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Effects of mercury contaminated rice from typical chemical plant area in China on nitric oxide changes and c-fos expression of rats brain

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Abstract: China is one of countries with the highest mercury production in the world. The Guizhou Province in Southwestern China is currently one of the world's most important mercury production areas. In order to study the neurotoxicity of rice from Qingzhen Chemical Plant area and probe into the signal transduction molecular mechanism of injury in rat brain stimulation by mercury contaminated rice. The rats were exposed to mercury contaminated rice for 20 d. Both of the measurements of NO and NOS were processed according to the protocol of the kit. The effect of Hg contaminated rice on the expression of c-fos mRNA in rat brain and the expression of c-FOS protein in cortex, hippocampus were observed using reverse transcription polymerase chain reaction(RT-PCR) and immunocytochemical methods. The results showed the neural transmitter NO and NOS in brain were significantly change between exposure groups and control group; the mercury polluted rice induced significantly the expression of c-fos mRNA; the c-FOS positive cells in hippocampus and cortex of exposure groups were significant different from control group($p < 0.01$). It could be concluded that nitric oxide was involved in mercury contaminated rice induced immediate early gene c-fos expressions in the rat brain. Through food chain, local ecosystem and health of local people iave been deteriorated seriously by mercury. This serious situation will last a long period. In order to alleviate mercury pollution, more work needs to do.

Keywords: chemical plant; mercury contaminated rice; c-fos; NO; NOS

Introduction

Mercury(Hg), as one of the priority pollutant and hot topic in the front of environmental research in many countries, has been paid higher attention in the world since the middle of last century. China is the third largest mercury producer, Guizhou Province (24°30′—29°13′ N, 103° 1′—109°30′ E, 1100 m asl, subtropical humid climate) in south-western China is an important mercury production center. The cinnabar deposits in Guizhou, approximately 80000 tons, are approximately 70% of the total in China. Emissions of mercury from the province to the global atmosphere have been estimated to be approximately 12% of the world total anthropogenic emissions(Tan, 2000; Xiao, 1998; Horvat, 2003). A source of environmental Hg is emission from chemical industries. A chemical plant is located in the vicinity Qingzhen City and approximately 24 km from the capital city Guiyang. The chemical plant was built in 1965 and became operational in 1971. Mercury is used as a catalyst for the production of acetic acid. As a by-product acetaldehyde is also produced. From 1971 to 1986 over 12×10^3 t of acetic acid was produced and after 1987 the production exceeded 20×10^3 t. It is estimated that for the production of 1 t of acetic acid 0.77 kg of Hg is used(Horvat, 2003). Hg emissions from coal burning power plants also contribute to the contamination of this area. The data of previous research showed mercury has deteriorated local ecosystem and health of local people seriously (Horvat, 2003; Feng, 2002), and strongly suggested more research work in the future.

Neurotransmitter is a kind of media through which nervous system transfer signal and is known as the first messenger. NO is a new kind of neurotransmitter. It could transfer signal in the nerve centre and play an important role in immunity. Nitric oxide plays important roles in many physiological and pathophysiological processes as well as in the maintenance of neuronal communication, vascular regulation, and immune systems. A family of isozymes named NO synthase (NOS) participates in the formation of NO. Three distinct isoforms of NOS have been cloned, that is, neuronal NOS(nNOS), endothelial NOS(eNOS), and macrophage NOS(iNOS) (Kim, 2002; Golpon, 2003). The c-fos proto-oncogene is the member of the immediate early genes (IEGs). IEGs including c-jun and c-fos in neurons are easily induced by a variety of extra cellular stimuli. They are considered to link such acute stimuli with subsequent changes in gene expression and hence to act as third messengers during signal transduction (Hironaka, 2000; Ghanima, 2002). The objective of this paper was to study the neurotoxicity of rice from Qingzhen Chemical Plant area and probe into the signal transduction molecular mechanism of injury in rat brain stimulation by Hg contaminated rice and search the influence and the assessment of the contamination with mercury in typical area of Guizhou Province on the healthy of local citizen.

