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Expression of c-fos and oxidative stress on brain of rats reared on food from mercury-selenium coexisting mining area

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Abstract: Wanshan mercury mine is the largest mercury deposit in Guizhou Province of China, but there were few reports on mercury toxic effect in the mining area. In order to study the neurotoxicity of food from Wanshan mercury mine area and probe into the effect of food from Wanshan mercury miner area on the changes of brain oxidative damage and expression of c-fos gene. The rats were exposed to mercury contaminated food for 20 d. The content of malondialdehyde (MDA), superoxide dismutase (SOD), GSH-peroxidase (GSH-px) and Glutathione (GSH) in rat brain was measured, and the effect of mercury 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 levels of GSH, MDA, SOD and of GSH-dependent enzymes in the rat brain changed 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 oxidative stress signals could contribute to the induction of immediate early genes (IEGs); free radicals and their by-products might not only cause oxidative damage, but also influenced gene expression; IEGs c-fos participated in the toxicity process of brain injury by mercury polluted food.
Keywords: Wanshan mercury mine; mercury polluted food; oxidative damage; c-fos

Introduction

China is the third largest mercury producing country, and Guizhou Province is the most important mercury mine area in China, and also one of the most famous mercury deposits in the world. Mercury ore reserves amount to 80000 t (Feng and Hong, 1999; Xiao *et al.*, 1998). About 70% of total mercury produced in China is from Guizhou Province. Mercury mining in Guizhou has a history of more than 600 years (Xiao *et al.*, 1998). Wanshan mercury mine, which is a mercury-selenium coexisting mining, called "Mercury Capital", is the largest mercury deposit in China. 184000 t of mercury and 1540 t of cinnabar were produced from 1950 to 1990, and the total output was reported to be 26000 t during 1949 to 1981 in Guizhou (Horvat *et al.*, 2003). Due to the depletion of the deposit, Wanshan mercury mining and smelting company has been closed for several years, but Hg pollution in local environment still lasts and affects local ecosystem and health of local people in a long time. Although numerous studies have been carried out in Wanshan mercury mine, most of them merely focused on mercury distribution, characteristics, or environmental behaviors (Feng and Hong, 1999; Xiao *et al.*, 1998; Horvat *et al.*, 2003; Tan *et al.*, 2000). Few attempts have been taken to study the effects of mercury pollution on public health.

Oxidative stress can cause cellular injury by the oxidation of lipids, proteins, and nucleic acids. Under normal physiological conditions, cells require both sustained antioxidant defense mechanisms to counter the steady-state generation of reactive oxygen species (ROS) during normal cellular metabolism, and inducible antioxidant defense mechanisms to counter acute oxidative challenges (Varadarajan *et al.*, 2000). Oxidative stress has been suggested as an important mechanism by which mercury exerts initial neurotoxic effects (Cho and Park, 2000; Shimojo *et al.*, 1996). Mercury can give rise to free radicals that induce lipid, protein, or DNA oxidation and enhance lipid peroxidation in several organs, as measured by the thiobarbituric acid reaction for MDA, and reduce GSH level (Cheng *et al.*, 2005a). 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 (Cheng *et al.*, 2005b). In order to assess the actually toxic effects of rice on ecosystem, in particularly the possible health effects on local population in Wanshan area. The rats were exposed to mercury contaminated rice for 20 d. The changes of oxidative damage and expression of c-fos in rats brain were investigated.

1 Materials and methods

1.1 Animals and procedures

Sprague-Dawley rats (purchased from Shanghai Animal Experimental Center, Chinese Academy of Sciences, weighing 135—140 g) were housed

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separately and maintained on a 12-h light /12-h dark cycle, ambient temperature maintained at 22℃ with free access to food and water. The rats were divided into four groups, all groups were exposed to rice for 20 d. Each group had 6 rats, the males were 3, the females were 3: (1) the control group (SCG) was fed on the rice which was purchased in Shanghai market, concentrations of total mercury and selenium in the rice was 0.004 and 0.050 mg/kg, respectively. (2) the Wanshan mercury mining group (WMM) was fed on the rice produced in Wanshan mercury mining area, concentrations of total mercury, organic mercury (MeHg) and selenium (Se) in the rice were 0.133, 0.033 and 0.800 mg/kg, respectively; (3) the Se-Hg group (SMG) was fed on the Shanghai rice amalgamated with Na_2SeO_4 and $HgCl_2$ so as to simulate the rice produced in the Wanshan mining plant, the mount of $HgCl_2$ was 0.13 mgHg/kg and the mount of Na_2SeO_4 was 0.80 mgSe/kg; (4) the $HgCl_2$ group (MCG) was fed on the Shanghai rice amalgamated with $HgCl_2$ so as to contrast with the Se-Hg group, the amount of $HgCl_2$ was 0.13 mgHg/kg. Vitamin mix and Corn oil and minerals were added to all diet in order to ensure to the reasonable nutrition (Ji *et al.*, 2005). After exposure for 20 d, rats were anaesthetized with 10% ketamine clorhydrate (0.5 ml/100g weight) before perfusion via the ascending aorta with 0.1 mol/L phosphate buffered saline. The brains were dissected quickly out of the skull.

