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Rice From Mercury Contaminated Areas in Guizhou Province Induces *c-jun* Expression in Rat Brain[1]

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Objective Mercury (Hg), as one of the priority pollutants and also a hot topic of frontier environmental research in many countries, has been paid higher attention in the world since the middle of the last century. Guizhou Province (at N24° 30′-29° 13′, E103° 1′-109° 30′, 1 100 m above the sea level, with subtropical humid climate) in southwest China is an important mercury production center. It has been found that the mercury content in most media of aquatics, soil, atmosphere and in biomass of corns, plants and animals, is higher than the national standard.The present study aims to explore the influence of mercury pollution on the health of local citizens. **Methods** The effect of rice from two mercury polluted experimental plots of Guizhou Province on the expression of *c-jun* mRNA in rat brain and *c-jun* protein in cortex, hippocampus and ependyma was observed using reverse transcription polymerase chain reaction (RT-PCR) and immunocytochemical methods. **Results** The results showed that the mercury polluted rice induced expression of *c-jun* mRNA and its protein significantly. Selenium can reduce Hg uptake, an antagonism between selenium and mercury on the expression of *c-jun* mRNA and *c-jun* protein. **Conclusion** *c-jun* participates in the toxicity process of brain injury by mercury polluted rice, the expression of *c-jun* mRNA in brain, and *c-jun* protein in rat cortex and hippocampus can predict neurotoxicity of mercury polluted rice. People should be advised to be cautious in eating any kind of Hg-polluted foods. To reveal the relationship between c-*jun* induction and apoptosis, further examinations are required.

Key words: Guizhou; Mercury polluted rice; *c-jun*; Gene expression; Early prediction; Exposure

INTRODUCTION

Mercury is one of the most highly bioconcentrated toxic trace metals in human food chain, and many national and international agencies and organizations have listed mercury as a target of possible emission control[1-2]. The accumulation, toxicity and effects of mercury have been documented in scientific literature and extensively reviewed. The most relevant results of mercury poisoning in most animal groups are its adverse effects on reproduction, immune response, neurological impairment, damage to organs including the central nervous system[3-4], the liver[5] and kidney[6].

Guizhou Province (at N24° 30′-29° 13′, E103° 1′-109° 30′, 1 100 m above the sea level, with subtropical humid climate) in southwest China is an important mercury production center. The cinnabar deposits in Guizhou Province, approximately 80 000 tons, account for approximately 70% of the total amount in China[7-8]. Emissions of mercury from Guizhou Province to the global atmosphere have been estimated to be approximately 12% of the world total anthropogenic emissions[9]. In addition[10-12], Guizhou Province is one of the major coal production provinces in China. Approximately 80% of the total energy consumption by a population of 34 million is provided by coal combustion. In recent years annual coal burning was above 8×10^6 tons without flue gas cleaning or emission control actions. The mean concentration of mercury in coal is 0.255 mg /kg. The mercury content in most media of aquatics, soil, atmosphere and in biomass of corns, plants and animas, was higher than the national standard. It is

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why the present study aims at the influence of mercury pollution on the health of local citizens.

MATERIALS AND METHODS

Rice From Experimental Fields

Rats were fed with rice collected from the experimental plots in Guizhou Mercury Mine (GMM) located in Wanshang City with high content of mercury, and Guizhou Chemical Plant (GCP) where Hg is used as a catalyst for the production of acetaldehyde. Experimental field of GMM was irrigated with water leached from ignition and mining residues. GCP is located in the vicinity of Qingzhen City and is approximately 24 km from the capital city of Guiyang where wastewater is discharged into the channel to irrigate our experimental rice field. Concentrations of total mercury and selenium in rice are presented in Table 1. Total mercury (T-Hg) in rice was detected by the AMA-254 liquid/solid mercury analyzer (Milestone, Italy). Selenium（Se） was determined using a Hitachi fluorescence spectrophotometer F-450 (Hitachi, Japan). At each sampling station the hull was removed from the grain to obtain white rice. Evidently, the concentration of total mercury in the rice (control) purchased in Shanghai market in absolute values was much lower than that in GMM and GCP. The atomic weights of mercury and selenium are 200.59 g/moL and 78.96 g/mol, respectively. The molar ratio of selenium: mercury was calculated for different areas. In GCP, the molar Se:Hg ratio was 1.19 close to 1:1. In GMM, however, selenium was present in a substantial surplus, being 15.28 times higher than the mercury concentration on a molar basis.