1 Materials and methods

1.1 Animals and procedures

Sprague-Dawley rats (purchased from Shanghai Animal Experimental Center of China Science Institute, 135—140 g) were housed separately and maintained on a 12 h light/12 h dark cycle, ambient temperature maintained at 22℃ with free access to food and water. Animals were randomly divided into 3 groups, each group had 7 rats(five rats for statistical analysis). (1) The control group(SCG): SCG were fed on the rice which was purchased in Shanghai market, concentrations of total mercury and selenium in the rice were 0.004 and 0.050 mg/kg respectively; (2) the Qingzhen Chemical Plant group (CPG): CPG were fed on the rice produced in Qingzhen, concentrations of total mercury, methyl mercury(MeHg) and selenium in the rice were 0.155, 0.075 and 0.130 mg/kg respectively; (3) the methyl mercury group(MMG): MMG were fed on the Shanghai rice amalgamated with MeHg so as to simulate the rice produced in the Qingzhen Chemical Plant, the amount of MeHg was 0.16 mgHg/kg. Vitamin mix and corn oil and minerals were added to all diet in order to ensure the reasonable nutrition (Domene, 2001). After exposure for 20 d, rats were anaesthetized with 10% ketamine clorhydrate (0.5 ml/100 g weight) before perfusion via the ascending aorta with 0.1 mol/L phosphate buffered saline. The brains were dissected quickly out of the skull. One part of tissue was post-fixed in the buffer of 4% paraformaldehyde solution for 24 h, washed in water, dehydrated and then embedded in paraffin. They were cut in arrowy aspect(the thickness was 5 μm). Five sections per rat (five rats per treatment) were used to examine the c-fos expression. The other part of tissue was rapidly removed and immediately frozen by immersion in liquid-nitrogen cooled and stored at −70℃ refrigerator until assayed.

1.2 NO and NOS assays

NO amount was determined by enzymatic reduction assay, and total

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NOS enzyme activity was measured by the L-arginine to L-citrulline conversion assay. Both of the measurements of NO and NOS were processed according to the protocol of the kit (Nanjing Jiancheng Bioengineering Institute, China).

1.3 RT-PCR

We removed the tissue sample from storage, placed it on a clean surface, and then cut it; determined the weight of the pieces to be used, and placed it into a suitably sized vessel for homogenization. Total RNA was isolated using the RNeasy protocol(Qiagen, Germany). The concentration of RNA in each sample was determined by photo spectroscopy. An aliquot of total RNA(0.5 μg) from each sample was used for cDNA synthesis. RT-PCR was performed using Qiagen one step RT-PCR kit (Qiagen, Germany). RT-PCR was carried out on a Touchgene Gradient PCR system(Touchgene Gradien, England). The sequences of primers (Qiagen, Germany) used for analysis are listed below:

c-fos I ATGATGTTCTCGGGTTTCA, c-fos II TGACATGGTCTT-CACCACTC; GAPDH I ATGGAAGAAGAAATCGCCGC, GAPDH II ACACGC-AGCTCGTTGTAGAA.

PCR product of the c-fos and GAPDH were 348 bp and 287 bp respectively. Samples(10 μl) of PCR products were separated on a 2% agarose gel containing ethidium bromide using a DNA molecular weight marker for comparison purposes. After electrophoresis, the expression of c-fos and GADPH mRNA was indicated by measuring the density of the respective specific bands using the Electrophoresis Documentation and Analysis System along with the Tanon(Shanghai, China) Image Analysis Software Program(Ver. 3.61). We determined the amount of mRNA expression by dividing the densitometry value of the mRNA RT-PCR product by that of the GAPDH product and the control was set as 1.0 (Wieland, 2000; Mercier, 2001).

