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Abnormal Expression of 8-Nitroguanine in the Brain of Mice Exposed to Arsenic Subchronically

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Abstract: To provide molecular toxicological evidences for exploring the mechanism of arsenic-induced neurotoxicity the accumulation of arsenic (As), the formation of 8-nitroguanine (8-NO_2-G) were examined in brain tissue of mice exposed to arsenic. And the gene expressions of inducible NOS (iNOS), superoxide dismutase 1 (SOD1) and peroxiredoxin 2 (Prdx2) were also analyzed by GeneChip. In the result, the concentration of As in the brain tissue of mice was 4.00, 13.70, 21.48 and 29.88 ng/g in the controls and experimental groups exposed to 1, 2 and 4 mg/l As_2O_3, respectively and increased in dose-response manner. Nervous cells in the brain of mice exposed to As showed disappearances of axons, vacuolar degeneration in cytoplasm and karyolysis, whereas no such pathological changes were observed in the control group. Weak immunoreactivity against 8-NO_2-G was observed in the brain tissue of mice given 1 or 2 ppm arsenic trioxide. More intensive immunoreactivity was found in cells at 4 ppm and it was mainly distributed in cytoplasm. The expressions of SOD1 and Prdx2 were down-regulated in the brain of mice exposed to As, but iNOS expression was not disturbed by As exposure. No the 8-NO_2-G immunoreactivity or abnormal expressions of these genes in brain tissue were observed in controls. These results indicate that As induces high expression of 8-NO_2-G in brain tissues of mice and that RNA in the cells may be modified by overproduced reactive nitrogen species.

Key words: As_2O_3, Reactive nitrogen species (RNS), 8-Nitroguanine (8-NO_2-G), RNA damage, Neurotoxicity

Introduction

Arsenic (As) is a well-known carcinogen and a notorious health killer on the earth. The regional As toxicosis is becoming a global problem of public health. An anthropogenic source of arsenic exposure stems from the widespread use of arsenical drugs in food-animal production in the United States and China. This use results in residual contamination of food products from animals raised with the drugs, as well as environmental contamination associated with disposal of wastes from these animals[1]. Chronic exposure to As resulting in neurotoxicity to nervous system has been receiving more and more attention. The intelligence quotient (IQ) of children in As-rich region was found to be lower than that of children in low As area, and the difference was remarkable[2]. It was shown in animal experiments that As could pass through blood-brain barrier and invade the brain parenchyma, and there was a noticeable correlation between the extent of As exposure and the concentration of As in the brain of guinea pigs and rats[3]. Chaudhuri *et al*. discovered that even if the As concentration fell into the provisional guideline issued by World Health Organization's (WHO), As could impair the oxidation-reduction equilibrium, enhance the peroxidation level of cephalopin, decrease the glutathione concentration and render the brain tissue vulnerable

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to radical attack resulting in abnormal apoptosis of neural cells[4]. However, the mechanism of As-induced neurotoxicity is unclear to date. Some researches implied that As exposure induced overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the body, which then inflicted nucleic acid damage to the neural cells[5]. 8-nitroguanine (8-NO_2-G) is a mutagenic nitrative DNA lesion caused by reactive nitrogen and oxygen species, and now has been used as a potential biomarker of inflammation-related cancers[6].

In the present study, we examined the accumulation of As, the formation and distribution of 8-nitroguanine, and morphological changes in the brain tissue of mice fed with drinking water containing 1, 2 or 4 ppm of As trioxide using ICP-MS and immunohistochemical method, respectively. The gene expressions of superoxide dismutase 1 (SOD1) and peroxiredoxin 2 (Prdx2) as antioxidant enzymes, and inducible nitric oxide synthase (iNOS) were also analyzed by GeneChip. The aim of this study is to provide toxicological evidences for exploring the mechanism of As-induced toxicity to the central nervous system.

