Arsenic-induced inhibition of hippocampal neurogenesis and its reversibility

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Arsenic exposure can result in damages of the neurological system. The present study aimed at whether cell proliferation and neurogenesis in the adult mouse hippocampus were affected after arsenic exposure and whether they could recover after exposure cessation. Mice were randomly placed into 3 groups. The first group received distilled water alone for 4 months (control group); the second group received 4.0 mg/L As2O3 through drinking water for 4 months (arsenic group); the third group received 4.0 mg/L As2O3 for 2 months and then changed to distilled water for another 2 months (recovery group). Serum and cerebellum arsenic concentrations of the arsenic group were significantly elevated, and then decreased to normal after the change of arsenic to water in the diet. After a four-month administration, the hippocampal number of proliferative cells and the percentage of new mature neurons decreased in the arsenic group as compared with the control group, however, increased significantly in the recovery group when compared with the arsenic group, and restored to the control level. There were no significant differences for apoptosis in different groups. Obvious histopathological ameliorations were observed in the hippocampus of the recovery group. The inhibition of hippocampus cell proliferation and neurogenesis by arsenic is reversible after the arsenic administration was terminated.

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1. Introduction

Arsenic is a common environmental and occupational contaminant distributed worldwide. It may exist in the well water or food. Millions of people consumed arsenic contaminated drinking water in the countries of Argentina, Bangladesh, China, India and Mexico (Ratnaike, 2003). The health of workers with high occupational exposures, such as employees in the semiconductor manufacturing industry, was badly affected (Chen, 2007). Since 1990s, arsenic trioxide is recognized as a highly effective drug for the treatment of acute promyelocytic leukemia (APL) (Wang and Chen, 2008). As2O3 and its metabolites can be detected in the plasma and cerebrospinal fluid of APL patients during treatment (Au et al., 2008; Kiguchi et al., 2010). Chronic arsenic exposure can result in multisystem diseases and the neurological system is a major target. The common neurological manifestation is peripheral neuropathy, however, central nervous system is involved (Ratnaike, 2003; Grandjean and Landrigan, 2006). Long term exposure to arsenic led to increased prevalence of cerebral infarction in adults (Chiu et al., 1997), impaired pattern memory and switching attention in adolescence (Tsai et al., 2003). In Mexican children, verbal intelligence quotient and long-term memory were decreased (Calderon et al., 2001). Children exposed to arsenic from drinking water were associated with low intellectual function and verbal comprehension in Bangladesh (Wasserman et al., 2004, 2011). In Swedish copper smelter workers, subclinical nerve injuries were detected (Lagerqvist and Zetterlund, 1994). In arsenic exposed animals, decreased spatial memory and locomotor behavior were observed (Itoh et al., 1990; Martinez-Finley et al., 2009; Luo et al., 2009; Rodriguez et al., 2010). Therefore, many papers, including ours, have demonstrated that arsenic exposure leads to neurological damages (Pryor et al., 1983; Rao and Avani, 2004; Piao et al., 2005; Ma et al., 2010; Flora et al., 2005; Vahidnia et al., 2007).

Adult neural stem cells (NSCs) can self-renew and differentiate into all types of neural cells, including neurons and glia cells. Adult neurogenesis has been demonstrated in the brain of mammals. The subgranular zone (SGZ) of the hippocampus dentate gyrus and the subventricular zone (SVZ) of the lateral ventricles are two areas for NSCs existence (Schiemer and Gage, 2004; Zhao et al., 2008; Ming and Song, 2011). Pathological conditions can influence adult...
neurogenesis, for example, stroke induces neurogenesis (Darsalia et al., 2005), and inflammation inhibits neurogenesis (Monje et al., 2003).

We are interested in whether cell proliferation and neurogenesis in the adult hippocampus are affected after arsenic exposure. In the present study, arsenic treated mice were used as animal models. The concentrations of arsenic in serum and cerebrum were detected by inductively coupled plasma mass spectroscopy (ICP-MS). The number of bromodeoxyuridine (BrdU)\(^+\) cells was used as the marker for cell proliferation, and the percentage of BrdU\(^+\)/NeuN\(^+\) cells was applied to evaluate neurogenesis in hippocampus. Meanwhile, the hippocampal cell proliferation and neurogenesis after arsenic removal was observed.

