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Differential expression of serum proteins in rats subchronically exposed to arsenic identified by iTRAQ-based proteomic technology——14-3-3 ζ protein to serve as a potential biomarker

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Arsenic is a multi-system toxicant. However, the mechanism of arsenic toxicity is not fully clarified and lacks of effective protein biomarkers could be used for arsenic poisoning. This study was to investigate the differentially expressed proteins in serum of rats subchronically exposed to arsenic. Sixty male rats were randomly divided into four groups, and the dose of sodium arsenite in drinking water for each group was 0, 2, 10, and 50 mg/L, respectively. The exposure lasted for 12 weeks. An Isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomic approach was used to identify the differentially expressed proteins in serum between control and 50 mg/L groups. A total of 201 serum proteins were identified by iTRAQ, of which 12 were significantly changed by arsenic exposure with two up-regulated and ten down-regulated. One down-regulated protein 14-3-3 ζ, an abundant protein expressed in the brain, was verified by ELISA assay using serum samples and by immunohistochemical, real-time PCR, and western blot methods using brain tissues in four groups. Our work provided valuable insight into the serum protein changes in rats exposed to arsenic, and indicated that 14-3-3 ζ may serve as a useful biomarker for nervous damage caused by arsenic poisoning.

Introduction

Arsenic is a naturally occurring metalloid, which is widely distributed throughout the environment in water, food, and air. Arsenic exposure is a public health problem worldwide. Many countries have areas where arsenic in drinking water is at high levels, such as Bangladesh, India, China, the United States, Argentina, Mexico, etc. [1] Millions of people in the world, including more than 3 million in the United States, more than 70 million in Bangladesh and adjoining West Bengal, India, are exposed to high levels of arsenic in drinking water.[2] In China, to now our best estimate of population exposed to arsenic in drinking water over 10 μg/L are 6 million, of these nearly 1.9 million are over 50 μg/L (data unpublished).

The main clinical manifestations of chronic arsenicosis are skin depigmentation, hyperpigmentation, palmoplantar keratosis, and even cancer.[3] In addition to the typical skin lesions, chronic arsenic exposure can even cause damages of cardiovascular, neurological,

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respiratory, hematopoietic, immune systems and so on, [4, 5] indicating that the hazards of chronic arsenic exposure to health are systemic in nature. In recent years, the neurotoxicity effect of arsenic has caused extensive concern of the researchers. It has been well reviewed that arsenic exposure induced neurological dysfunction, causing human or animal behavior change and cognitive impairment.[6, 7] Neurotoxic effects amounting to behavioral deficits and alterations in locomotor activity have been observed in adult male rats following exposure to arsenic.[8] Significant hearing loss and influence on reduce the intelligence[9] and disorders on learning memory[10] have been reported in children who live in the chronic high-arsenic exposure area. However, the precise mechanism of arsenic toxicity has not been completely elucidated, also lacks of biomarker are effective in early screening and diagnosis for arsenic-induced damage of internal organs, including the brain.

A large number of reports have revealed that 14-3-3 ζ is an important regulatory protein, which has been implicated in several neurological diseases such as epilepsy, bipolar disorder, mental retardatio and schizophrenia et al. Taken together with the findings that this gene is consistently down-regulated in post-mortem brain samples at the mRNA [11] and protein levels, [12] these data collectively identify 14-3-3 ζ as a potential risk factor for neurodevelopmental disorders. In summary, arsenic has neurologic toxicity, and 14-3-3 ζ plays an important role in the nervous system injury.

Proteomics is an emerging discipline which studies a certain type of cell, tissue or body fluid composition and functions of all proteins in a large-scale, high-throughput, and systematic way.[13] Isobaric tags for relative and absolute quantitation (iTRAQ) technology is a new isotope labeling technique in vitro launched by Applied Biosystems Inc. in 2004,[14] characterized by high throughput, high sensitivity, accuracy, and the combination with mass spectrometry online.[15, 16] Coupled with liquid chromatography-tandem mass spectrometry, iTRAQ can simultaneously isolate and identify hundreds of proteins to get "full set of information" to a maximum degree.[17, 18] In recent years, iTRAQ technology is widely used in identifying potential biomarkers in neurodegenerative diseases,[19, 20] lung squamous cell cancer,[21] gastrointestinal tract cancer,[22] and breast cancer,[23] et al. To the best of our knowledge, iTRAQ-based proteomic technology has not been used in the field of arsenic poisoning.

So, in this study, an iTRAQ-based proteomic technology was applied to screen the differential expressed serum proteins in rats subchronically exposed to arsenic. As one of our interests was to identify the

biomarkers involved in the nervous toxicity of arsenic poisoning, the down-regulated expression of a high abundant protein in brain, 14-3-3 ζ was further verified in both serum and brain tissue using proper methods.

