Consumption of Mercury-contaminated Rice Induces Oxidative Stress and Free Radical Aggravation in Rats[1]

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Objective To study the oxidative stress induced by consumption of mercury-contaminated rice in rats, and to assess the possible public health risk of mercury contamination in Wanshan mining area. **Methods** Sprague Dawley rats were fed the mercury-contaminated rice produced from Wanshan area for 90 days. The antioxidant status and the free radicals in rat serum were evaluated. **Results** High mercury accumulation in organs of rats fed the mercury-contaminated rice confirmed the server pollution of mercury in Wanshan mining area. The intensity of electron spin resonance (ESR) signal increased by 87.38% in rats fed the rice from Wanshan compared with that in the control rats fed the rice from Shanghai, suggesting that chronic dietary consumption of rice from mercury mining area could induce an aggravation of free radicals. Feeding the mercury-contaminated rice was associated with significant decreases in the antioxidant enzymatic activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) and concentration of serum nitric oxide (NO), but it had no effect on serum nitric oxide synthase (NOS) activity. Feeding the mercury-contaminated rice raised the level of serum malonyldialdehyde (MDA), indicating the occurrence of oxidative stress. **Conclusion** The long-term dietary consumption of mercury-contaminated rice induces the aggravation of free radicals and exerts oxidative stress.

Key words: Electron spin resonance; Free radicals; Mercury; Oxidative stress; Rice

INTRODUCTION

Mercury is toxic to both animals and humans, targeting particularly the central nerve system, and inducing pathological changes of the nervous system including the increased membrane permeability and neuronal protein production[1]. Many national and international agencies have made considerable efforts on monitoring mercury contamination[2-3]. China is the third largest mercury producer, and Guizhou Province is the major mercury mining area. The total output was reported to be 26 000 tons during 1949 to 1981, and the peak annual mercury emission from mining and refining to the atmosphere has reached 11 tons[4-5]. Wanshan Mercury Mine, known as the "Mercury Capital" in China, is the largest cinnabar deposit in Guizhou. Exploration of mercury in Wanshan mining area has led to severe environmental pollution and deteriorated local ecosystems[6]. Scientific surveys in this area showed that most samples of aquatic, soil, and biomass had much higher mercury content than the national standard[7].

The impact of mercury on ecosystems and in particular on public health is greatly concerned. Oxidative stress has been suggested as one of the important mechanisms by which mercury exerts its initial neurotoxic effect[8]. Although previous studies have been carried out in Wanshan mercury mining area, most of them merely focused on the distribution, characteristics, and environmental behaviors of mercury[9-11]. The effects of mercury pollution on public health in Wanshan mercury mining area have rarely been studied. In order to assess the health effects of mercury contamination on local population in Wanshan area, the present study was therefore to investigate the oxidative stress in rats fed the rice from Wanshan mining area. Free radicals, malonyldialdehyde (MDA), superoxide dismutase

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(SOD), glutathione peroxidase (GSH-Px), nitric oxide (NO) and nitric oxide synthase (NOS) were determined to elucidate the ecotoxicity of mercury contamination in Wanshan area. This study may have implication for public health risk assessment of mercury contamination in Wanshan mining area.

METHODS AND MATERIALS

Rice and Feeding

Rats were divided into Wanshan group (WG) and Shanghai group (SG). WG Rats were fed the rice produced from Wanshan mercury mining area. The rice was irrigated with water leached from ignition and mining residues, thus high mercury content was detected. SG rats were the control group fed the rice purchased from Shanghai market, which had a much lower mercury content compared with that from Wanshan area. Concentrations of total mercury (T-Hg) and methylmercury (MeHg) in rice are presented in Fig. 1. T-Hg in rice was detected by the AMA-254 liquid/solid mercury analyzer (Milestone, Italy), and MeHg was determined by National Institute for Minamata Disease (Minamata, Japan) using the GC-ECD method.

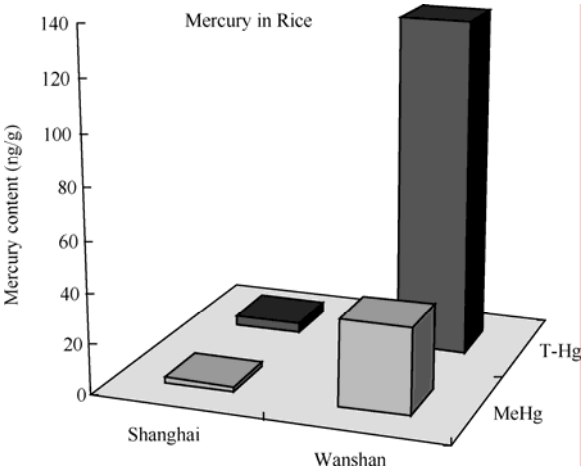

FIG. 1. Mercury and methylmercury in rice obtained from Shanghai and Wanshan mercury mining regions.