2. Materials and methods

2.1. Animals and treatments

90 male Kunming mice weighting from 18 to 22 g were purchased from animal center of Dalian Medical University. These animals were randomly placed into 3 groups, 30 mice for each group. The first group received distilled water alone for 4 months (control group), the second group received 4.0 mg/L As\(_2\)O\(_3\) (Beijing Chemical Works) through drinking water for 4 months (arsenic group), the third group received 4.0 mg/L As\(_2\)O\(_3\) through drinking water for 2 months and then changed to distilled water for another 2 months (recovery group). In our experiments, the mice were housed in cages under 12 h light/dark cycles with ad libitum access to food and water. All animal procedures conformed to the Animal Guideline of Dalian Medical University. For dividing cell labeling, mice were injected with BrdU (50 mg/kg, i.p.) twice daily 12 days before perfusion. For RNA extraction and arsenic detection, the mice were sacrificed by decapitation. For histology analysis, mice were anesthetized i.p. with 200 mg/kg pentobarbital sodium and transcardially perfused of normal saline solution, followed by ice-cold 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were removed and fixed in the paraformaldehyde medium for hematoxylin and eosin (HE) staining. For immunofluorescence detection, brains were transferred to 30% sucrose in 0.1 M PBS for 24 h. 40-\(\mu\)m coronal sections were cut on a freezing microtome and stored in cryoprotective solution (30% sucrose, 30% ethylene glycol, 1% polyvinyl pyrrolidone, 0.05 M PBS, pH7.4) (Garza et al., 2008) until processing immunofluorescence.

2.2. Detection of total arsenic concentration

Cerebrum samples were first placed into Teflon beaker and digested once with ultra-pure HNO\(_3\). 7500cc ICP-MS (Agilent, USA) was used for analysis of serum or tissue total arsenic (m/z = 75) no matter its oxidation states. Ar (1.2 L/min) was used as reaction gas. Ge (1.0 mg/L, m/z = 72) was used as internal standard in this mode. The concentration of arsenic was satisfactorily measured without interference. The calibration range of arsenic was from 0 to 100 \(\mu\)g/L.

2.3. Immunofluorescence

For neuron-specific nuclear protein (NeuN) and BrdU double-labeled immunofluorescence (Iosif et al., 2006), free-floating sections were denatured in 1 M HCl for 30 min at 65 C, and blocked the endogenous biotin using Endogenous Biotin-Blocking Kit (Invitrogen, Eugene, OR), then incubated with 5% donkey serum for 2 h at room temperature, next incubated overnight with biotinylated mouse anti-NeuN antibody (1:100, Chemicon, Temecula, CA) at 4 C. Sections were rinsed and incubated in streptavidin Alexa Fluor 488 (1:400, Invitrogen, Eugene, OR) in the dark for 2 h the next day. After washing and donkey serum blocking, sections were incubated in rat anti-BrdU antibody (1:100, AbD Serotec, Kidlington, UK) at 4 C overnight, then rinsed and incubated for 2 h with cyanine 3-conjugated donkey anti-rat IgG antibody (1:400; Jackson ImmunoResearch, West Grove, PA) the third day. Samples were mounted on glass slides, and coverslipped with fluorescence mounting medium (Dako), followed by observation under confocal microscope (TCS SP2, Leica, Mannheim, Germany).

2.4. Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay

The TUNEL assay was performed using In Situ Cell Death Detection Kit and Fluorescein (Roche, Mannheim, Germany). All the procedures were based on manufacturer’s protocol with slight modifications. In brief, free-floating brain sections were treated with permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 6 min on ice. Afterward, the sections were incubated for 80 min with TUNEL reagent mixture at 37 C, then stained with Hoechst 33342 (2, \(\mu\)g/ml in PBS) for 5 min. Finally, the sections were mounted with fluorescence mounting medium and visualized under confocal microscope (TCS SP5, Leica, Mannheim, Germany).

2.5. Microscopic analysis

All assessments were carried out by an observer blind to treatment. Brain histopathological changes were observed under Leica DM4000B microscope. The hippocampus immunoreactive cells were calculated in the granule cell layer (GCL) and within two cell diameters below this area in the SGZ (Iosif et al., 2006). Six serial coronal sections spaced 240 \(\mu\)m apart were examined in each animal for the number of BrdU-positive cells, and the data were presented as BrdU\(^+\) cell numbers/group. Colorization of double-labeled cells was confirmed under 40× confocal microscope. At least forty BrdU-positive cells from each animal were examined for NeuN double labeling in the SGZ/GCL. A minimum of 1000 Hoechst-positive cells from each animal were counted for TUNEL positive cells in the SGZ/GCL. Four animals were counted in each group.

2.6. Real-time PCR analysis

Three samples of the same group were pooled together. Hippocampus total RNA was purified with RNAasy mini kit (QiAGEN, Germany), and converted into first-strand cDNA with PrimeScript\textsuperscript{®} RT reagent Kit (TaKaRa, Japan). The amplifications were run with SYBR\textsuperscript{®} Premix Ex Taq\textsuperscript{TM} II kit (TaKaRa, Japan) using a Thermal Cycler Dice\textsuperscript{TM} Real Time System TP 800 (TaKaRa, Japan), and all the reactions were repeated three times. \(\beta\)-Actin was used for normalization. The PCR primers were as follows: 5’-CTGTGGAG-TCCGTCCTACCCGG-3’ and 5’-ACATGCCGGACCGGTTTGCCAGC-3’ for \(\beta\)-actin, 5’-CTGGAGAAGCCGTTGGAATCG-3’ and 5’-CTTGCCTTCATT-GTGGTCTGTG-3’ for Wnt3.