Materials and methods

Animals and treatment

Sixty healthy male Wistar rats weighing 170-200g were chosen as our study animals. Rats were obtained from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All rats were housed in an air-conditioned room with a temperature maintained at 20±2□, a humidity level of 50±15%, and a 12-h light/dark cycle. The animals were randomly divided into four groups (each 15 rats). Based on median lethal dose (LD50) of sodium arsenite through oral administration of rats is 41 mg/kg, about 1/5 of the LD50 was chosen as the high-arsenic exposure dose (approximately 50mg/L sodium arsenite in drinking water), the correspondent middle-arsenic dose is 1/5 of the high-arsenic dose (10mg/L sodium arsenite in drinking water), the correspondent low-arsenic dose is 1/5 of the middle arsenic dose (2mg/L sodium arsenite in drinking water), and the control group had no sodium arsenite in the drinking water. All rats were fed ad libitum with a standard rat chow and water. The rats were weight weekly and the arsenic exposure lasted for 12 weeks. Then the rats were fasted overnight and sacrificed by chloral hydrate anesthesia. The urine of rats was collected at 12 weeks and stored at -20 ℃ for use. Blood was collected and serum was separated to small pack, stored at -80 ℃ until use. All experiments were approved by the Animal Ethics Committee of Harbin Medical University and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996, USA).

Arsenic level assay

The levels of total arsenic in urine were determined using hydride generation atomic fluorescence spectrometry (HG-AFS) according to a national standardized method in China (GB/T 5009.11-2003) as described before.[24]

Serum sample preparation

Twelve serum samples were randomly selected from control and high-arsenic groups, respectively. Then equal volumes from every four individuals of each group were pooled. So six distinct pooled samples were yield, and named as C1, C2, C3 in control group and E1, E2, E3 in high-arsenic group, respectively. To deplete the high-abundance proteins of each serum pool, Multiple Affinity Removal Column (H-14) (Agilent, USA) was used according to the manufacture's instruction, before which samples were diluted with H-14 buffer (1:5) and filtrated with 0.22μm membrane (Corning Incorporated, USA). Serum pools were then desalted with ultrafiltration filter (5 kDa cutoff, Sartorius, Germany). The total protein content of each pooled serum was quantified with a Bradford

protein assay kit (Bio-Rad, USA) and split into 100μg each tube, then stored at -80□ for further analysis.

iTRAQ labeling

Details of serum pretreatment before iTRAQ reagents labeling are described in Supplementary materials. Subsequently, a 100 μg peptide mixture from each serum pool was labeled with 8-plex iTRAQ reagents according to the manufacturer's instructions (Applied Biosystems, USA). Sample C1, C2, C3, E1, E2, and E3 were labeled with reagent 113, 114, 115, 116, 117, and 118, respectively.

Capillary high performance liquid chromatography separation and MS/MS analysis

Samples were separated with a capillary high performance liquid chromatography system, Easy nLC1000 (Thermo Fisher Scientific, USA). Details of buffers, columns, and the condition of segmented gradient separation are described in Supplementary materials. The Q-Exactive (Thermo Finnigan, USA) mass spectrometer was used to perform data acquisition. Instrument parameters were set as in the Supplementary materials.

Protein identification, quantification, and bioinformatics

Raw files of the resulting MS/MS spectra were searched against the uniprot rat databases (20130308, 41,766 sequences) with MASCOT software (version 2.2, Matrix Science, London, U.K.). The details of parameters were set as in the Supplementary materials. Relative levels of protein quantification across multiple samples were analyzed by Protein Discoverer 1.4, and the sum of total six channel signals was set as the reference. Statistical analysis was conducted using t-test. A threshold of 1.2-fold change and a *p* value below 0.05 were considered as significant changes. The functional classifications of the total and differentially expressed proteins were performed using BLAST2TO (version2.8) and pathway annotation using KEGG Automatic Annotation Server (http://www.genome.jp/tools/kaas/).

Enzyme-Linked Immunosorbent Assay

Rat serum samples were diluted in proper concentration. The expression of 14-3-3 ζ in serum was detected among four groups using ELISA assay kit (catalog number SEJ809Mi, USCN, Wuhan, China) according to the manufacturer's instructions.