To meet nutrient requirements, vitamin mix, corn oil and cornstarch were added to the rice[12]. The rice was cooked and added with other feed ingredients before feeding to the rats.

Animals

Sprague-Dawley rats weighing 135-140 g (Shanghai Animal Experimental Center, Chinese Academy of Sciences, Shanghai, China) were housed separately in an animal room at 22℃ with a 12-12 h light-dark cycle. Too active and dull rats were excluded from the experiment. The rats were allowed free access to food and water. The day, before the rice was given, was marked as day zero. After feeding the rice for 90 days, the rats were sacrificed and blood samples were collected and saved for the biochemical analyses and electron spin resonance (ESR) measurement. Brain, liver, and kidney were also collected to determine their mercury and methymercury contents.

Biochemical Analyses

The level of lipid peroxides in rat serum was determined spectrophotometrically by measuring the pink-colored product of the thiobarbituric acid-reactive substance complex, as previously described by Ohkawa *et al.*[13].

NO amount was determined using an enzymatic reduction assay. In brief, total NOS enzyme activity was measured by monitoring conversion of L-arginine to L-citrulline. The measurements of NO and NOS were undertaken according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China).

SOD activity was assayed by the inhibition of pyrogallol autoxidation at 25℃, and followed kinetically at 420 nm[14]. One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of pyrogallol autoxidation.

The activity of GSH-Px was determined by 5,5'-dithionbis (2-nitrobenzoic acid) (DTNB) photometric method[15]. Briefly, 400 μL homogenate was mixed with 400 μL of 1.0 mmol/L GSH, and incubation at 37℃ for 5 min followed by addition of 200 μL of 1.25 mmol/L H_2O_2. After incubation at 37℃ for 3 min, 4 mL of 5% trichloroacetic acid was added. After centrifugation, the supernatant was collected and mixed with 2.5 mL of disodium hydrogen phosphate, 0.12 mL of 4 mol/L.0 NaOH and 500 μL of DTNB. The absorbance of the sample was recorded against the blank at 422 nm using a Unico UV-2102 PCS spectrophotometer (Unico UV-260, Unico, China). To correct the spontaneous reactions in the absence of enzyme, blanks were run without sample and then subtracted from the assay values.

Free Radical Measurements With Electron Spin Resonance

All ESR measurements were conducted using a Bruker EMX-8 spectrometer (Bruker Instruments Inc., Germany) and a flat cell assembly. The whole blood was centrifuged at 10 000 g for 10 min. An aliquot of serum (400 μL) was transferred into a test tube and DMPO was added to a final concentration of 100

mmol/L. The reaction mixture was then transferred to a flat cell for ESR measurement using a modulation frequency of 100 kHz, modulation amplitude of 3.5 G, microwave power of 19.97 mW, time constant of 20.48 ms and a scan rate of 100 G/41.94 s. The ESR spectrum of the sample was measured at room temperature within 5min. The relative amount of radicals in the test samples was calculated by the relative signal intensity rate=$[(h_x–h_0)/h_0] \times 100\%$, where h_x and h_0 were the ESR signal intensities of WG serum and SG serum, respectively.

Statistical Analysis

All results were expressed as $\bar{x} \pm s$. Statistical analysis was performed using SPSS v. 11.0. $P < 0.05$ was considered statistically significant.

RESULTS

Mercury Distribution

Chemical analysis of the rice showed that both mercury and methymercury contents in Wanshan sample were much higher than those in Shanghai sample. Total mercury content in Wanshan rice was 133 ng/g, 25.39% of which was methymercury. It was about 2 magnitudes higher than the value of Shanghai rice. This result confirmed the heavy contamination of Hg in Wanshan mining area.

A significant increase of the total mercury content was found in all organs of the rats in Wan Shan group compared with those in the controls (Table 1). The highest accumulation of total mercury occurred in the kidney, followed by liver and brain. The mean methymercury concentrations and relative standard deviation in organs are also shown in Table 1. The accumulation pattern of methymercury in organs examined in the present study had a same trend as the pattern of total mercury, but the portion of methylmercury to total mercury in kidney, liver, and brain was in a decreasing order.

MDA, NO and Enzymatic Activities

The levels of MDA, NO, NOS, and antioxidant enzymatic activities (GSH-Px, SOD) in the WG and SG rats are listed in Table 2. Although NOS activity in serum had no obvious change in WG group, the amount of NO significantly decreased. The antioxidant enzymatic activities of SOD and GSH-Px significantly decreased, suggesting that the scavenge ability of these two antioxidant enzymes was inhibited after rats were fed the mercury-contaminated rice for 90 days. The level of MDA increased in the serum of WG group compared to the control, indicating the occurrence of oxidative stress in WG rats.