1.2 Analytical method for mercury and selenium

Mercury analysis was carried out by an AMA-254 solid/liquid mercury analyzer (Milestone, Italy) with an absolute detection limit of 0.01 ng. Se was determined by hydride generation-atomic fluorescence spectrometry technique (Titan, China).

1.3 Oxidative damage

The content of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione-peroxidase (GSH-px) and glutathione (GSH) in brains was measured as described by Cheng *et al.* (2005b). Briefly, the level of lipid peroxides in brains was determined by spectrophotometry of the pink-colored product of the thiobarbituric acid-reactive substances complex. GSH content was measured by the modified Beutler methoh. SOD activity was assayed by the inhibition of pyrogallol autoxidation at 25℃, and was followed kinetically at 420 nm. The activity of GSH-px was determined by 5,5'-dithionbis (2-nitrobenzoic acid) (DTNB) photometric method.

1.4 Expression of c-fos

The expression of c-fos mRNA in brains were observed using reverse transcription polymerase chain reaction (RT-PCR) methods. Total RNA was isolated using the RNeasy protocol (Qiagen, Germany). RT-PCR was performed using Qiagen onestep RT-PCR kit (Qiagen, Germany). The sequences of primers (Qiagen Germany) used for analysis were listed below (Cheng *et al.*, 2005a): c-fos I ATGATGTTCTCGGGTTTCAA, c-fos II TGACATGGTCTTCACCACTC; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) I ATGGAAGAAGAAATCGCCGC, (GAPDH) II ACACGCAGCTCGTTGTAGAA. 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 (Cheng *et al.*, 2005a). The expression of c-FOS protein in cerebral cortex, hippocampus was observed using immunocytochemical methods. Primary antibody and secondary antibody were purchased from Santa Cruz biotechnology. 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).

1.5 Statistical analysis

All results were expressed as means ± *SD*. Statistical analysis was performed using SPSS (v. 11.0). Significance level was defined as $P< 0.05$.

2 Results and discussion

2.1 Oxidative damage in rat's brains

The results of MDA, reduced GSH and antioxidant enzymatic activity (GSH-px, SOD) are listed in Table 1. The levels of GSH, MDA, SOD and of GSH-dependent enzymes in the brains of MCG had significant difference compared with control group ($P<0.01$, $P<0.05$). The level of MDA was clearly increased of WMM compared to the control groups. The activities of GSH-px in SMG were clearly decreased compared to the control groups. But the level of GSH, MDA, SOD and of GSH-px in WMM had no statistically significant changes compared to its simulating group SMG. It showed that Se had strong protective capability to the oxidative damage induced by mercury.

2.2 Expression of c-fos

The relative expression level of c-fos mRNA is shown in Fig.1. It indicates that the expression of c-fos mRNA in brains of WMM and SMG and MCG was very significant difference from rats of SCG ($P<0.01$). The expression of c-fos mRNA in WMM had no significant difference compared with its simulating group SMG ($P<0.05$). The expression of c-fos mRNA in MCG increased obviously and had significant

Table 1 Levels of GSH, MDA, SOD and of GSH-dependent enzymes in the brain of rats exposed to rice for 20 d

Group	MDA, nmol/ml	GSH-px, U/L	GSH, μmol/L	SOD, U/ml
SCG	2.12±0.22	21.60±1.31	9.63±0.66	130.31±4.19
WMM	2.80±0.52*	22.14±2.81	9.43±0.82	125.27±16.56
SMG	2.00±0.42	18.22±0.74**	9.34±0.97	129.78±3.94
MCG	2.69±0.26**	12.06±2.26**	11.39±0.96*	113.53±12.77*

Notes: The superscripts indicate significant differences between exposure samples and control; * $P<0.05$; ** $P<0.01$; every value represented as mean values±*SD* ($n=6$)

difference compared with its simulating group SMG ($P<0.01$). Very few c-FOS positive cells could be detected in the hippocampus, cerebral cortex of SCG. While in WMM, SMG and MCG mercury polluted rice induced cerebral cortex and hippocampus produced a significant number of c-FOS positive cells. Fig.2 shows that c-FOS positive cells in hippccampus and cerebral 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 cerebral cortex. The expression of c-FOS protein in hippocampus and cerebral cortex of WMM and MCG was higher than that exposed to its simulating SMG. It showed the antagonisis between selenium and mercury on the expression of c-fos mRNA and c-FOS protein.