Immunohistochemical analysis

The samples from cerebrum cortex of each rat were fixed in 4% paraformaldehyde and embedded in paraffin. The cerebrum cortex of the tissue was cut at 4 μm and placed on poly-L-lysine coated slides. Brain tissue sections from each group were dewaxed and dehydrated in a graded series of ethanol concentrations. Then the slides were immersed in 0.01 mol/L sodium citrate buffer (pH6.0) and pretreated in a microwave oven for 15 min. Activity of endogenous peroxidase was quenched using 3% hydrogen peroxide (H_2O_2) for 15 min at room temperature. The sections were blocked

with normal goat serum for 30 min. Sections were then incubated with anti-14-3-3 ζ antibody (1:100 dilution; catalog number Ab51129, Abcam, UK) overnight at 4°C. After washing with PBS, sections were incubated with biotinylated horseradish peroxidase-conjugated secondary antibody (Boster Bio-Engineering, China) for 30 min at 37°C. DAB chromogenic microscopic observation in tan particles is positive. Sections were counterstained with hematoxylin for termination reaction. Photos were taken using microscope Olympus BX-53 (Japan). Approximately 1000 cells in five randomly chosen fields in the whole granular cell layer of cerebrum cortex from each rat were counted at 400X magnification in a masked manner. The percentage of positive cells was then calculated using the following formula.

The percentage of positive cells = positive stained cells /(positive stained cells + negative stained cells) ×100%

Real time PCR analysis

Total RNA was extracted from the cerebrum cortex of rats with Trizol Reagent. Reverse transcription and real time PCR were performed by using Takara PrimeScript™ RT reagent kit and *SYBR Premix Ex Taq*™ kit according to the manufacturer's instructions. The forward primer for 14-3-3 ζ was 5′-AAAAGCAGCAGATGGCTCGA-3′ and reverse 5′-TTCTGGCTGCGAAGCATTG-3′. The forward primer for β-actin was 5′-GACCAGAGGCATACAGGGACAA-3′ and reverse 5′-CTGAACCCTAAGGCCAACCG-3′. Data were analyzed using the $2^{-\triangle\triangle CT}$ method.[25]

Western blot analysis

Appropriate amounts of cerebrum cortex were lysed in Cell lysis buffer for Western and IP (Beyotime Institute of Biotechnology, China) on ice, then the homogenates were centrifuged at 14,000×g for 5 min and the supernatant was obtained. The total protein concentration was quantified using a bicinchoninic acid assay (BCA) kit (Beyotime Institute of Biotechnology, China). An equal amount of protein for each sample in loading buffer was heated at 100℃ for 5 min and was separated by electrophoresis in 10% SDS-PAGE gels, and then transferred onto PVDF membranes (Millipore Corporation, USA). After reacting with anti-14-3-3 ζ antibody (1:500 dilution; catalog number 7413S, CST, America), and anti-β-actin antibody (1:1000 dilution; Zhongshan Golden Bridge Biotechnology, China), the membrane was then incubated with alkaline phosphatased secondary antibody (1:1000 dilution; Zhongshan Golden Bridge Biotechnology, China). The immunoreactivity was visualized using Tanon Gis-2020 imaging system (Shanghai, China).

TdT-mediated dUTP Nick-End Labeling staining

The apoptosis of neuron in cerebrum cortex was evaluated by TdT-mediated dUTP nick-end labeling (TUNEL) staining using an InSitu Cell Death Detection Kit (Roche, Germany). Rat paraffin sections were prepared as immunohistochemical analysis. The procedure of TUNEL assay was performed according to the manufacturer's instructions with slight adjustment. Briefly, the sections were incubated with TUNEL reaction medium (30-μL each) at 37 °C for1 h in a humidified chamber. Then 30-μL converter POD was added to each section and incubated at 37 °C for 30 min (POD was diluted with Tris-Hcl PH=7.5, dilution ratio was 1:16). DAB chromogenic microscopic observation in brown staining of the nucleus is apoptosis positive. The quantification of TUNEL-positive cells was analyzed as immunohistochemical analysis but only approximately 500 cells of each section were counted.

Statistical analysis

SPSS19.0 statistical software package was used for data analysis. Except for urinary arsenic level which was compared by Kruskal-Wallis H test among four groups, other indexes were analyzed by one-way ANOVA analysis. Nemenyi test was used for two-group comparison after Kruskal-Wallis H test, while LSD test was used after one-way ANOVA analysis. *P* value less than 0.05 was considered statistically significant for all analysis in this study.

Results

Body weight and urinary arsenic level of rats

Body weight and urinary arsenic level of rats at the end of the experiment are provided in table 1. The doses and period of arsenic exposure in this study had no significant effect on the body weight of rats as compared with control group. However, the levels of arsenic in urine were significant different among four groups and between every two groups. The levels of arsenic in urine increased gradually as the doses of arsenic exposure increased.