TABLE 1

Total Mercury and Methymercury Concentrations (ng/g Wet Weight) in Organs of Rats ($\bar{x} \pm s$)

Organs	Total Mercury (ng/g)		Methymercury (ng/g)		Methymercury (%)	
	WG	SG	WG	SG	WG	SG
Brain	22.76±3.37**	2.61±0.29	20.43±0.44**	2.56±1.03	89.76	98.08
Liver	137.33±27.03**	8.15±1.96	60.10±20.11	3.28±0.68**	43.77	40.30
Kidney	703.45±46.15**	88.66±22.31	189.35±44.04**	6.43±0.06	26.92	7.26

Note. The superscripts indicate significant differences between WG samples and controls $^{**}P<0.01$; Every value represents as $\bar{x} \pm s$ (n=7).

TABLE 2

Levels of GSH-Px, SOD, MDA, NO, and of NOS in Rat Serum ($\bar{x} \pm s$)

	GSH-Px (U/mgprot)	SOD (U/mL)	MDA (nmol/mL)	NO (μmol/L)	NOS (U/mL)
WG	172.10±6.56**	31.1±3.29**	10.81±0.96**	7.05±1.50*	32.67±0.59
SG	183.36±1.98	39.12±2.73	6.57±0.47	11.11±1.59	29.38±2.89

Note. The superscripts indicate significant differences between WG group and the control. $^{*}P<0.05$. $^{**}P<0.01$. The data were expressed as $\bar{x} \pm s$ (n=7).

ESR Results

In our study, ESR spectra of the spin adducts obtained from rat sera are presented in Fig. 2. The principal spin adduct had hyperfine splitting constants of α_H =22.7±1.62G and α_N = 15.5±0.5 G. Based upon these constants, it appeared that the principal spin adduct came from the trapping of a methyl radical. However, the intensity of the 6-line signal was not equivalent, which was resulted from

coupling with a DMPO-OH adduct. Also, the splitting of α_H' in the spectra showed existence of the DMPO-OOH adduct. The intensity of ESR signal increased 87.38% in serum of WG compared with that of the control group, which was inversely related to the enzymatic activity inhibition shown in Table 2. The result suggested that chronic dietary consumption of mercury-contaminated rice could induce an aggravation of the free radical products.

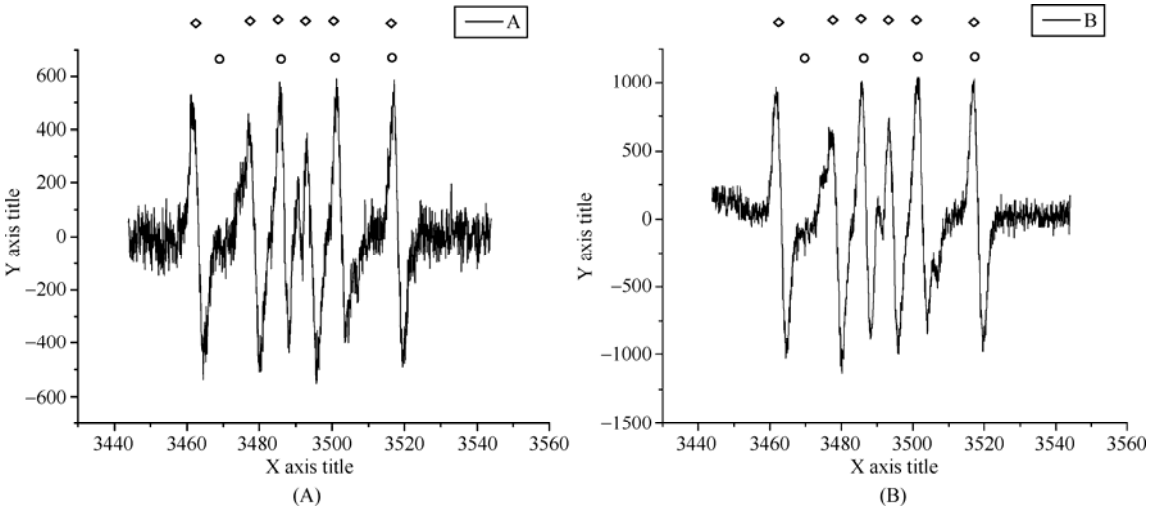

FIG. 2. ESR spectra of SG (A) and WG (B) rat serum within 5 min of the reaction.