Animals and Procedures

Sprague-Dawley rats (purchased from Shanghai Animal Experimental Center, Chinese Academy of Sciences), weighing 135-140 g, were housed separately and maintained on a 12 h light / 12 h dark cycle, with the ambient temperature maintained at 22℃ with free access to food and water. Rats were raised for several days before experiment. Too active ones were excluded from experiments. Rats were divided into Shanghai group (control group, SCG), Guizhou Chemical Plant group (GCP), Guizhou Mercury Mine group (GMM). Each group had 7 rats (five rats for Statistical analysis), SCG was fed on the rice purchased in Shanghai market for 30 days. GCP was fed on the rice produced in Guizhou Chemical Plant experiment plots for 30 days. GMM was fed on the rice produced in Guizhou Mercury Mine experiment plots for 30 days. Vitamin mix, corn oil, and minerals were added to all diets in order to ensure reasonable nutrition[13]. Each rat was needed to be fed on 50 g/d (it was enough). Rats were weighted at 9:00 a.m. every other day, and the day was marked before feeding as the zero day. Thirty days after exposure, rats were anaesthetized with 10% ketamine clorhydrate (0.5 mL/100 g) before perfusion via the ascending aorta with 0.1 mol/L phosphate buffered saline. The brains were dissected 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 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-jun* expression. The other part of tissue was rapidly removed and immediately frozen by immersion in liquid-nitrogen and stored at -70℃ until being assayed.

Reverse Transcriptase Polymerase Chain Reaction

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 onestep RT-PCR kit (Qiagen, Germany), and on a Touchgene Gradient PCR system (Touchgene Gradient, England). Thirty PCR cycles consisted of denaturing at 94℃ for 30 s, annealing at 60℃ for 50 s, and extension at 72℃ for 60 s. The sequences of primers (Qiagen Germany) used for analysis are listed in Table 2. Samples (10 μL) of PCR products were separated on a 2% agarose gel containing ethidium bromide using a DNA molecular weight marker for comparison. After electrophoresis, the expression of *c-jun* and GADPH mRNA was indicated by measuring the density of 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[14-15].

Immunocytochemistry

Five μm-thick paraffin sections were removed from tissues, deparafinized and dehydrated. The sections were subjected to an immunohistochemical staining procedure on microscopic slides and the results were visualized using the avidin-biotin-peroxidase method[16-17]. In brief, the sections were

incubated for 20 minutes in 1-3 drops of serum block, serum was aspirated from slides, 1-3 drops of pre-diluted primary antibody were immediately added (Santa Cruz Biotechnology, santa Cruz), incubated for 2 hours, rinsed with PBS and washed in PBS twice for 2 minutes each on a stir plate. Excess liquid was aspirated from slides, incubated for 30 minutes in 1-3 drops of biotinylated secondary antibody (Santa Cruz Biotechnology, santa Cruz). It was washed as above, incubated for 30 minutes in 1-3 drops of HRP-streptavidin complex, then washed as above and 1-3 drops of HRP substrate mixture were added, developed for 30 seconds-10 minutes, or until desired stain intensity developed. It was rinsed with demonized H_2O and transferred to a demonized H_2O for 2 minutes on a stir plate, counterstained, dehydrated and mounted onto slides. An addition set of control slides was tested to ensure the specificity of the primary antibody. These slides were included in each step of the staining protocol with the following exceptions: during the primary antibody incubation, a mixture containing only TBS, NGS, and 10% Trition was applied. Later analysis of these slides revealed virtually no staining. 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.

Statistical analysis[18-19] of data included the mean, SD, and SEM. Statistical comparisons between exposed and control groups were made with paired Student's *t*-test. An ANOVA test was also applied for each group (five different treatments) in each location analyzed. Significance level was defined as $P<0.05$.

RESULTS

Mercury Concentrations in Rat Brain

Total mercury in brains is shown in Fig. 1, the decreasing order of mercury concentration was GMM>GCP>SCG (Fig. 1). The accumulation of mercury in brains of GCP and GMM was significantly different from that in rat brains of SCG ($P<0.01$). The concentration of mercury in rat brains of GMM was about 2.33 times greater than that in brain of GCP. It has been shown that rats exposed to GCP, having the highest mercury burdens (Table 1), were able to detoxify mercury by a specific chemical mechanism involving selenium. According to Koeman *et al.* (1973)[20-21], the molar ratio Se/Hg^0 (Hg^0=Hgtot-HgMet) in the rice of Guizhou Chemical Plant was approximately 1. Elemental Se (Se^0) may interact chemically with Hg in a similar fashion, forming a Hg-Se complex. The possibility of the extension of elemental Se^0 into Se^{2-} might also be considered for the interaction of Hg^{2+} and Se^{2-} to form the Hg-Se complex. Fig. 2 shows the growth of rat weight, the increasing order of rat weight was GCP>GMM>SCG. In a low dose sphere, mercury could accelerate the growth of rats. But when the dose was very high, mercury would suppress the growth. When Se was added, the suppression was alleviated. The effect of mercury on growth was related to the dose and species of mercury.