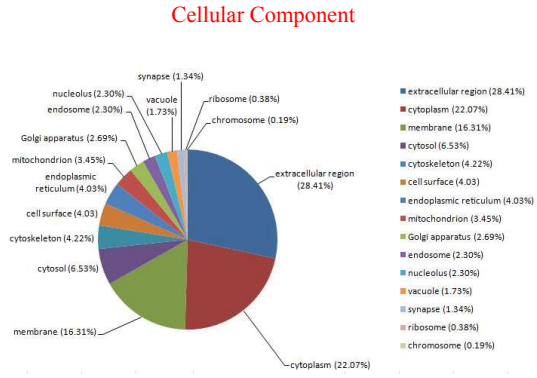
Table 1 Body weight and urinary arsenic level among four groups

	control	2 mg/L $NaAsO_2$	10 mg/L $NaAsO_2$	50 mg/L $NaAsO_2$
Body weight Mean ± SD (g)	458.8 ± 47.7	437.4 ± 35.1	440.7 ± 28.5	442.7 ± 46.8g
Urinary As Median (range) (mg/L)	0.012 (0.006-0.037) [a]	0.088 (0.048-0.361) [b]	0.790 (0.454-2.808) [c]	11.245 (1.640-19.458) [d]

[a, b, c, d] Values labeled with different letters are significantly different between every two groups, $p<0.05$.

Spectrum and Go analysis of identified proteins of rat serum

Total 1742 peptides in rat serum were identified covering 209 proteins, of which 201 proteins simultaneously existed in six channels with quantitative information and were included in following analysis.



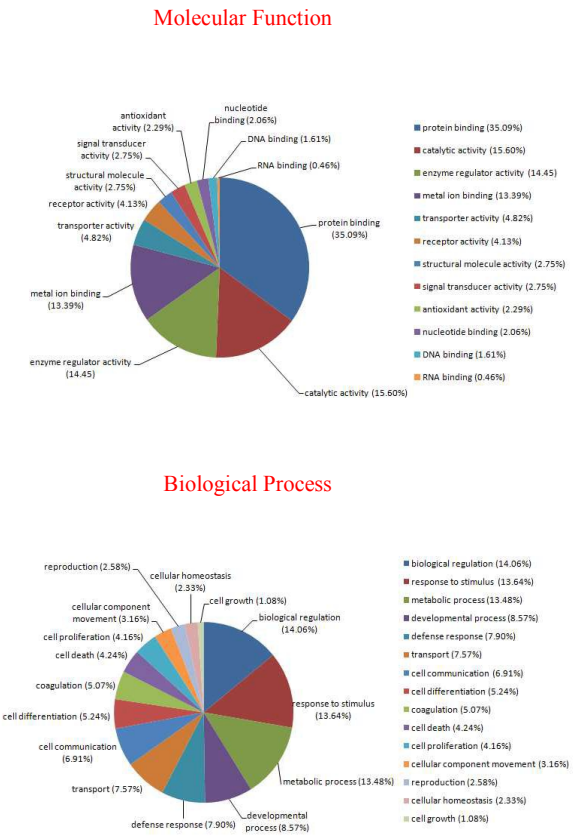


Fig. 1 Go classification of the identified proteins of rat serum: cellular component, molecular function, and biological process.

Functional information of these 201 proteins was annotated according to their cellular component, molecular function, and biological processes using BLAST2TO (version2.8) (Fig.1). In the cellular component analysis, we found that most of the rat serum proteins were associated with extracellular region, cytoplasm, membrane, and cytosol. Molecular function-based analysis demonstrated that most of these proteins were found to play a role in protein binding, catalytic activity, enzyme regulator activity, and metal ion binding. The main biological processes of these proteins were biological regulation, response to stimulus, metabolic process, development process, defense response, and transport.

Differential proteins identification and pathway annotation

On the basis of screening rules for differentially expressed proteins, 12 proteins were significantly changed by arsenic exposure, including 2 up-regulated and 10 down-regulated. Details of differentially expressed proteins are in Table 2. Among the twelve differential expressed serum proteins, two were hypothetical proteins which were excluded for the following bioinformatic pathway annotation. Pathway annotation by Kegg analysis showed that the differentially expressed proteins are involved in Rap1 signaling pathway (Rap1, Profilin), Platelet activation (Rap1, Fermt 3), PPAR signaling

Table 2 The list of serum differentially expressed protein in rats induced by subchronic arsenic exposure through iTRAQ identification.