Materials and Methods

Chemicals

Arsenic trioxide, HNO_3 and H_2O_2 were purchased from Sigma Chemical Company (St. Louis, USA). Mouse monoclonal anti 8-NO_2-G were obtained from Japan Institute for the Control of Aging (Fukuroi, Japan). Diaminobenzidine (DAB) color reagent kit and UltrasensitiveTM S-P kit came from MAIXIN-Bio (Fuzhou, China).

Animals and treatment

Thirty two mature, healthy Kunming mice were provided by Experimental Animal Center, Dalian Medical University. China. These mice were randomly segregated into four groups and each group had 8 mice with half male and half female. One group received drinking water alone (control), the other three groups received 1, 2, or 4 ppm arsenic trioxide (Wako Pure Chemical Industries, Osaka, Japan) respectively, through drinking water *ad libitum* for 60 d. They were caged under a 12 h dark-light cycle in standard conditions of temperature (18–22°C) and humidity (50%). After the last administration, the thoraxes of mice were opened and the tissue fixation (10% formalin) was injected via a needle inserted into the left ventricle of the heart, and then, the brains were removed and placed in fixative. The animal experiment was performed in accordance with the Animal Guideline of Dalian Medical University and in agreement with the Ethical Committee of Dalian Medical University.

Treatment of test samples

Samples (0.2–0.3 g, wet base) of brain in each mouse were taken by microbalance. These samples were put into Teflon (100 ml) beaker and added 10 ml HNO_3 (ultra-pure, Kanto chemicals) to this vessels. The samples with HNO_3 were settled during 1-d, and then were carried out initial acid digestion for 1 h. After the HNO_3 digestion, the samples were cooled at room temperature and 10 ml H_2O_2 (Atomic absorption spectroscopy grade, Wako chemicals) was added into the HNO_3 predigested samples. These samples were digested 2-h again, and then were concentrated up to 10 ml. Finally, test volume of samples was adjusted to 20 ml with Mili-Q water.

Analysis of As

ICP-Ms (Agilent 7500CE) was used for an analysis of As (M/z=75) with chemical reaction mode. He (4.5 ml/l) was used as reaction gas. By this reaction gas mode, the concentration of As was satisfactorily measured without interference by ICP-MS. Ga (1 mg/l, M/z=71) was used as an internal standard in this mode instead of common Y or In. The calibration range of As was from 0 to 20 μg/l.

Morphological observation on the brain tissue of brain

The formalin-fixed brain tissues were embedded in paraffin, sliced at 5 μm, mounted on glass slides coated with poly L-lysine, and subjected to hematoxylin and eosin staining according to the routine histopathological methods. Histopathological changes were observed under a light microscopy.

Measurement of 8-NO_2-G formation in the brain

Sixty days after As exposure, mice were deeply anesthetized by intraperitoneal injection of sodium pentobarbital, placed in a supine position, and the thorax was opened through a bilateral incision. A catheter was inserted into the left ventricle, the right atrium was incised, and physiologic saline was infused until the perfusate from the right atrium was bloodless. The saline was followed by 4% paraformaldehyde. Then, the brain was removed and placed in fixative. The fixed frozen brain sections (3-μm thickness) were incubated in 1% skim milk for 30 min, followed by incubation with mouse monoclonal anti-8-nitroguanine antibody (1.5 μg/ml, Dojin Chemicals, Kumamoto, Japan) overnight and then with Alexa 594-labeled goat antibody against mouse IgG (1:4, Molecular Probes, Eugene, OR, USA) for 3 h, and then with diaminobenzidine (DAB) chromogen solution. Finally, the sections

were counterstained with hematoxylin and mounted in xylene-based mountant. Five fields randomly selected were observed at each section and the optical density value of 8-NO_2-G was analyzed quantitatively using image analyzer (Image-Pro Plus 4.5, Media Cybernetics) after background value was deducted.