2.3 Total Hg contents in rat's brain

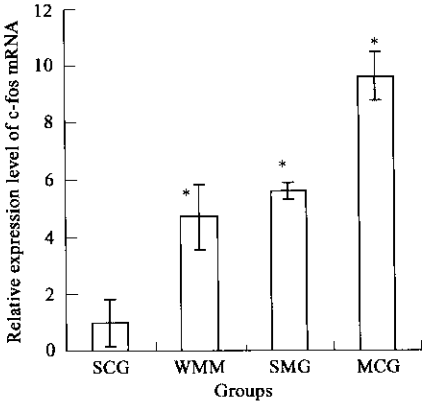

Fig.1 Relative expression level of c-fos mRNA
Relative level of c-fos gene expression for each sample was normalized against GAPDH mRNA signal and the control was set as 1.0; each column and bar represents the mean ±*SD*, $n=6$ rats in each group at each time point; *$P<0.01$ compared to control

Total mercury in rat brains is shown in Table 2, the decreasing order of mercury concentration was MCG>WMM>SMG>SCG. The accumulation of mercury in rat brains which exposed to rice of MCG, WMM and SMG for 20 d was very significantly different from rats which exposed to rice of SCG ($P<$

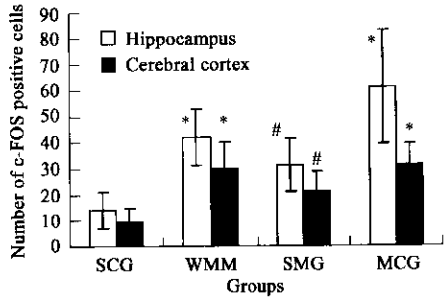

Fig.2 Number of c-FOS-positive cells in rat hippocampus and cerebral cortex
Each column and bar represents the mean ±*SD*, $n=6$ rats in each group at each time point; #$P<0.05$ compared to control; *$p<0.01$ compared to control

0.01). SMG had no significant influence on the total mercury concentrations in brains than SCG ($P>0.05$), while lower significance than MCG ($P<0.05$). The accumulation was relative to the Hg species and the accumulation course. Se showed an effect on the accumulation and Se could reduce the Hg uptake.

Mercury is a persistent pollutant, which means

Table 2 Mercury concentrations in rat brain

Group	SCG	WMM	SMG	MCG
Total mercury, μg/kg	4.7±0.4	24.0 ±3.6**	5.38±0.5**	37.5 ±0.5**

Notes: ** $P<0.01$, compared to control group; every value represented as mean values±*SD* ($n=6$)

that it is very resistant against degradation in the environment. It is known to concentrate in the fats of animals and to build up in the breast milk of nursing mothers or passed to children during pregnancy. It is highly toxic, causing a range of adverse effects, including death, disease and birth defects among humans and animals. Specific effects can include cancer, allergies and hypersensitivity, damage to the central and peripheral nervous systems, reproductive disorders, and disruption of the immune system. Mercury poisoning was first recognized in Minamata, Japan around 1960. Hundreds of fisherfolk and their families were severely poisoned during the 1950s by mercury that bioaccumulated in fish as a result of release of mercury to the bay from a local chemical plant (Gochfeld, 2003). Mercury accumulation in rice samples collected from Wanshan mercury mining area was significantly higher than that of control group. Exposure of the local population to mercury may occur due to consumption of mercury concentrated food (rice and fish, etc.). It should be noted that in general it is believed that Hg in rice does not pose a significant source of exposure in humans. Indeed in a few studies Hg was determined in rice; the levels were rather low (Horvat *et al.*, 2003). This suggests that in mercury contaminated sites, the source of mercury is not primarily related to fish consumption, but other

foods need to be evaluated as well. For example, epidemics of organic mercury poisoning from consumption of grain treated with organo- mercurial fungicides have also occurred in Iraq and Guatemala. A family in New Mexico was poisoned by eating pork from their pigs, which they had fed on fungicide-treated grain (Gochfeld, 2003). However, it was noted that Se contents in Wanshan samples also significantly exceeded that of Shanghai samples, in which the concentrations of Se rarely exceed 0.05 mg/kg. Se is an essential trace mineral and has experimentally been shown to reduce the toxic response in the nervous system associated with exposure to mercury, but the efficacy of Se as an antidote against mercury toxicity in humans is controversial (Horvat *et al.*, 2003). It is, however, notable that in rice samples of Wanshan with high mercury levels. Se is present in substantial surplus to mercury on a molar basis, indicating an association between mercury and Se. The accumulation of Se in Wanshan samples may associate with the higher environmental background of Se. The significant correlations of mercury and Se in Wanshan samples suggested that Se most likely plays an important role in the metabolism and toxic effects of mercury.

