV method was developed and applied to analyzing amino acid neurotransmitters of aspartate, glutamate, glutamine, homocysteine, serine, glycine, γ-aminobutyric acid and taurine in brain tissues of immature rats after the treatment of realgar. Significant changes of these amino acid neurotransmitters were observed in realgar treated groups. Negative correlations were found between the levels of some amino acids and the contents of arsenic in brain tissues. The result indicates that the neurotoxicity induced by realgar is associated with its effects on amino acid neurotransmitters.

1. Introduction

Realgar has been used as a traditional Chinese medicine for thousands of years. Except the major component of sparingly soluble tetra-arsenic tetra-sulfides (As_4S_4), realgar has up to 10% of arsenic being present as non-sulfur bound species, such as arsenate (As^V) and arsenite (As^{III}), which is well-known for its acute and chronic toxicity [1]. In recent years, some realgar poisoning cases have been reported due to overdose, long-term use and improper processing [1–4]. Acute arsenic exposure was reported to cause problems of memory, difficulties in concentration, mental confusion and anxiety [5–7]. Therefore, the neurotoxicity from realgar or realgar-containing traditional medicines was paid most interest by human beings. However, at present, the neurotoxicity induced by realgar is less reported.

Amino acids have a wide variety of physiological and potentially pathophysiological roles during central nervous system (CNS) development [8–11]. Moreover, amino acid neurotransmitters are closely linked to the vulnerability of the immature brain to neuronal injury [12–14]. Data from studies performed in immature rats indicate that these animals may be especially susceptible to elevated concentrations of aspartate (Asp), glutamate (Glu), glutamine (Gln) and γ-aminobutyric acid (GABA) [15–18]. Therefore, the investigation of these amino acids and others is important to understand the evolution of developmental brain damage.

Currently, the effect of realgar on amino acid neurotransmitters has not been reported. In this paper, alterations of eight amino acid neurotransmitters (aspartate, glutamate, glutamine, homocysteine, serine, glycine, γ-aminobutyric acid and taurine) were measured by high performance liquid chromatography with precolumn derivatization in brain tissues of immature rats after intragastric administration of realgar. The study is fundamental to the understanding of neurotoxicity induced by realgar.

2. Materials and methods

2.1. Reagents

The standards of eight amino acid neurotransmitters and derivative reagent (dansyl chloride) were obtained from Sigma Chemical Corporation (St. Louis, MO, USA). Standard reference materials of inorganic arsine (iAs) (GBW 08611), methyl-arsenic (MMA) and dimethyl-arsine (DMA) were obtained from Tri Chemical Laboratories Inc. (Yamanashi, Japan), which contain 1000 ppm of iAs, MMA and DMA, respectively. All other reagents were of analytical-reagent grade and arsenic free (smaller than 0.01 ppm). Chromatographic-grade water was produced by a Milli-Q system.

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2.2. Animal

Thirty-two 3-week old Sprague–Dawley rats (50 ± 10 g) obtained from China Medical University were housed at (24 ± 1) °C and relative humidity maintained at (50 ± 5)%, under artificial lighting (12 h light/dark cycle). The Sprague–Dawley rats pups were randomly divided into four groups with eight rats in each group, and i.g. administrated with 0.3 g/kg (n = 8), 0.9 g/kg (n = 8) and 2.7 g/kg (n = 8) bodyweight realgar suspended in 0.5% (w/v) sodium carboxymethylcellulose (CMC-Na) or CMC-Na only (control, n = 8) once a day for consecutive 2 weeks. Then the animals were anaesthetized with 10% chloral hydrate, decapitated and brains were extracted and placed in chilled saline solution (0.9%) and stored in −70 °C until analysis.

2.3. Determination of arsenic in brain tissue

Levels of arsenic species (iAs, MMA and DMA) in tissue were determined by atomic absorption spectrophotometry with a equipment of arsenic pretreatment system (AA-6800, ASA-2SP, Shimadzu Co., Kyoto, Japan). The arsenic speciation was based on the well-established hydride generation of volatile arsines, followed by cryogenic separation in liquid nitrogen and determined by atomic absorption spectrophotometry (HG-AAS). Arsenic species were measured by the method reported previously [19]. Briefly, 0.05 g of brain samples were taken to make homogenate by adding 2 mL of deionized water, then mixed with 1 mL of 3 mol/L H_2SO_4. Samples were digested in a focused microwave field for 10 min in a 10 mL polymethylpentene test tube. Digested samples were assayed using the method based on the hydride generation of volatile arsines, followed by cryogenic separation (in liquid nitrogen) and final detection of iAs, MMA and DMA was by HG-AAS. Arsenic was detected at 193.3 nm.