Microarray analysis

Brain tissue was separated and total RNA of each was isolated with TRIzol agent (Invitrogen, USA), followed by purification with RNeasy mini kit (QIAGEN, Germany). The synthesis and purification of cDNA were performed with Affymetrix one-cycle cDNA synthesis kit and Affymetrix GeneChip Sample Cleanup Module. The synthesis and purification of biotin labeling of cRNA were carried out with GeneChip IVT Labeling Kit and GeneChip Sample Cleanup Module. Hybridization was performed at 45°C for 16 h using GeneChip hybridization oven 640. After chip scanning, data were obtained and analyzed using computer workstation loaded with Affymetrix GeneChip Operating Software. A fold change cutoff of 2-fold was set to identify genes for further study. All the data of the inducible NOS (iNOS), superoxide dismutase 1 (SOD1) and peroxiredoxin 2 (Prdx2) gene expressions were the means of two tests.

Statistical analysis

Values were expressed as mean ± SE for 8 mice in each group, and significance of the differences between mean values were determined by one-way analysis of variance (ANOVA) followed by the Scheffe's test for multiple comparison using Statistical Package for Social Sciences 11.5 (SPSS 11.5) computer package.

Results

Concentration of As in the brain tissue of mice

The concentrations of As in the brain tissue of mice are shown in Table 1. They were 4.00, 13.70, 21.48 and 29.88 ng/g in the controls, low, middle and high groups exposed to As, respectively. concentrations of As in the three experimental groups were significantly higher than that in the control group ($p<0.01$) and increased in a dose-response manner.

Table 1. Concentrations of arsenic in brain tissue of mice in the 4 groups

Groups	Treatment As_2O_3 (mg/l)	No. of mice	Concentrations of arsenic (Mean ± SE, ng/g)
Control	Drinking water	8	4.00 ± 0.43
Low	1	8	13.70 ± 0.51[a]
Middle	2	8	21.48 ± 0.67[a, b]
High	4	8	29.88 ± 1.27[a, b, c]

[a]$p<0.01$, compared with control group by the Scheffe's test; [b]$p<0.01$, compared with Low group by the Scheffe's test; [c]$p<0.01$, compared with Middle group by the Scheffe's test.

Pathological observation of the brain tissue in mice exposed to As

Under light microscope, nervous cells in the brain of mice exposed to 4 ppm arsenic trioxide showed disappearances of axons, shrinkage of cells, remarkable vacuolar degeneration in cytoplasm and karyolysis (Fig. 1d); whereas relatively light pathological changes were showed in mice exposed to 1 or 2 ppm arsenic trioxide (Fig. 1b, c). No the above mentioned phenomena were observed in the control group (Fig. 1a).

Expression of 8-NO_2-G in brain tissue of mice exposed to As

8-NO_2-G immunoreactivity in the brain tissue of mice is shown in Fig. 2. Weak 8-NO_2-G immunoreactivity was observed in the brain tissue of mice given 1 or 2 ppm arsenic trioxide (Fig. 2b, c). More intensive immunoreactivity was found in the cells (Fig. 2d) at 4 ppm and it was mainly distributed in cytoplasm neighboring the plasmic membrane, but not in nucleus.

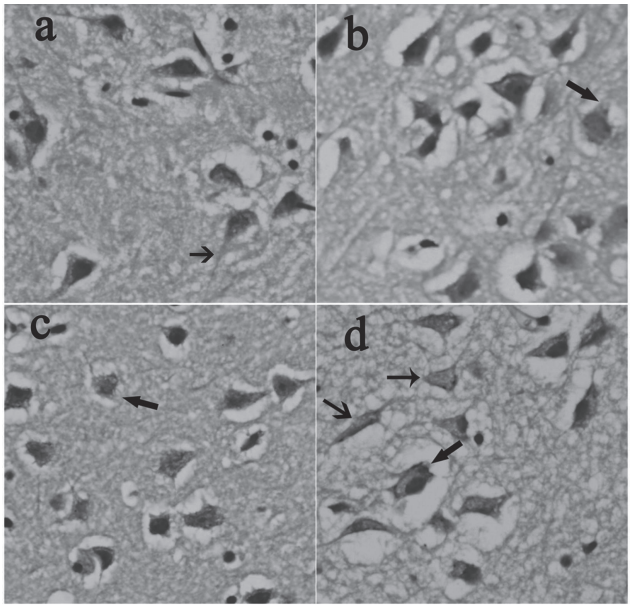

Fig. 1. Histopathological changes in the brain tissue of mice in the 4 groups.