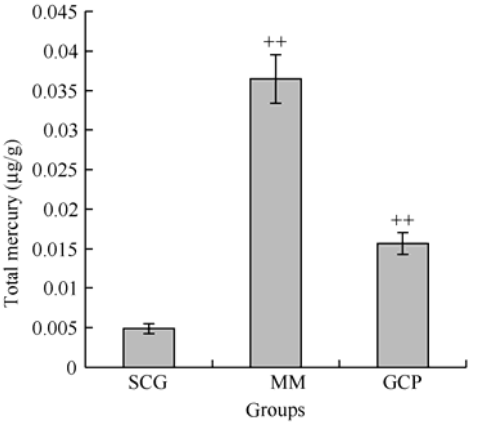

FIG. 1. Hg concentrations in the brains of rats exposure for 30 days . $^{+}P<0.05$, compared to the control group; $^{++}P<0.01$, compared to the control group. Each column and bar represents x+s. n=7 rats in each group at each time point.

TABLE 1

Se and Hg Content of Rice in Different Areas

Concentrations (ng/kg)	Shanghai	GMM	GCP
Total Mercury	-	133	155
Organic Mercury	-	33	130
Selenium	-	800	75

Note. - Below the limit of detection.

TABLE 2

Nucleotide Sequence and Size of Expected PCR Products for Oligonucleotide Primers Used for RT-PCR

Gene	Sequence	PCR Product (bp)
GAPDH	5′-ATGGAAGAAGAAATCGCCGC-3′ 5′-ACACGCAGCTCGTTGTAGAA-3′	287
c-jun	5′-ATGACTGCAAAGATGGAAAC-3′ 5′-TTGAAGTTGCTGAGGTTGGC-3′	530

c-jun mRNA Expression

Fig. 3 shows the expression of *c-jun* mRNA. The control group (SCG) had no light strip while the other groups (GCP and GMM) had different extents of light strip. The relative expression level of *c-jun* mRNA is shown in Fig. 4. Fig. 4 shows that the expression of *c-jun* mRNA in brains of GMM and GCP was significantly different from that of SCG ($P<0.01$). This was the same as the situation of the content of mercury in the brain 30 days after exposure (Fig. 1). The mercury polluted rice induced significant expression of *c-jun* mRNA. Compared to the GMM, the expression of *c-jun* mRNA in GCP was decreased significantly ($P<0.05$). It showed the antagonism between selenium and mercury on the expression of *c-jun* mRNA in the rats of GCP. In GCP, the molar Se:Hg ratio was 1.19 close to 1:1.

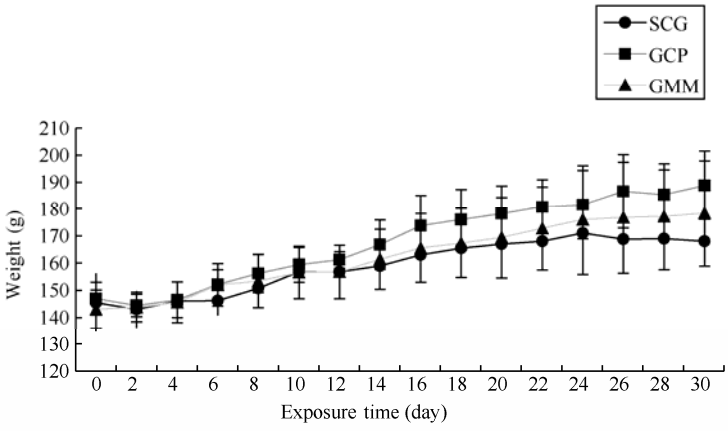

FIG. 2. Changes of weights of rats. n=7 rats in each group at each time point.