Accession	Protein	Gene symbol	Cov%	No. of unique peptides	MW（kD）	Calc. pI	E/C*
Q6P9U7	L-lactate dehydrogenase	Ldha	3.7	1	29.81	9.06	0.71
P20673	Argininosuccinate lyase	Asl	1.08	1	51.52	6.40	1.30
O08813	Rap 1A	Rap1a	10.64	1	10.79	4.41	0.76
P63102	14-3-3 ζ/δ	Ywhaz	5.71	1	27.75	4.79	0.69
Q5XIF6	Tubulin α-4A	Tuba4a	3.35	1	49.89	5.06	0.67
B2GVB9	Fermt3	Fermt3	1.35	1	75.57	7.08	0.76
P62963	Profilin-1	Pfn1	16.43	2	14.95	8.28	0.78
P06866	Haptoglobin	Hp	51.59	19	38.54	6.54	0.74
P04638	Apolipoprotein A-II	Apoa2	52.94	4	11.43	6.65	1.40
Q8K3R4	Adiponectin	Adipoq	3.69	1	26.39	5.74	0.75
V9GZ85	Protein LOC100361457	LOC100361457	44.92	5	41.59	5.71	0.79
D3ZUU6	Protein LOC100912012	LOC100912012	5.94	1	22.29	5.44	0.76

* relative expression, E: high dose arsenic group, C: control group. The ratio of E/C greater than 1.2 indicates up-regulation of more than 1.2 times, and the ratio less than 0.83 indicates down-regulation of more than 1.2 times.

pathway(Apolipoprotein A-II, Adiponectin), Hippo signaling pathway (14-3-3 ζ/δ), Type II diabetes mellitus (Adiponectin), Adipocytokine signaling pathway (Adiponectin), et al. Pathways above may be changed by arsenic exposure and involved in the toxic mechanism of arsenic poisoning. Representative MS/MS spectrums showing the peptide from 14-3-3 ζ/δ is given in Fig. 2.

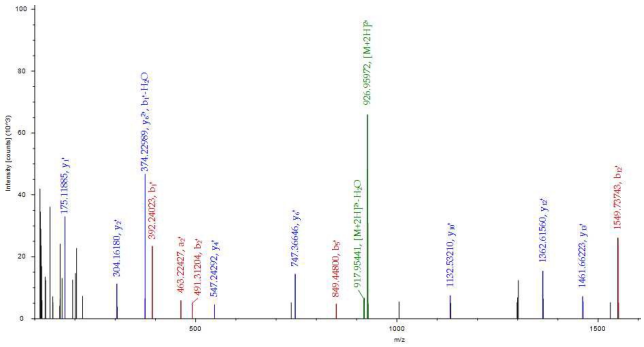


Fig. 2 Representative MS/MS spectrum showing the peptides from 14-3-3 ζ/δ (peptide sequence: SVTEQGAELSNEER).

Verification of 14-3-3 ζ expression in serum

The concentrations of serum 14-3-3 ζ in control, low-arsenic, middle-arsenic, and high-arsenic groups were 2.66±0.61, 2.36±0.38, 2.44±0.50, and 2.13±0.40 μg/mL, respectively. Result is shown in Fig. 3. The concentrations of serum 14-3-3 ζ in arsenic exposure groups tended to decrease, but only that of the high-arsenic group was significantly decreased as compared to control ($p<0.05$), which was consistent with the iTRAQ experiment.

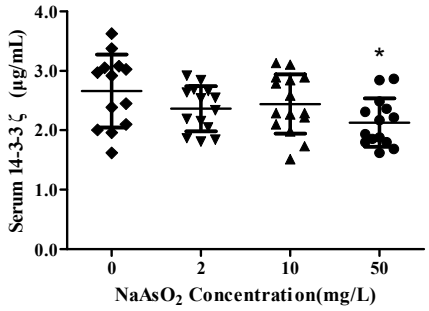


Fig. 3 Serum 14-3-3 ζ level of rats exposed to different level of arsenic analyzed by ELISA assay. * indicates $p<0.05$ vs control.

Verification of 14-3-3 ζ expression in the brain

The localization and expression of 14-3-3 ζ in cerebrum cortex was first analyzed by Immunohistochemical assay. Positive staining for 14-3-3 ζ was abundant in rat cerebrum cortex. 14-3-3 ζ was mainly found in the cytoplasm and nucleus. As shown in Fig. 4 A-D, the immunoreactivity of 14-3-3 ζ was gradually attenuated in the extra granular cell layer as arsenic level increased, resulting in a massive negative staining cells in high-arsenic exposure group. The statistical analysis indicated that the average percentage of immuno-positive staining cells for 14-3-3 ζ in control, low-arsenic, middle-arsenic, and high-arsenic groups were 59±4%, 49±2%, 41±2%, and 34±3%, respectively. Result is shown in Fig. 4 E. At last, the expression of 14-3-3ζ was further verified at transcription and translation levels using real time PCR and Western blot methods, which showed that the expression of 14-3-3 ζ in cerebrum cortex tended to be decreased by arsenic

exposure, but the significance only existed between high-arsenic and control groups（$p<0.05$）(Fig.5). This trend is similar as the protein expression in serum obtained by ELISA analysis.