a: control; b: 1 mg/l arsenic trioxide; c: 2 mg/l arsenic trioxide; d: 4 mg/l arsenic trioxide. Histopathological changes were examined by H&E staining. Original magnification × 200. ➔: representing axon; ➡: representing disappearance of axon; ➔: representing shrinkage of cells; ⟶: representing karyolysis.

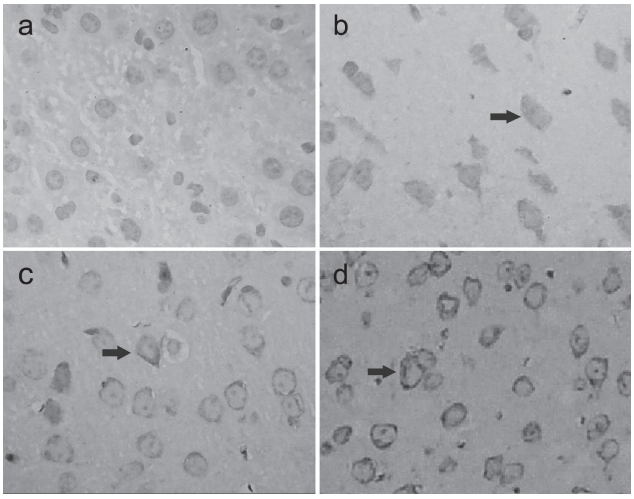

Fig. 2. 8-NO_2-G immunoreactivity in the brain tissues of mice in the 3 groups.

a: control, b: 1 mg/l arsenic trioxide; c: 2 mg/l arsenic trioxide; d: 4 mg/l arsenic trioxide. Each 8-NO_2-G-positive cells appeared brown (indicating by arrow), and had stained cytoplasm. Original magnification × 200.

Table 2. Mean optical density of 8-Nitroguanine immunostaining blots in the brain tissue of mice

Groups	As_2O_3 (mg/l)	No. of mice	Mean optical density (Mean ± SE)
Control	Drinking water	8	0.018 ± 0.002
Low	1	8	0.046 ± 0.009*
Middle	2	8	0.057 ± 0.010**
High	4	8	0.177 ± 0.003**

*p<0.05 or **p<0.01, compared with control by the Scheffe's test.

No immunoreactivity of 8-NO_2-G in brain tissue was observed in controls (Fig. 2a). Quantitative analysis of anti 8-nitroguanine staining blots showed that the average optic densities of immunostaining blots in the 3 experimental groups were significantly higher than that in the control group (p<0.05, or p<0.01) as shown in Table 2.

Expression levels of the SOD1, Prdx2 and iNOS genes in brain tissue of mice exposed to As

Gene microarray analysis was applied to study the gene expression profiles in the brain tissue of mice fed with arsenic. Some genes were found remarkably upregulated (by 1,000 fold for the highest) or down-regulated in pathological mouse groups compared with the normal mice (data not shown). Among these genes, the expressions of SOD1 and Prdx2 was 3.6 and 3.9 times lower respectively in brain tissue of mice exposed to 4 ppm arsenic trioxide than controls (Figs. 3, 4), whereas iNOS expression pattern seemed not noticeably disturbed by arsenic interference (Fig. 5). The results of these gene expressions were further confirmed by RT-PCR method (another paper).

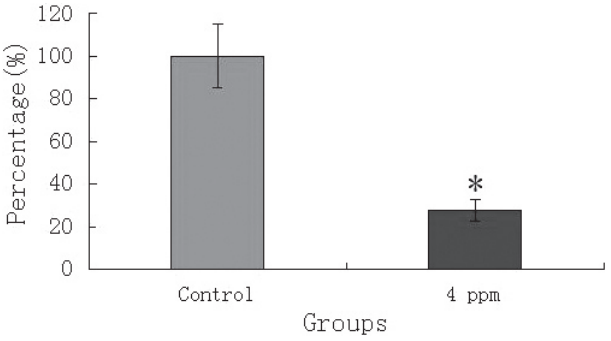

Fig. 3. Expression of SOD1 in the brain tissues of mice exposed to As.