1.4 Immunocytochemistry

Five μm thick paraffin sections were removed from tissues, then deparafinized and dehydrated them. The sections were subjected to an immunohistochemical staining procedure on microscopic slides and the results were visualized using the avidin-biotin-peroxidase method. In brief, the sections were incubated specimens for 20 min in 1—3 drops of serum block; aspirated serum from slides, immediately added 1—3 drops of pre-diluted primary antibody(Santa Cruz Biotechnology, Santa Cruz), incubated for 2 h; rinsed with PBS then washed in PBS twice for 2 minutes each on a stir plate, aspirated excess liquid from slides, incubated for 30 min in 1—3 drops of biotinylated secondary antibody (Santa Cruz Biotechnology, Santa Cruz); washed as above, incubated for 30 min in 1—3 drops of HRP-streptavidin complex; washed as above, added 1—3 drops HRP substrate mixture, developed for 30 s—10 min, or until desired stain intensity develops; rinsed with demonized H_2O and transferred to a demonized H_2O for 2 min on a stir plate; counterstained, dehydrated and mounted slides. An addition set of control slides was tested to ensure the specificity of the primary antibody (Kakishita, 2001; Staiger, 2000). Labeled sections were examined using bright field microscopy throughout the rostra-caudal extent of the striatum from each animal. Digitized brightfield images were obtained with a video camera attached to an item microscope and analyzed with Tanon (Shanghai, China) Image Analysis Software Program (Ver. 3.61). The program was used to measure the number of labeled cells.

1.5 Statistical analysis

Statistical analysis of data included the mean, S.D, and S.E.M. statistical comparisons between exposed groups and control groups were made with paired Student's *t*-test. An ANOVA test was also applied for each group (five different treatments) in every location analyzed. Significance level was defined as $p < 0.05$ (Robner, 2001; Won, 2000).

2 Results and discussion

2.1 The total mercury concentrations in rat brain

Total mercury(T-Hg) was detected by means of AMA-254 liquid/solid mercury analyzer(Milestone, Italy) with an absolute detection limit of 0.01 ng. Total mercury in rat brain is shown in Table 1.

Table 1 The mercury concentrations in rat brain

Group	SCG	CPG	MMG
Total mercury, $\times 10^{-3}$ mg/kg	4.7 ± 0.4	13.7 ± 0.3^{++}	480.8 ± 70.0^{++}

Notes: ++. $p < 0.01$, compared to control group; $n = 7$ rats in each group

The accumulation of mercury in rats brain which exposed to rice of MMG and CPG for 20 d was very significantly different from rats which exposed to rice of SCG($p < 0.01$). The content of total mercury in MMG increased obviously and have significant difference compared with its simulating group CPG($p < 0.01$). The accumulation was relative to the Hg species and the accumulation course. The uptake of MMG by brain was easier than that of CPG, which was probably eue to the higher affinity of MeHg. Selenium(Se) showed an effect on the accumulation and selenium could reduce the Hg uptake. The atomic weights of mercury and selenium are 200.59 and 78.96 g/mol, respectively. The molar rations of selenium: mercury were calculated for the different areas. In Qingzhen, the molat Se : Hg ratios were 1.19 close to 1 : 1. Previous studies suggested that the formation of 1 : 1 Hg-Se compounds may explain Hg detoxification by Se (Kosta, 1975; Byrne, 1995; Falnoga, 2000).

2.2 NO and enzymic activities

The results of NO and enzymic activities of NOS are listed in Fig. 1. The level of NO and NOS increased significantly in brain of exposure groups comparing with the control group. NO is the middle product of the arginine metabolizability cycle and is synthesized by NOS in the body only. We can find NO in nerve cell, blood vessel and other issues. Some researches showed that a great deal of NO could participate in the toxicity of MDA and could generate cell toxicity by combining with superoxide. Researches also indicated that meruсry could increase the activities of NOS and the content of NO in liver and kidney (John, 2001). The experiment showed that the exposure of mercury contaminated rice could increase the level of NOS and NO in brain.

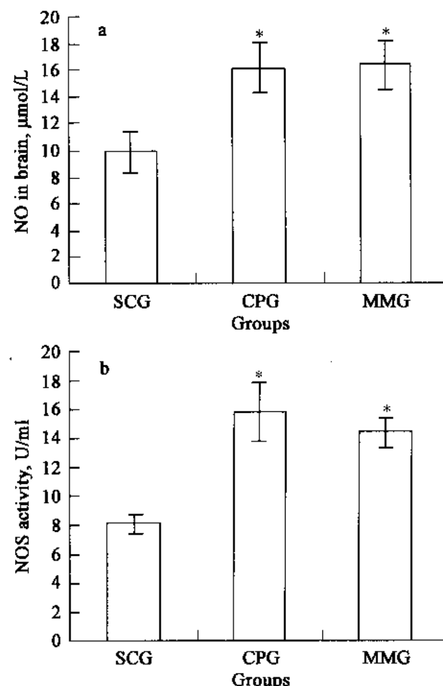

Fig. 1 Changes of NO and NOS in brain of rats exposure to mercury contaminated rice for 20 d
a: NO changes; b: NOS activity. Each column and bar represents the mean and *S.E.M.*, $n = 7$ rats in each group at each time point; * $p < 0.05$ compared to control

2.3 c-fos expression

Fig.2 shows that the expression of c-fos mRNA. The control group (SCG) was so weak that could not see the light strip while that of the other groups(CPG and MMG) had both different extent of light strip.