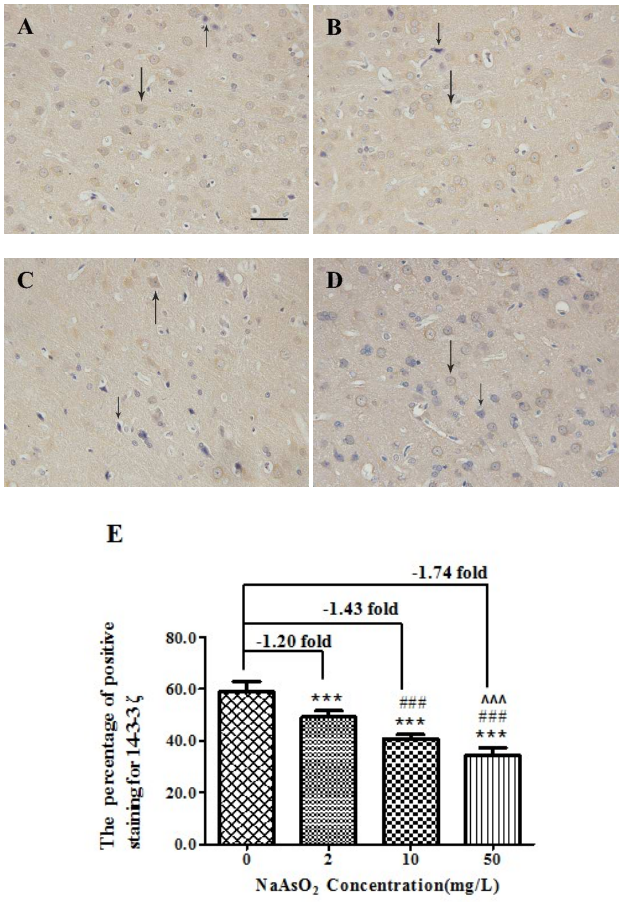


Fig. 4 The expression of 14-3-3 ζ in the granular cell layer of cerebrum cortex of rats in response to different level of arsenic exposure analyzed by immunohistochemical assay. (A)-(D) the expression of 14-3-3 ζ in control group, low-arsenic group, middle-arsenic group, and high-arsenic group, respectively. Magnification is 400x. Long arrow indicates positive staining cells. Short arrow indicates negative staining cells. Scale bar=40μm. (E) The quantification analysis of immunostaining for 14-3-3 ζ in four groups (n=6/group). *** designates significance from control at $p < 0.001$, ### designates significance from low-arsenic group at $p < 0.001$, ^^^ designates significance from middle-arsenic group at $p < 0.001$.

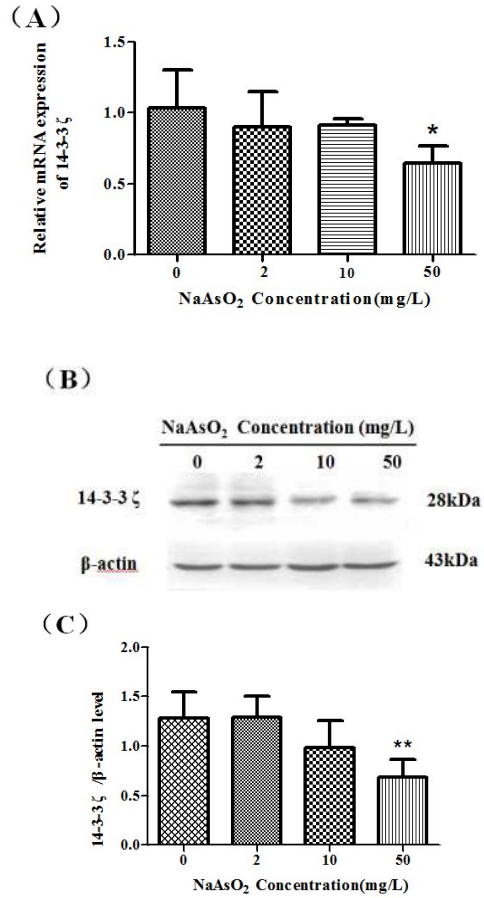
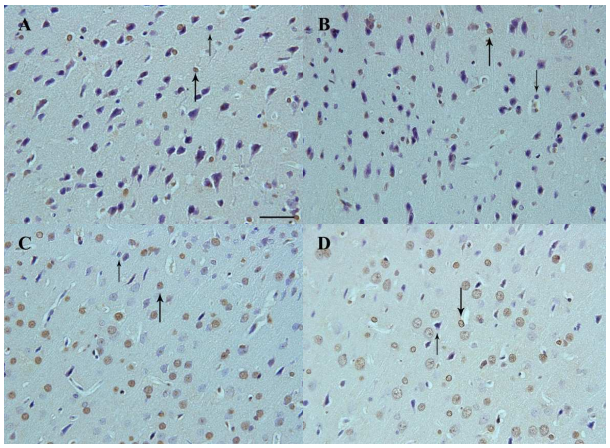


Fig. 5 The expression of 14-3-3 ζ in cerebral cortex of rats in different level of arsenic exposure groups analyzed by real time PCR and Western blot assays. (A) The expression of 14-3-3 ζ mRNA (normalized to β-actin) in cerebral cortex of rats in different level of arsenic exposure groups. n=6/group, *indicates $p<0.05$ vs control; (B) and (C) The expression of 14-3-3 ζ protein (normalized to β-actin) in cerebral cortex of rats in different level of arsenic exposure groups. n=6/group, **indicates $p<0.01$ vs control.