DISCUSSION

Mercury is widely distributed in food, water, and environment. It is generally accepted that the nervous system is a main target of mercury. Oxidative stress has been suggested as one of the important mechanisms by which mercury exerts initial neurotoxic effect. Mercury can give rise to free radicals that induce lipid, protein, and DNA oxidation[16]. This was reflected from the present observation that the thiobarbituric acid reactive substances increased in several organs, and the activity of antioxidant enzymes was reduced in rats fed the mercury-contaminated rice. Most likely, neurotoxicity of mercury may be due to alterations in membrane integrity via formation of free radicals and perturbation of antioxidant defense mechanisms.

The present study has confirmed the severe contamination in Wanshan mercury mining area. The total mercury and methymercury levels in all organs of WG rats were also much higher than those in the control group. The highest mercury and methymercury concentrations were found in kidney, followed by liver and brain. However, the ratio of methylmercury to total mercury in kidney, liver and brain was in a decreasing order, suggesting that brain was the target organ of methymercury, but the liver and kidney were the target organs of inorganic mercury because they were actively involved in the detoxification of heavy metals.

ESR spin trapping is the method of choices for detection and identification of free radical generation due to its specificity and sensitivity. The intensity of the signal is used to measure the amount of short-lived radicals trapped, and the hyperfine couplings of the spin adduct are generally characterized by the original trapped radicals. The present findings confirmed that mercury accumulation could induce an aggravation of free radicals. Meanwhile, ESR results demonstrated that the long-term consumption of mercury-contaminated rice greatly induced formation of R· , OH· radicals and superoxide anions. Because R· is liposoluble and its life is longer and the active sites of biomolecules are closely related with lipophilic phase, it is expected that R· has a greater toxicity than hydrophilic O_2· and OH· . The generation of free radicals is greatly involved in lipid peroxidation[17]. Lipid peroxidation is an important mechanism by which free radicals exert their toxic effects, and it is mainly composed of three steps: promotion, extension and termination of the free radical chain reaction. Reactive oxygen species are important promoters in the chain reaction, while R· radicals play an important role in chain extension.

Reactive oxygen species could strongly attack the antioxidant enzymes. They can inactivate antioxidant enzymes by attacking –SH group, thus resulting in significant decrease of the enzyme activity. The present results provided evidence that

the activities of antioxidant enzymes, SOD and GSH-px, were significantly decreased in WG rat serum. These two antioxidant enzymes 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[18]. The decrease activity of these two antioxidant enzymes would inevitably result in a higher oxyradical formation and a lipid peroxidation. This would increase oxidative stress, leading to increased formation of lipid peroxides, which could cause further tissue damage. The significantly increased MDA level indirectly confirmed the severe antioxidant status in WG rats.

NO is important in regulating physiological functions of the nerve system in organisms. NOS plays an important role in the production of NO. Changes in NO and NOS may indirectly indicate the effects of mercury on normal physiological functions of organisms. NO may have possible neurotoxic and/or neuroprotective roles intervened with each other. Exposure of cells to excess NO can cause covalent modifications of –SH groups and damage to iron-sulphur proteins in mitochondria due to NO derived oxidation products ($-N_2O_3$, -ONOO·, -ONOOH). However, Radi *et al.*[19] showed that NO and O_2· can antagonize each other's biological action, and NO can protect cells by removing O_2· resulting in an inhibition of lipid peroxidation in some O_2· dependent systems. Thus, NO has strong antioxidant actions over free radicals and metal-mediated processes[19-20]. Hogg *et al.*[21] found NO could clean superoxide radicals directly and thus terminated the peroxidatic reaction. Previous studies showed that long-term exosure to heavy metals was always accompanied with decreased NO and/or NOS levels in serum[22-26]. In the present study, after 90-day exposure, NO concentration in rat serum decreased significantly compared with the control, suggesting that NO in serum might have beneficial effects in metal exposure biological processes, and the possible antioxidant actions of nitric oxide in serum might have been alleviated.

The aggravation of free radicals causes tissue damage and results in progressive cell damage including lipid peroxidation, oxidative modification of proteins, and DNA alterations, leading to cell death and neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease. Therefore, the US Environmental Protection Agency has set a much stricter guideline for mercury daily allowance from the diet[27]. In this regard, a person weighing 60 kg should not consume more than 20 g rice from Wanshan mining area per day. The present study confirmed that daily intakes of Wanshan mercury-contaminated rice would induce aggravation of free radicals and inevitably result in oxidative stress. If the data could be transferred to humans, the local population in Wanshan area would be at a great risk of mercury contamination. It is recommended that a number of biomarkers such as peroxidation and functional damage in target organ tissues be used to monitor health status in the Wanshan population who consume the locally produced rice .

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