The relative expression level of c-fos mRNA is shown in Fig.3. Fig.3 shows the expression of c-fos mRNA in brains of CPG and MMG

was significantly different from rats of SCG($p < 0.01$). The expression of c-fos mRNA in MMG increased obviously and had significant difference compared with its simulating group CPG($p < 0.01$).

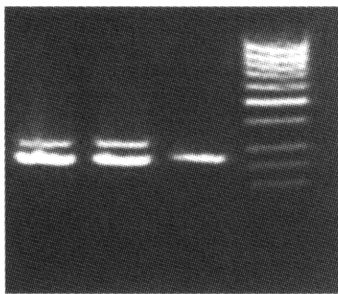

Fig.2 RT-PCR products of c-fos and GAPDH on a 2% agarose gels stained with ethidium bromide
Lanes 1, 2, 3 represent CPG, MMG and SCG respectively; DNA marker (Sangon, China) sizes were 50, 100, 150, 200, 250, 300,350, 400, 450, 500, 550, 600, 650, 700, 750 bp from bottom to top

Very few c-FOS positive cells could be detected in the hippocampus, cortex of SCG. While in CPG and MMG, mercury polluted rice induced cortex and hippocampus produced a significant number of c-FOS positive cells. Fig.4 shows that the c-FOS positive cells in hippocampus and cortex of exposure groups were significant different from control group ($p < 0.01$). In exposure groups, the expression of c-FOS protein in hippocampus increased more obviously than in cortex. The expression of c-FOS protein in hippocampus and cortex of MMG was higher than that exposed to its simulating group CPG, there was difference notability between them($p < 0.05$).

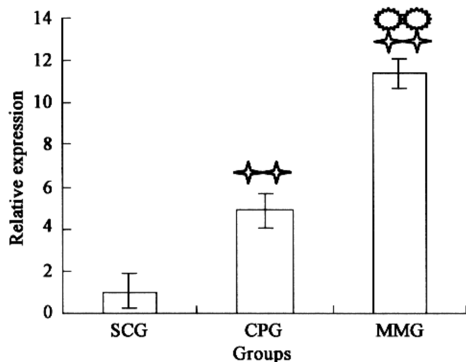

Fig.3 Relative expression level of c-fos mRNA. The relative level of c-fos gene expression for each sample was normalized against GAPDH mRNA signal and the control was set as 1.0. Each column and bar represents the mean and $S.E.M.$, $n = 7$ rats in each group at each time point. ✦✦ $p < 0.01$ compared to control. Compared between Qingzhen groups to its simulation group, ∞ $p < 0.01$

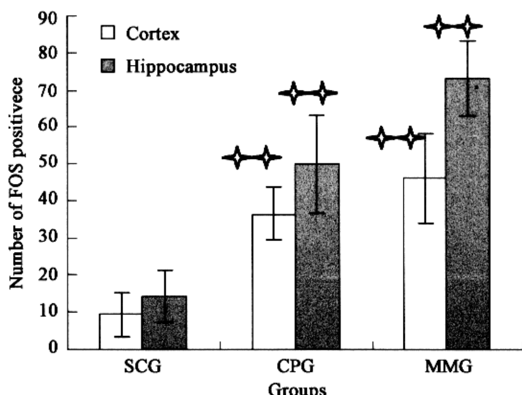

Fig.4 Number of c-FOS-positive cells in rat hippocampus and cortex. Each column and bar represents the mean and $S.E.M.$, $n = 7$ rats in each group at each time point, ✦✦ $p < 0.01$ compared to control.