Control group: the group received drinking water alone; 4 ppm group: the group received 4 mg/l arsenic trioxide. Gene expression of superoxide dismutase 1 (SOD1) was analyzed by GeneChip.

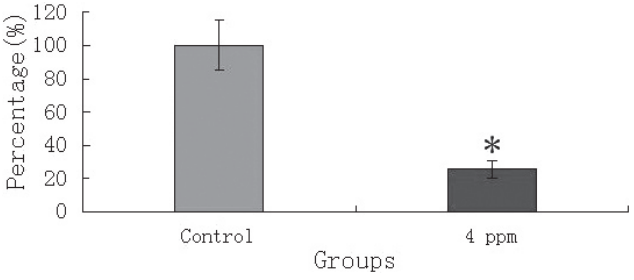

Fig. 4. Expression of Prdx2 in the brain tissues of mice exposed to As.

Control group: the group received drinking water alone; 4 ppm group: the group received 4 mg/l arsenic trioxide. Gene expression of peroxiredoxin 2 (Prdx2) was analyzed by GeneChip.

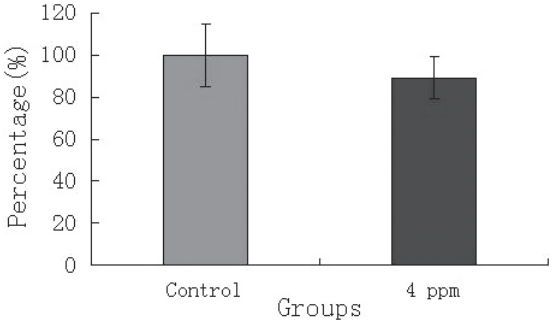

Fig. 5. Expression of iNOS in the brain tissues of mice exposed to As.

Control group: the group received drinking water alone; 4 ppm group: the group received 4 mg/l arsenic trioxide. Gene expression of inducible NOS (iNOS) was analyzed by GeneChip.

Discussion

Epidemiological studies have demonstrated that As causes neurotoxicity including impairments of learning and concentration[7] and deterioration in pattern memory and switching attention in humans[8]. Rodriguez *et al*. reported that As exposure decreased locomotor activity and induced behavioral disorders in the rats[9]. Chattopadhyay *et al*. observed that As resulted in abnormal changes in brain cell membrane, lost ground matrix, inhibited neural networking and apoptotic in natal mice[10]. In previous study, we also found that environmentally relevant levels of As exposure induced learning and memory impairment and pathological changes in the brain tissues *in vivo*[11, 12]. The above literatures indicated that brain tissue may be an important target of As-neurotoxicity. However, the mechanisms by which As exerts a toxic effect on central nervous system are still unclear.

In the present study, the concentration of As was analyzed in the brain tissue of mice exposed to As. Concentrations of As in the experimental groups were significantly higher than that in the controls ($p<0.01$) and increased in a dose-response manner. These results implied that subchronic exposure to As may lead to its accumulation in brain tissue of mice. The morphological observations in nerve cells of the As-treated mice showed disappearances of axons, shrinkage of cells, remarkable vacuolar degeneration in cytoplasm and karyolysis. It indicated that the subchronic exposure to As can result in pathological changes in brain cells of mice.