2.7. Statistical analysis

Data are presented as means ± SEM. For data collected at the two-month point, comparisons were performed using Student’s unpaired t-test. For data collected at the four-month point, comparisons were performed with one-way ANOVA followed by post hoc Bonferroni-Dunn test (Iosif et al., 2006) or LSD test. Statistical Package for Social Sciences 11.5 (SPSS 11.5) computer package was applied for analysis. Differences were considered significant when p < 0.05.

3. Results

3.1. Arsenic concentration in mouse serum and cerebrum

After a two-month exposure to arsenic, the concentrations of arsenic in control mouse serum and cerebrum were 28 ± 9.4 and
31 ± 5.0 ng/g, respectively. In the arsenic group, the concentrations of arsenic in serum and cerebrum were 42 ± 6.2 and 60 ± 8.5 ng/g, respectively. Cerebrum arsenic level of treated group was significantly higher than that in control one (p < 0.05) (Fig. 1). After another two-month feeding, the arsenic concentration continued high in the arsenic group (61 ± 3.2 ng/g in serum, 74 ± 6.3 ng/g in cerebrum) and decreased significantly in the recovery group (25 ± 4.4 ng/g in serum, 23 ± 2.8 ng/g in cerebrum, p < 0.01). There were no significant differences between the recovery and control groups (31 ± 3.5 ng/g in serum, 22 ± 2.4 ng/g in cerebrum). These results indicated that arsenic was deposited in the brain after exposure, and excreted after it was removed from the drinking water.

3.2. Histopathological changes in the brain tissue

Pathological findings in the mouse hippocampal neurons were observed. No abnormal histological changes were observed in the control group. After exposure to arsenic for 4 months, karyopyknosis was apparent. Obvious ameliorations were observed in the hippocampus of recovery group (Fig. 2). Relatively slight pathological changes were observed in neurons from the 2-month arsenic group (picture not shown).

3.3. The inhibition of cell proliferation by arsenic and its reversibility

BrdU is used to label new cells because it is a thymidine analog and can incorporate into DNA when the cell divides. After a two-month arsenic administration, the number of BrdU+ cells in the SGZ/GCL was 104.2 ± 5.4 cells/section, which was decreased but with no significant change as compared with the control group (120.7 ± 12.6 cells/section, Fig. 3). After a four-month administration, the numbers of BrdU+ cells in the control, arsenic and recovery groups were 111.8 ± 5.6, 84 ± 2.3, and 128.5 ± 4.8 cells/section, respectively.
control group \(1.3 \pm 0.088 \times 10^{-4}\), Fig. 6). After a four-month administration, the levels of Wnt3/\text{actin} mRNA in the control, arsenic and recovery groups were \(1.4 \pm 0.088, 0.75 \pm 0.071\), and \(0.64 \pm 0.085 \times 10^{-4}\), respectively (Fig. 6). The Wnt3 expression in the arsenic group was significantly decreased as compared with the control group \((p < 0.01)\). However, the Wnt3 level was not restored in the recovery group.

4. Discussions

The arsenic concentration in drinking water for our research was 4.0 mg/L, which was the same dose as our previous papers (Piao et al., 2005; Ma et al., 2010). The elevated arsenic levels in the mice cerebrums of the arsenic group indicated that arsenic could deposit in the brain after its uptake in drinking water. In the recovery group, arsenic excreted, and the cerebral arsenic content decreased to normal after consumption of distilled water, which provides the basis that it was arsenic that contributed to the following alterations in mouse brain. Meanwhile, obvious cell membrane and nucleus pathological findings in the hippocampus were observed after arsenic exposure.

Chronic arsenic toxicity can lead to multisystem disease. Skin changes, such as hyperpigmentation and keratosis, are irreversible after the patients moved from high arsenic content region to the area with low arsenic level. However, respiratory damages and peripheral neuropathy may relieve after exposure cessation (Sengupta et al., 2008). Whether manifestations of central nervous system are reversible or not after chronic exposure has not been reported in arsenic. There are primarily two types of cells in the central nervous system, one is neuron carrying out information processing and transmission, and the other is glia performing supportive roles. Therefore, we paid more attentions to neurons, especially those newly formed mature neurons in the hippocampus where NSCs exist.