Neuroapoptosis in the brain of rats exposed to different level of arsenic

TUNEL results showed that some positive neuroapoptosis appeared in cerebrum cortex of control group, and significant more TUNEL positive cells were found in arsenic exposure groups. The proportion of apoptotic cells increased gradually with arsenic concentration increased. These findings implied that apoptosis in the brain was induced by arsenic exposure.



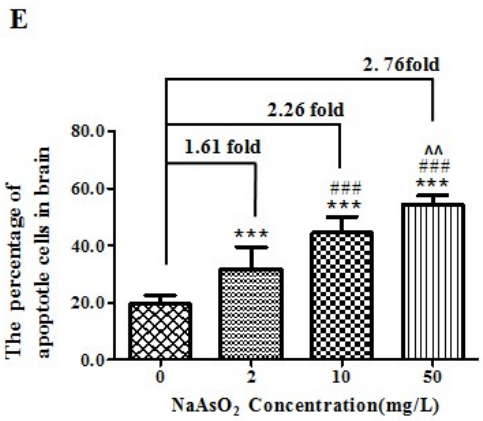


Fig. 5 Apoptosis in the granular cell layer of cerebrum cortex of rats in response to different level of arsenic exposure analyzed by TUNEL staining. (A)-(D) Neuroapoptosis in control group, low-arsenic group, middle-arsenic group, and high-arsenic group, respectively. Magnification is 400x. Long arrow indicates apoptotic staining cells. Short arrow indicates non-apoptotic cells. Scale bar=40μm. (E) The quantification analysis of TUNEL staining in four groups (n=5/group). *** designates significance from control at $p < 0.001$, ### designates significance from low-arsenic group at $p < 0.001$, ^^ designates significance from middle-arsenic group at $p < 0.01$.

Discussion

Serum is an attractive sample for identifying potential protein biomarkers because of easy accessibility. Moreover, it is an archive of information on the physiological and pathological conditions.[26] Compared with conventional techniques for the detection of specific already known proteins, the advantages of proteomics technology are to separate and identify proteins with characteristics of high throughout, high sensitivity, low detection limit, and helpful to discover new disease related proteins.

In recent years, several proteomic studies aimed to screen the differential proteins of arsenic exposure using samples of cells, animals, or human being.[27-31] Limitations of these reports are the defects of the proteomic technique themselves as well as the absent verification experiment, which is usually an obstacle for proteomic research, thus reduce the reliability of the previous proteomic results. As compared to animal models, the arsenic exposure information of residents living in rural China is usually hard to evaluate due to change of water source and also the arsenic exposure is likely compounded with exposure to pollution, poor diet, and low socioeconomic status, et al. Thus, in the present study, iTRAQ technology coupled with liquid chromatography-tandem mass spectrometry was used to screen the potential serum protein biomarkers of arsenic exposure in rats model flowed by verification experiments to conform the results of former proteomic test.

This study was comprised of two stages, a discovery stage and a verification stage. The discovery stage

consisted of control and high-arsenic exposure groups, while control and three different levels of arsenic exposure groups were included in the verification stage. Only two groups were involved in the discovery stage in consideration of the expense of proteomic test. The dose-response relationship between arsenic exposure and the expression of differential serum proteins are expected to be presented at the verification stage. In the discovery stage, a total of 201 serum proteins were identified, of which 12 were significantly changed by arsenic exposure, including 2 up-regulated and 10 down-regulated. According to the literature review, four differential proteins, including L-lactate dehydrogenase, Haptoglobin, Adiponectin, and 14-3-3 ζ were ever reported to be associated with arsenic exposure,[32-37] while others were not. As one of our interests was to identify the proteins involved in the nervous toxicity of arsenic poisoning, the down-regulated expression of 14-3-3 ζ , an abundant protein in the brain, was further verified in both serum and brain tissue using proper methods.