In the study area rice is eaten with almost every meal and provides more caloric than any single food. Exposure of the local population to Hg may occur due to inhalation of Hg present in air and consumption of Hg contaminated food and water. A very important factor in the impacts of mercury to the environment is its ability to build up in the organisms and up along the food chain. Although all forms of mercury can accumulate to some degree, methylmercury is absorbed and accumulates to a greater extent than other forms. Inorganic mercury can also be absorbed, but is generally taken up at a slower rate and with lower efficiency than is methylmercury. The biomagnification of methylmercury has a most significant influence on the impact on animals and humans. According to the USEPA(1997), effects can occur at a dose of 0.18 mg/kg body weight per day or 1.1 mg/kg methylmercury in diet. Death may occur in species at 0.1—0.5 mg/kg body weight per day or 1.0—5.0 mg/kg in the diet. Smaller animals(for example, minks, monkeys) are generally more susceptible to mercury poisoning than larger animals (for example, mule deer or harp seals). The average concentration of organic mercury in rice of Guizhou Chemical Plant was approximately 0.130 mg/kg. This means that an average person weighing 60 kg could consume rice was very limited per day.

Several previous studies have shown that there is a close relationship between NO and c-fos expression in the central nervous system(Wu, 2000; Jahng, 2004). NO is involved in many path ways which lead to c-fos expression. For example, nitroglycerin, which forms NO *in vitro*, is able to induce c-fos expression in various brain nuclei (Tassorelli, 1995). A NOS inhibitor can prevent stress activation of c-fos expression in neurons of the hypothalamic paraventricular nucleus in rats(Amir, 1997). In the spinal cord, NO mediates c-fos expression induced by mechanical noxious stimulation applied in the periphery. NOS is co-localized with c-fos within spinal cord dorsal horn neurons following formalin injection in the hind paw of rats. Both the non-selective NOS inhibitor, L-NAME, and the selective NOS inhibitor, 7-NINA, reduce formalin-evoked c-fos expression in the dorsal horn of the rat spinal cord. A NOS inhibitor can also reduce the c-fos expression in the rat lumbar spinal cordinduced by carrageenan injection in the periphery(Hunter,1995; Tseng, 1997).

The proto-oncogene c-fos is one of several immediate-early genes that can be rapidly induced in many kinds of cells following various forms of stimulation. The c-FOS transcription can be activated in neurons by nerve growth factor, cAMP, acetylcholine and voltage-dependent calcium channels in PC12 cells. The c-FOS protein expression detected by immunohistochemistry has been used as a marker for neuronal activation in the central nervous system. It has been found that c-fos mRNA can be induced within 30 min following noxious stimulation, and the protein expression detected by immunocytochemistry reaches its peak between 1 and 3 h after stimulation(Wu, 2000; Jahng, 2004; Uma, 2002). The c-FOS protein has been shown to play a causal role in the activation of apoptosis. It has been reported that the administration of cadmium to the experimental animals induces apoptotic cell death in the proximal tubules, testis, and liver. In cultured LLC-PK1 renal epithelial cells, it was also found that incubation with cadmium results in DNA fragmentation (Matsuoka, 2000; Habeebu, 1998). To reveal the relationship between c-fos induction and apoptosis, further examinations are required.

3 Conclusions

The accumulation of mercury in rat brains of Qingzhen Chemical Plant group was very significantly different from rats of Shanghai group (control group; $p < 0.01$); from the clinic observation the control group show less clam than the exposure groups; the neural transmitter NO and NOS in brain were significantly change between exposure groups and control group; the mercury polluted rice induced significantly the expression of c-fos mRNA; the c-FOS positive cells in hippccampus and cortex of exposure groups were significant different from control group($p < 0.01$); the antagonisis between selenium and mercury on the exposure process. It could be concluded that IEG(c-fos) participated in the toxicity process of brain injury by mercury polluted rice. Nitric oxide is involved in mercury contaminated rice induced immediate early gene c-fos expressions in the rat brain. To reveal the relationship between c-fos induction and apoptosis, further examinations are required. Qingzhen

Chemical Plant areas is polluted seriously by mercury, mercury content in rice is higher than that of control samples, and much higher than the tolerance limit for food(GB2762-81-94: ≤0.020 mg/kg). Through food chain, local ecosystem and health of local people have been deteriorated seriously by mercury. This serious situation will last in a long period. In order to alleviate mercury pollution, more work needs to do.

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