2.4. Determination of amino acid neurotransmitters in brain tissue

2.4.1. Preparation of amino acid standards

Stock solution of eight amino acid neurotransmitters was prepared at concentration of 1 mmol/L in Na_2CO_3–$NaHCO_3$ buffer solution at pH 9.5. The stock solution was then serially diluted with Na_2CO_3–$NaHCO_3$ buffer solution to provide working standard solutions.

2.4.2. Sample preparation

Brain tissue samples (0.1 g) were weighted and homogenized in 2 mL of acetonitrile. The final homogenate was centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was transferred and evaporated to dryness at 40 °C under a gentle stream of nitrogen.

2.4.3. Derivatization procedure

The dried residue was reconstituted in 400 μL of Na_2CO_3–$NaHCO_3$ buffer solution (pH 9.5). An aliquot of 200 μL dansyl chloride (10 mmol/L) was added and vortex mixed. The solution was incubated in dark at 65 °C for 25 min and cooled to room temperature.

2.4.4. Chromatographic conditions

Amino acid analysis was carried out on a Waters 600 separations module with a built in vacuum degasser and a Thermo ODS column (250 mm × 4.6 mm i.d., 5 μm). The mobile phase was composed of Na_2HPO_4–NaH_2PO_4 buffer solution (pH 7.2, A) and acetonitrile (B) with a flow rate of 1.0 mL/min. The column was eluted with a linear gradient of 14–20% B over 0–7 min, 20–27% B over 7–14 min and held at 27% B for 11 min. The detection was performed at 254 nm and the injection volume was 20 μL.

2.4.5. Method validation

The calibration curve was prepared by spiking 0.1 g mixed brain tissue sample (mixture of brain tissues from 6 rats in control) with 100 μL of the amino acid working solutions. The limit of quantification (LOQ) was defined as the lowest concentration of amino acid standards that could be determined with sufficient precision and accuracy (precision of 20% and accuracy of 80–120%). Intra-day precision (% relative standard derivation, *RSD*) was evaluated on the same day, whereas inter-day precision was evaluated on three separated days. Recovery (%) was calculated by comparing the concentrations of spiked brain homogenates with those of the controls. The stability of eight amino acids was evaluated at room temperature for 8 h and the stability of brain samples was evaluated at −70 °C for 14 days.

2.5. Quantification of amino acid neurotransmitters in brain tissues

The contents of eight amino acid neurotransmitters in brain tissues of control and realgar treated groups were determined under the experimental conditions mentioned above.

2.6. Statistic analysis

The significance of differences between control and realgar treated groups were assessed by an unpaired Student's-t test. Results are expressed as the mean ± SD for amino acid neurotransmitters contents. In all cases, the differences between treatment groups were considered significant when $P < 0.05$. To investigate the relationship between amino acid neurotransmitters and the contents of arsenic and its metabolites accumulated in brain tissues, bivariate correlation analyses were performed using the contents of TAs, MMA, DMA and the contents of eight amino acid neurotransmitters in brain tissue samples from low, middle and high-dose realgar treated groups using SPSS software (13th version).

3. Results and discussion

3.1. Arsenic contents in brain tissues

The detection limits of the HG-AAS method for iAs, MMA and DMA were 1 ng. The calibration curves for iAs, MMA and DMA are $y = 148.7x + 211.7$ ($r = 0.9965$), $y = 159.4x + 42.97$ ($r = 0.9953$) and $y = 48.9x + 266.6$ ($r = 0.9973$), respectively, in the content range of 1.56–12.5, 1.56–12.5 and 7.81–62.5 ng/mL. The contents of iAs, MMA, DMA and TAs (iAs + MMA + DMA) in brain tissues of realgar treated groups are significantly increased compared with controls ($P < 0.05$). The mean contents of iAs, MMA, DMA and TAs in brain tissues of control, low, middle and high-dosage realgar treated groups are displayed in Table 1. Dose-related trend was found between total brain arsenic contents and realgar given to rats ($P < 0.05$).