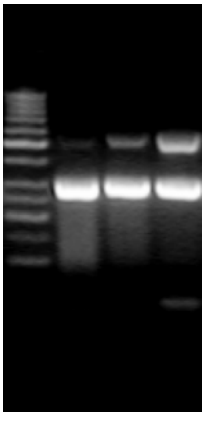

FIG. 3. RT-PCR products of *c-jun* and GAPDH on a 2% agarose gel stained with ethidium bromide. Lanes 1, 2, 3: GCP, GMM and SCG. DNA marker (Sangon, China) sizes were 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750-bp from bottom to top.

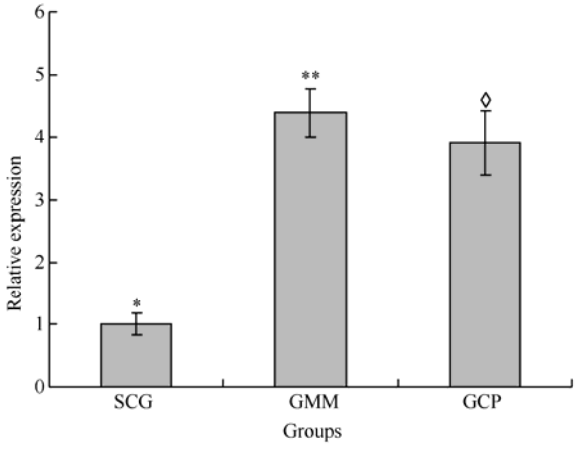

FIG. 4. Relative etxpression level of *c-jun* mRNA. The relative level of *c-jun* gene expression for each sample was normalized against GAPDH mRNA signals, and the control was set as 1.0. Each column and bar represents mean and SEM., n=7 rats in each group at each time point. $^{*}P<0.05$ compared to control, $^{**}P<0.01$ compared to control. Compared between neighbor exposure groups, $^{\diamond}P<0.05$.

c-jun Protein Expression

Few *c-jun* positive cells could be detected in the hippocampus, cortex and ependyma of SCG. In GMM and GCP, mercury polluted rice could induce cortex and hippocampus to produce a significant number of *c-jun* positive cells. Some *c-jun* positive cells could be detected in ependyma. The number of positive cells in the ependyma could not be counted easily. So we only calculated the number of labeled

cells in hippocampus and cortex. Fig. 5 shows that the *c-jun* positive cells in hippccampus and cortex of exposure groups were significantly different from that of control group (P<0.05, P<0.01). In both exposure groups the expression of *c-jun* protein in hippocampus increased more obviously than in cortex. The expression of *c-jun* between exposure groups had no notable difference.

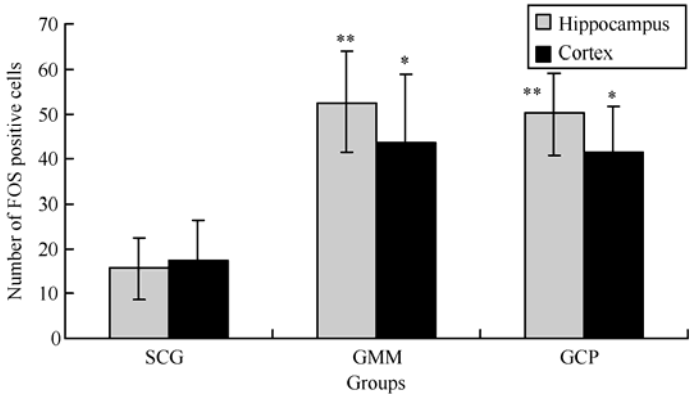

FIG. 5. Number of JUN-positive cells in rat hippocampus and cortex. Each column and bar represent the mean and S.E.M., n=7 rats in each group at each time point. $^{*}P$<0.05 compared to control, $^{**}P$<0.01 compared to control.