The 14-3-3 proteins, also called tyrosine 3/tryptophan 5 monooxygenase, are a family of homologous proteins that consist of seven isoforms (β/α, γ, ζ/δ, ε, η, σ, and τ) in mammals, in which α and δ are the phosphorylation of β and ζ.[38] They are ubiquitously expressed in various types of tissues, but the highest expression is in the brain, where they make up approximately 1% of the total soluble proteins. [39] The 14-3-3 proteins act as molecular adapters which interact with key signaling molecules and thereby regulate various cell functions including proliferation, differentiation, metabolism, and apoptosis.[40] Although much remains to be learned and understood, 14-3-3 proteins have been implicated in a variety of neurological disorders based on evidence from both clinical and laboratory studies, suggesting their involvement for diagnosis and intervention of some specific neurological diseases. [41, 42] Among all the 14-3-3 isoforms, particular interest has emerged on 14-3-3 ζ because this isoform is apt involved in neurodegenerative, neurologic, and neuropsychiatric diseases and also because 14-3-3 ζ may function to oppose endoplasmic reticulum stress, an important trigger of apoptosis.[43]

As refer to the relationship between the expression of 14-3-3 proteins and arsenic exposure, studies indicated that arsenic trioxide treatment was showed to down-regulate the expression of Isoform long of 14-3-3 protein β/α in the HaCaT human keratinocyte cell line[36] and the expression of 14-3-3 proteins (Isoform short of 14-3-3 protein β/α, ζ/δ, γ) in Multiple myeloma (MM) cell line U266.[37] Furthermore, overexpression of 14-3-3 ζ in MM cells attenuated ATO-induced cell death, whereas RNAi-based 14-3-3 ζ knock-down enhanced

tumor cell sensitivity to the ATO induction.[37] In our study, the expression of 14-3-3 ζ was also down-regulated by 50mg/L arsenic exposure on rats lasting for 12 weeks, which was proved by both serum iTRQA-based proteomic test and later verification experiments in serum and cerebral cortex. These observations in vivo and in vitro implicate 14-3-3 isoforms, especially 14-3-3 ζ, as potential molecular targets of arsenic action. Arsenic is proved to be a neurotoxicant, which can cause neuronal morphological changes, apoptosis, necrosis, and nervous system abnormalities, resulting in behavioral changes and cognitive impairment in both human being and animals.[6, 7] TUNEL assay in this study showed a dose-response relationship between the proportion of apoptotic cells in the brain of Wistar rats and arsenic exposure. Data of our research indicated that14-3-3 ζ may contribute to the higher apoptosis in the brain of rats exposed to arsenic exposure and play a critical role in the process of nervous system damage. So, 14-3-3 ζ may serve as a potential biomarker for arsenic poisoning. But unexpectedly, except for immunohistochemical analysis, the dose-response relationship between arsenic exposure and the expression of 14-3-3 ζ was not distinct since the 14-3-3 ζ levels in low-arsenic and medium-arsenic exposure groups were not significantly altered as compared to control in the experiments of verification stage, which may due to the short period of arsenic exposure in our study.

Further studies targeting at arsenic exposure populations are needed to confirm the value of serum 14-3-3 ζ as a potential biomarker of arsenic poisoning. Besides 14-3-3 ζ, if other 14-3-3 isoforms will be regulated by arsenic exposure and if the regulation occurs in specific localizations of the brain should also be considered. In addition to 14-3-3 ζ, other differentially expressed proteins identified by iTRAQ-based proteomic technique in this study deserve to be further verified in the future to clarify the full scan of damage effect caused by arsenic exposure.

Conclusions

Immunohistochemical analysis

In summary, an iTRAQ-based proteomic technology was successfully applied to explore the differentially expressed serum proteins in rats subchronically exposed to arsenic in this study and a spectrum of twelve differential proteins was found, which provided valuable clues for studing the molecular mechanism of arsenic poisoning. The expression of 14-3-3 ζ, a down-regulated protein by arsenic exposure, was further verified using ELISA, immunohistochemical, real time PCR, and Western blot methods, indicating that 14-3-3

ζ may be a potential biomarker for the nervous damage caused by arsenic poisoning.

Conflict of interest

The authors declare that there are no conflicts of interest.

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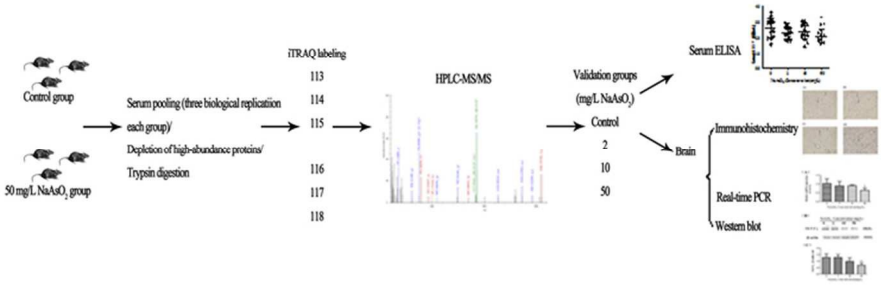

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