3.2. Optimization of the derivatization procedure

The main factors affecting derivatization yields (pH value of buffer, reaction time, temperature and concentration of derivative reagent) were investigated in our study to make the derivatization reaction completely. The optimum derivatization condition is described in Section 2.4.3 and under the optimum derivatization conditions, the derivatization reaction rate of eight amino acid neurotransmitters were all 100%.

Table 1
Contents of iAs, MMA, DMA and TAs in brain tissues of control, low, middle and high-dosage realgar treated rats.

Group	iAs (ng/g)	MMA (ng/g)	DMA (ng/g)	TAs (ng/g)
Control	*n.d.*	*n.d.*	*n.d.*	*n.d.*
Low dosage	*n.d.*	140 ± 22	1597 ± 228	1720 ± 246
Middle dosage	*n.d.*	299 ± 89	3687 ± 846	3986 ± 924
High dosage	*n.d.*	435 ± 112	3896 ± 943	4332 ± 1047

n.d.: not detected.

3.3. Method development and validation

In our study, an improved HPLC–UV method was established. Compared with the original method [20], some improvements were made. Dansyl-Cl was chosen as the derivatization reagent, which has been used extensively for the analysis of amino acids at trace level [21], and the derivatization reaction conditions were optimized. Simpler mobile phase was applied and the gradient elution program was improved to get better resolution.

Fig. 1A illustrates the peaks of eight amino acid standards. Fig. 1B shows an application of the method for brain analysis. The chromatogram shows the well-resolved peaks of eight amino acids and other unknown substances. The method showed a good overall intra-day (5.7–11.8%) and inter-day (4.2–12%) validation. The calibration curves for all eight amino acids yielded good linearity with correlation coefficients of >0.99 in the range of 0.0525–108 μg/g (Table 2). Furthermore, 73.4–105.8% recovery was obtained by spiking known amounts of a mixture of amino acid standards into the brain homogenates. The eight amino acid neurotransmitters were stable at room temperature for 8 h and the brain samples were stable at −70 °C for 14 days.

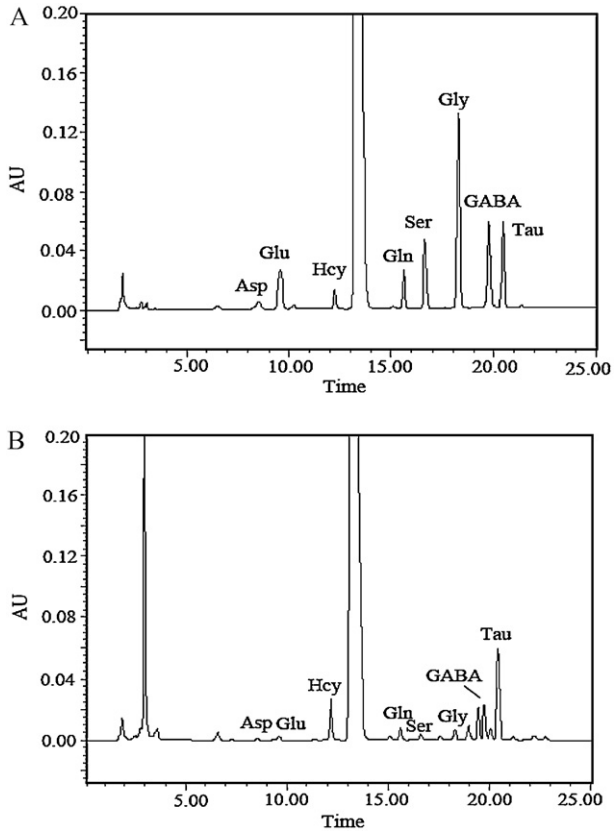

Fig. 1. Typical chromatograms of eight amino acid neurotransmitters standard solution (A) and a brain tissue sample from control group (B).