DISCUSSIONS

Mercury is a silvery, liquid metal at room temperature. It is sometimes referred to as one of the "heavy metals." Mercury enters the environment either from natural sources such as volcanoes and the weathering rocks, or from unintentional releases of this element by burning fossil fuels and smelting metals, producing chlorine and caustic soda and wiring devices and switches for electric lights, as well as from measuring and control instruments. Other mercury can also escape from the environment when items containing mercury are broken or thrown away. Whether the items are dumped in sewers, garbage cans or burned, some of the mercury would eventually be emitted into the atmosphere and enters lakes or oceans. Subsequently, mercury (Hg) is one of the priority pollutants and also a hot topic of the frontier environmental research in many countries, and has been paid higher attention in the world since the middle of last century. Evaluation of the effect of pollutants on environment has changed from organism, organs and tissues to cells, hypo-cells and even molecular genes. No matter how complexly the pollutants affect the ecosystem or individuals, or how serious the ultimate effect is, the earliest reaction must begin at the molecule level in cells and is reflected on individuals, species and ecosystem. So this earliest reaction in the molecular gene level is important for forecasting environmental pollution. *c-jun* is an immediate-early gene that has been implicated in the conversion of short-term stimuli into long-term changes in the phenotype of cells by regulating gene expression, and is therefore used as a functional marker of neural activation[22-23]. In normal condition, *c-jun* gene has low expression in nerve cells and takes part in cell growth, cell polarization and message transfer. The outer stimulators such as pollutants, the cold or cerebral ischemia could induce the expression of *c-jun*. The *jun* family contains three proteins (*c-jun*, *jun*-B and *jun*-D) and the Fos family contains five proteins (c-Fos, Fos-B, Fra-1 and Fra-2). Proteins can cross-dimerize with any of the three *jun* proteins, forming heterodimers, but cannot dimerize themselves. *jun* proteins, however, can also form homodimers, but *jun*-Fos heterodimers are formed much more efficiently than *jun-jun* homodimers and bind to DNA with a higher affinity. The activating protein 1 (AP-1) is an immediate early transcription factor composed of multiple protein families such as the Jun, Fos and ATF. AP-1 is an important transcription factor in multiple cellular processes as diverse as cell proliferation, differentiation and apoptosis.

In 1997, the US Environmental Protection Agency (US EPA) established a new guideline for methylmercury in the diet containing 0.1 microgram of mercury per kilogram of body weight per day (0.1 ug/kg/day) (US EPA, 1997). This is 4.7 times higher than the World Health Organization (WHO) standard of 0.47 μg/kg/day. The average concentration of organic mercury in rice of Guizhou Chemical Plant and Guizhou Mercury Mine was approximately 0.085 mg/kg (Table 1). In practical terms this means that a person weighing 60 kg could only consume approximately 12 g of rice per day.

The accumulation of mercury in brains of rats exposed to Guizhou Chemical Plant and Guizhou

Mercury Mine was both significantly different from those exposed to Shanghai rice (Fig. 1). The accumulation was related to the Hg species and the accumulation course in the groups exposed to Hg-polluted rice was slower than those exposed to MMC. The uptake of MeHg by brains was easier than that of $HgCl_2$, which was probably due to the higher affinity of MeHg. Selenium (Se) showed an effect on the accumulation and selenium could reduce Hg uptake (Fig. 1). We determined the levels of both *c-jun* mRNA and *c-jun* protein in rat brains exposed to different rice and found that polluted rice could induce the expression of *c-jun* gene. Increased *c-jun* mRNA and its protein product were observed after exposure for 3 days. Moreover, they increased significantly after 30 days. The expression could keep a long time (Figs. 4 and 5). These phenomena suggested that people should be advised to be cautious in eating any kind of Hg-polluted foods. Thus it could be seen that *c-jun* induction took place much earlier than cell apoptosis caused by mercury. So, the expression changes of IEG *c-jun* in rat brain can be used as an early warning for the neuotoxicity of mercury. Compared with the expression intensity of *c-jun* protein in different regions of hippocampus and cortex as well as ependyma, it could be concluded that more sensitive expression occurred in hippocampus and cortex, but not in ependyma. This demonstrated that hippocampus and cortex could be selected as the regions for the early warning on mercury toxicity.

Previous studies have shown that *c-jun* gene expression was increased after exposure to other heavy metals. Arsenite[24-25] was found to induce c-*jun* gene expression in the HeLa cell line in response to a 50 mmol/L exposure. Cadmium[26-27] was also found to induce *c-jun* gene expression in the LLC-PK1 cell line, porcine renal epithelial cells, in response to 1 h exposure to 20 mmol/L, however it also induced c-*myc* gene expression. On the other hand *c-jun* protein has been shown to play a role in the activation of apoptosis. It has been reported[28-30] that the administration of cadmium to the experimental animals could induce apoptotic cell death in proximal tubules, testis, and liver.

This research is important as it provides a new method for studying the effects of a toxicant, allowing to more accurately assess what might occur in the human body when this toxicant is introduced. Although a definitive conclusion cannot yet be drawn regarding cell fate from the findings of our research, it does show a discrete reproducible response that may be able in the future to give insights into the effects of chronic low-level exposures. To reveal the relationship between *c-jun* induction and apoptosis, further examinations are required.

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