The antioxidant enzymes, SOD and GSH-px are active scavengers of free radicals. GSH-px catalyzes the reduction of lipid and hydrogen peroxides to less harmful hydroxides. SOD catalyzes the transformation of superoxide radicals to H_2O_2 and O_2, and is the first enzyme to deal with oxy-radicals (Ji *et al.*, 2005) . They are both involved in protecting against potential cell injury and neuropathological conditions. In this study, increased activities of the antioxidants, GSH-px and SOD, were found in the brain of exposure group rats which indicates the generation of hydrogen peroxides and superoxide radical anion. Changes of the enzyme activities might indicate the presence of oxidative stress, because these two major antioxidant defense enzymes were inducible enzymes that could be induced by a slight oxidative stress due to compensatory response. Generally, activities of antioxidant enzymes increased at low-intermediate doses as a counteractive response of slight oxidative stress, and inhibited at high doses as a result of higher oxyradical formation. GSH redox cycle is one of the most important intracellular antioxidant systems. GSH is an essential compound for maintaining cell integrity because of its reducing properties and participation in the cell metabolism. Variations in GSH levels under laboratory exposure to mercury contaminants have been observed, and the responses were variable for different subjects and different experimental conditions. High expression of MDA is regarded as an indicator of lipid peroxidation. An increase in MDA production is probably a consequent result of augmented activity of ROS. The unchanged MDA level of rats exposed to mercury contaminated rice may be related to the increase in the activity of GSH-px and SOD. Moreover, the increase of GSH content give an additional cellular reducing protect through the -SH group.

We determined the levels of both c-fos mRNA and c-FOS protein in brains of rats exposed to different rice. It was found that mercury contaminated food could induce the expression of c-fos gene very significantly ($P<0.05$, $P<0.01$). The expression of c-fos had been identified as a signal for cell death. Hg had been found to induce apoptosis in a number of cell types. Thus it could be seen that c-fos induction took place much earlier than apparent cell apoptosis and oxygen damage caused by mercury. Free radicals and their by-products may not only cause oxidative damage, but also influence gene expression, particularly growth factor-inducible genes (Masaya *et al.*, 1999). Oxidative stress generating experiments such as superoxide produced by the xanthin/xanthine oxidase system, or direct administration of hydrogen peroxide onto cultured cells induce the expression of several immediate early genes (IEGs) including c-fos. It has been shown that growth factor-mediated c-fos expression is reactive oxygen species ROS dependent. Tumor necrosis factor and basic fibroblast growth factor induced ROS production and act as a common signal to stimulate c-fos gene expression (Lo and Cruz, 1995). But exact mechanisms underlying ROS-dependent c-fos expression is not clear. Oxidative stress signals can contribute to the induction of immediate early genes. Support for this possibility has been provided by studies where the degree and duration of c-fos expression are modified in mice over expressing Cu-Zn/SOD, and by *in vitro* experiments showing a redox-dependent regulation of AP-1 (active protein) activity (Maria *et al.*, 2003). In the present study we have used MeHg as an oxidative stressor, as its neurotoxic effect on the central nervous system has been repeatedly attributed to an induction of oxidative unbalance. Our data indicate that, following a MeHg injection, c-fos is strongly induced in the cerebral cortex, hippocampus after exposure for 20 min (Cheng *et al.*, 2005). The role of c-fos expression under pathophysiological conditions is still unclear. In kainate neurotoxicity, c-fos expression has been alternatively interpreted as a marker of neuronal death, an unspecific index of death, a marker of cells sensitive to kainate neurotoxicity, and a regulator of neuronal cell survival. In this model of neurotoxicity, c-fos expression does not indicate specific propensity of cell populations towards death or survival, but it may reflect a selective sensitivity to mercury-induced oxidative disorder.

3 Conclusions

The accumulation of mercury in rats brains which exposed to rice of MCG, WMM and SMG for 20 d was very significantly different from rats which exposed to rice of SCG ($P<0.01$). The levels of GSH, MDA, SOD and of GSH-dependent enzymes in the brains changed between exposure groups and control group; mercury polluted food induced significantly expression of c-fos mRNA and the c-FOS protein. The significant correlations of mercury and selenium in Wanshan samples suggested that Se most likely plays an important role in the metabolism and toxic effects of mercury. Free radicals and their by-products may not only cause oxidative damage, but also influence gene expression.

Abbreviations:

AP-1:	Active protein-1;	MeHg:	Methyl mercury;
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase;	RT-PCR:	Reverse transcription polymerase chain reaction;
GSH:	Glutathione;	ROS:	Reactive oxygen species;
GSH-px:	GSH-peroxidase;	SCG:	Control group;
IEGs:	Immediate early genes;	SOD:	Superoxide dismutase;
MCG:	$HgCl_2$ group;	SMG:	Se-Hg group;
MDA:	Malondialdehyde;	WMM:	Wanshan mercury mining group

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