3.4. Contents of amino acid neurotransmitters in brain tissues

Fig. 2 shows the contents of eight amino acid neurotransmitters in brain tissues of immature rats untreated and treated with realgar. Fig. 2 illustrates that levels of eight amino acid neurotransmitters in realgar treated groups are different from those in controls. The levels of serine (Ser), glycine (Gly) and GABA are significantly elevated in low dose realgar treated group compared with controls; while the levels of Hcy, Gln and Ser are obviously decreased in middle and high dose realgar treated groups compared with controls. Moreover, the level of Asp is lower in high dose realgar treated group than that in control group. From Fig. 2, it can be seen that the change trends of eight amino acid neurotransmitters in low dose realgar treated group are different from those in middle and high dosage group, which may indicate different neurotoxic mechanism of low dose and middle/high dose realgar on immature rats.

3.5. Relationship between amino acid neurotransmitters and arsenic content in brain tissues of realgar treated rats

No correlation was found between amino acid neurotransmitters and contents of TAs, MMA, DMA in brain tissues of low dose realgar treated group. Negative correlation was found between the levels of GABA and MMA contents in brain tissues of middle dose realgar treated rats. Negative correlation was also found between the levels of Asp and TAs, DMA; Hcy and MMA, DMA; Gln, Ser and TAs; GABA and MMA, DMA in high dose realgar treated group. The results indicate that the effect of realgar on amino acid neurotransmitters is associated with arsenic accumulation in brain tissues (Table 3).

3.6. Amino acid neurotransmitters and neurotoxicity

In low dose realgar treated group, significant increases of Gly, GABA and Ser were found in brain tissues of immature rats. GABA is the chief inhibitory neurotransmitter in the mammalian CNS. However, in developing brain, GABA plays an excitatory role. This action appears to be instrumental in signaling and controlling proliferation, migration and maturation of neurons. Once neuronal maturation is complete, GABA activity becomes inhibitory [22]. Gly is the second major inhibitory neurotransmitter in the CNS. In the brain, Gly acts as a co-agonist with Glu on N-methyl D-aspartate (NMDA) receptors and in this situation potentiates the effect of Glu, that is, it facilitates excitation rather than act as an inhibitor [22]. Elevated levels of GABA and Gly found in low dose realgar treated group may indicate excitotoxicity of low dosage realgar on immature rat brain. Ser is an immediate precursor of Gly. The levels of Ser are elevated when increased cellular proliferation is taking place [10]. In addition, it may function as an endogenous agonist of the Gly site on the NMDA receptor [23,24]. Taken together, this may indicate that Ser has an important role in NMDA-mediated excitotoxicity and optimal NMDA receptor functioning. It is likely that serious dysfunction would occur in the brain when Ser biosynthesis, catabolism and Ser/Gly metabolism are compromised or altered [25]. Therefore, elevated Ser levels induced by realgar may

Table 2
Calibration curves and limit of quantification for eight amino acids neurotransmitters.

Amino acid neurotransmitters	Range (μg/g)	Equation	r	LOQ (μg/g)
Asp	0.0665–13.3	$y = 636.2x - 676.8$	0.9965	0.0333
Glu	0.0735–14.7	$y = 742.6x - 268.5$	0.9995	0.0368
Hcy	6.75–108	$y = 84.49x + 9039$	0.9986	0.0135
Gln	1.46–29.2	$y = 723.5x - 401.5$	0.9980	0.0365
Ser	0.0525–10.5	$y = 920.8x - 379.9$	0.9961	0.0263
Gly	0.0875–17.5	$y = 3223x - 4152$	0.9964	0.0175
GABA	5.15–82.4	$y = 1477x - 3071$	0.9990	0.0258
Tau	6.25–100	$y = 1080x + 21{,}526$	0.9963	0.0313

Table 3
Correlation between amino acid neurotransmitters and the contents of arsenic and its metabolites accumulated in brain tissues.

Amino acids	Low dosage			Middle dosage			High dosage		
	TAs	MMA	DMA	TAs	MMA	DMA	TAs	MMA	DMA
Asp	−0.38	−0.33	−0.38	−0.64	−0.64	−0.64	−0.67*	−0.34	−0.70*
Glu	−0.22	−0.16	−0.22	−0.36	−0.33	−0.36	−0.59	−0.60	−0.58
Hcy	−0.77	−0.70	−0.78	−0.44	−0.43	−0.44	−0.78	−0.64*	−0.79*
Gln	−0.20	−0.10	−0.21	−0.07	−0.22	−0.06	−0.87*	−0.79	−0.87
Ser	0.21	0.23	0.21	−0.63	−0.46	−0.64	−0.72*	−0.60	−0.72
Gly	−0.33	−0.27	−0.34	−0.30	−0.24	−0.30	−0.64	−0.63	−0.63
GABA	−0.51	−0.46	−0.51	−0.58	−0.72*	−0.56	−0.91	−0.92*	−0.90*
Tau	0.70	0.75	0.69	−0.61	−0.26	−0.64	−0.65	−0.68	−0.64

* $P < 0.05$.