The conditions of nucleic acid in brain cells of mice exposed to As was also evaluated with immunostaining of 8-nitroguanine, which is one of the major product formed by the reaction of guanine and $ONOO^-$ (the most important reactive nitrogen species) and has been used as a popular biomarker for RNS pressure[13]. It was shown that in the nucleus, where DNA resides, very little 8-nitroguanine was present; whereas in the cytoplasm, the occupational location of RNA, a much stronger 8-nitroguanine signal was detected. It is suggested that RNA in the brain cells may be damaged by As intervention. It has been reported that compared with RNA, DNA has the same ability to be nitrated by RNS, but the stability of 8-nitroguanine in DNA is much less and can be spontaneously cut off from the DNA chain leaving the corresponding sites abasic instead. The half life of 8-nitroguanine in DNA was estimated as about 1 h at 37°C and pH 7.0–7.5, whereas 8-NO_2-G present in RNA is relatively stable, with only about 5% of the modified nucleoside lost during 6 h of incubation under similar conditions[14]. The causal abasic sites in DNA have been proved to be the major locations of DNA breakage. These data explain why there was no 8-NO_2-G in nucleus, while DNA rupture was produced. It suggested that detection of 8-NO_2-G being used as an indicator of DNA damage should be analyzed cautiously. In our previous study, the expression of 8-hydroxy-2-deoxyguanosine (8-OH-dG) as a marker of oxidative DNA damage was also observed in the brain tissue of mice exposed to As. We found high amount of 8-OH-dG accumulating in the nuclear region, where 8-NO_2-G is barely able to be detected[12, 15]. These results indicated that nucleic acid including DNA and RNA was vulnerable to be attacked by ROS and RNS.

The occurrence of 8-nitroguanine is the consequence of the overproduction of nitric oxide (NO) and the causal increase of RNS in the cell. It has been used as biomarker of RNS/ROS damage in the studies of inflammation-related hepatic carcinogenesis[16]. The RNS, such as nitrogen oxide and peroxynitrite ($ONOO^-$), are formed by the reaction between nitric oxide and oxyradicals[17, 18]. It was reported that As can induce increase in level of oxyradicals in nervous tissue through inhibiting the activity of superoxide dismutase (SOD) as antioxidant enzyme[19–21]. In present study, we also found that the gene expression of SOD1 was significantly downregulated in brain tissue of mice exposed to As by Gene microarray or RT-PCR analysis. It indicated that As can promote indirectly reaction of NO with superoxide (O_2^-) due to increasing oxyradical levels and result in overproduction of $ONOO^-$. It has been reported that As exposure can induce NO production[10]. Moreover, the overproduction of NO is believed to be associated with activation of iNOS, an isoform of NOS[22]. Ding *et al*. also reported that blocking iNOS activity with specific inhibitors abolished increased NO production[23]. Waalkes *et al*. observed that sodium arsenate upregulated iNOS and induced 3-nitrotyrosine in Swiss mice[24]. These results indicated that continuous exposure to As may induce NO overproduction in tissue cells via activation of iNOS expression. However, in the present study it is surprising to find that the NOS gene expression levels in brain tissue did not change noticeably after exposure of mice to arsenic by microarray or RT-PCR analysis. It was reported that arsenite inhibited iNOS gene expression in cytokine-stimulated human liver cells[25], in rat pulmonary artery smooth muscle cells[26] and in hepatocytes[27]. Hauser *et al*. also reported that pretreatment with sodium arsenite reverses the expression of iNOS protein induced by cecal ligation and puncture in rats[28]. These literatures and our results indicated that As may have a complicate effect on the expression of iNOS depending on cell-

type, As species and dose. Prdx2 is an antioxidant thiol peroxidase that reduces hydrogen peroxide, peroxynitrite, and hydroperoxides[29]. Therefore, it is important in both oxidative and nitrosative stresses protection. In the present study, gene expression level of Prdx2 was lower in the brains of mice after subchronic As exposure and the results of RT-PCR analysis confirmed the change measured by microarray. It indicated that As may also promote formation of NO and overproduction of $ONOO^-$ via inhibiting Prdx2 activity.

In the following study, we are going to study the possible pathway of production of 8-nitroguanine and RNS in the neuron cells, and the real role of 8-nitroguanine in cellular events, the clarification of which will help to understand the molecular mechanism of As-induced neurotixicity.

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