BrdU can incorporate into the newly synthesized DNA of dividing cells, accordingly, the BrdU assay can be used to detect the newly produced cells or measure the cell proliferation. BrdU positive cells in the SGZ/GCL can be newly formed neural stem/progenitor cells, glia or neurons. After a two-month administration, the significant declined percentage of BrdU+/NeuN+ cells in the arsenic group proved newly formed mature neurons was inhibited. BrdU positive cells in the arsenic group were decreased, but there were no significant differences as compared with the control. Apparent histopathological findings and cellular changes were showed after a two-month arsenic administration. Therefore, the two-month point was chosen to change the administration protocols, half of the mice treated with arsenic were switched to consumption of distilled water (recovery group) for another two months. After a four-month administration, the numbers of BrdU positive cells in the arsenic group decreased significantly, it might be the proliferation inhibition of one or several kinds of newly formed cells by arsenic, and the percentage of BrdU+/NeuN+ cells was persistently low in the arsenic group. After the change of arsenic to distilled water, the number of BrdU+ cells and percentage of BrdU+/NeuN+ cells in the recovery group increased as compared with the arsenic group, and restored to the level of the control group, which indicated the arsenic-induced proliferation and neurogenesis inhibitions were reversible under current conditions. Obvious pathological ameliorations in the hippocampus of the recovery group demonstrated further the histological improvement. Our data revealed that adult neurogenesis could be recovered after stopping exposure of certain environmental and occupational contaminants. It is interesting to know if adult neural stem/progenitor cells are involved in the arsenic poisoning and the recovery. In the future, more researches will be done to explore
whether the newly formed neural stem/progenitor cells are inhibited, or their differentiations are affected.

The TUNEL assay detects DNA strand breaks generated during apoptosis. There was no significant change for apoptosis in different groups. For our research, the cell number was mainly influenced by the cell proliferation in SGZ/GCL regions, and long term inhibition of proliferation without compensation will produce notable brain dysfunction (Balu and Lucki, 2009). It has
been reported that irradiation can inhibit adult hippocampal neurogenesis completely and impair hippocampus-dependent memories (Madsen et al., 2003; Snyder et al., 2005; Winocur et al., 2006a). In our studies, less than 50% BrdU/NeuN cells were inhibited, and latencies of each group to reach platform were similar using Morris water maze (Supplementary data, Fig. S1), therefore, memory tests that can identify mild neurogenesis inhibition will be considered in the future.

Arsenic trioxide is also a chemotherapy drug that induces apoptosis and differentiation in APL patients. During the period of treatment, the blood concentration of arsenic trioxide in patients can vary from (0.25–0.5 × 10⁻⁶ M) to (1–2 × 10⁻⁶ M) according to different protocols (Wang and Chen, 2008), which is compatible with the 42–61 ng/g (0.56–0.81 × 10⁻⁶ M) serum arsenic level in our arsenic group. Although no obvious neurological side effects were observed in arsenic treated APL patients due to the possible short-term administration, three cases of encephalopathy following arsenic trioxide treatment for metastatic urothelial carcinoma were reported (Lin et al., 2008). One research found that 61% of women receiving chemotherapy (5-fluorouracil, doxorubicin and cyclophosphamide) for breast cancer exhibited decline in cognitive function (attention, learning, and processing speed), and approximately 50% patients who experienced declines showed improvement after one year recovery, whereas 50% were unchanged (Wefel et al., 2004). Growing data showed that the incidence of long-term cognitive decline induced by chemotherapy ranged from 16% to 75% (Argyriou et al., 2011). Cognitive dysfunctions were also reported in animal models after chemotherapy (Winocur et al., 2006b; Reiriz et al., 2006; Joshi et al., 2005), and neural progenitor cells were found to be vulnerable to 5-fluorouracil (Han et al., 2008). Accordingly, many concerns raised about the cognitive recovery after chemotherapy. Our researches provided the experimental evidence of brain neuron regeneration after removal of arsenic, however, more data are required to know whether this phenomenon exists in cancer patients after chemotherapy.

It has been proved Wnt/β-catenin signaling plays a role in the adult hippocampal neurogenesis (Lie et al., 2005). In our study, the relative mRNA level of Wnt3 in the hippocampus of different groups was measured using real-time PCR. The Wnt3 expression was inhibited in the arsenic group, and there was no restoration in the recovery group, consequently, it was unlikely that Wnt/β-catenin signaling stimulated neurogenesis in this situation. Therefore, further experiments are required to unravel the signaling pathways through which the neural stem/progenitor cells proliferate and differentiate after arsenic exposure and removal.

In conclusion, arsenic inhibited the cell proliferation and neurogenesis in the hippocampus, and this inhibition was reversible after the arsenic was removed. Uncovering the mechanism for inhibition reversibility might reveal specific strategies to prevent and treat the neural damages.

**Conflict of interest statement**

None.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2012.04.020.

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