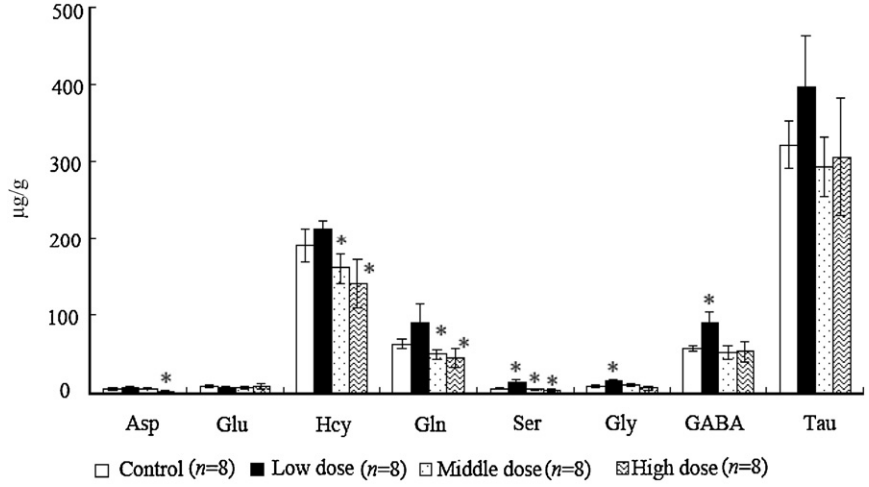

Fig. 2. Levels of Asp, Glu, Hcy, Gln, Ser, Gly, Tau and GABA in control and realgar treated groups.

be indicative of excitotoxicity and increased cellular proliferation in response to injury in the brain.

In middle and high dose realgar treated groups, obvious decrease of Hcy, Gln and Ser were found and a lower level of Asp was also found in high dose realgar treated group. Glu and Asp are the most abundant free amino acids in the mammalian brain and are classed as excitatory neurotransmitters that are released in a Ca-dependent manner. They can activate NMDA receptors, which have been implicated in activities ranging from learning and memory to development and specification of nerve contacts in a developing animal. In our study, significant decrease of Asp was observed in high dose realgar treated group which may partially be contributed to the low ability of learning and memory of realgar treated rats. Ser can optimize NMDA receptor functioning and decrease of Ser may also be associated with the low ability of learning and memory of realgar treated rats. Gln is an essential precursor of the amino acid neurotransmitters glutamate and GABA, as well as an important energy metabolite in brain. Glu and Gln may be interconverted through activity of glutamine synthetase (GS) or glutaminase. Decrease of Gln in middle and high dose realgar treated group indicate a disturbance of Glu–Gln cycle, which has been reported to be disturbed by arsenic [26]. Hcy is a non-essential sulfur-containing amino acid that is derived from methionine metabolism. The recent interest in Hcy in neuropsychiatry stems from an increasing appreciation of Hcy as a neurotoxin [27] and a risk factor for cerebrovascular disease. Hcy is reported to be involved in arsenic methylation [28], and negative correlations of MMA and DMA with Hcy were observed in brain in our study. The decrease of Hcy may be due to increased arsenic methylation metabolites accumulation in realgar treated rats.

4. Conclusions

An improved HPLC–UV method for the simultaneous measurement of amino acid neurotransmitters was successfully developed and applied to measure eight major amino acid neurotransmitters in brain tissues of immature rats treated with realgar. To the best of our knowledge, we are the first to report the levels of Asp, Glu, Hcy, Gln, Ser, Gly, GABA and Tau in brain tissues of realgar treated rats. Several results are obtain: (a) significant changes of amino acid

neurotransmitters were observed in realgar treated groups compared with controls; (b) the neurotoxic mechanisms of low dose and middle/high dose realgar on amino acid neurotransmitters in immature rats are different; (c) the effect of realgar on amino acid neurotransmitters is associated with arsenic accumulation in brain tissues.

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