Effect of methylmercury on some neurotransmitters and oxidative damage of rats

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Abstract: In order to study the molecular mechanism of injury in rat organs induced by methylmercury, and the relationship between neurotransmitter and oxidative damage in the toxicity process of rat injury by methylmercury was studied. The control group was physiological saline of 0.9%, the concentration of exposure groups were 5 mg/(kg·d) and 10 mg/(kg·d) respectively. The content of AChE, ACh, NOS, NO, MDA, SOD, GSH-Px and GSH in different organs of rats were determined with conventional methods. The results showed that after exposure to methylmercury for 7 d, the mercury content in brain of exposure groups increased clearly and had significant difference compared with the control group($P < 0.01$). In rat's brain, serum, liver and kidney, the content of ACh and AChE were all decreased; the content of NOS and NO were all increased; the content of MDA was increased compared with the control group, the exposure groups had significant difference ($P < 0.01$); the content of SOD, GSH and GSH-Px was decreased compared with the control group, the exposure groups had significant difference($P < 0.01$). It could be concluded that methylmercury did effect the change of neurotransmitter and free radical. They participated in the toxicity process of injury by methylmercury. The damage of neurotransmitter maybe cause the chaos of free radical and the chaos of free radical may also do more damage to neurotransmitter vice versa.

Keywords: methylmercury; neurotransmitter; oxidative damage

Introduction

Mercury(Hg), as one of the priority pollutant and hot topic in environmental research, has been paid higher attention in the world since the middle of last century. Mercury plays an important role in our daily life such as produce electrical instrument, pesticide and bactericide. Mercury is a globally spread pollutant due to characteristics such as low melting and boiling points, conversions between chemical forms and participation in biological cycles. As a result of emission to environment, it could transform into methylmercury (MMC), a substance which is typically detected in tissue. Mercury and its compounds all have great toxicity to human body. The half-life of mercury in human body is about 29—60 d. And mercury mainly accumulated in kidney, liver and spleen. The typical symptom of inorganic mercury poisoning is shimmy, abnormal spirit and gingivitis. Methylmercury could easily accumulated in organism because of its high fat-soluble. And it could enter into the brain through the blood-brain barrier. The typical symptoms of MMC poisoning is abnormal in pace, difficult in pronounce, ataxia, deaf and slack up in eyesight. The MMC could also break through the placenta barrier to effect the growth of fetus. There are also serious mercury pollution in some areas of China. Therefore, research of mercury pollution and mercury toxicities is an urgent task in our country.

Neurotransmitter is a kind of media through which nervous system transfer signal and is known as the first messenger. Acetylcholine is one of the major neurotransmitters in the brain of importance for cortical activation, memory and learning. It has a major role in the control motor tone and movement and probably counterbalances the effect of dopamine. The synthesized process of ACh contains 3 substances: choline acetylase (ChAc); choline and AcCoA. Most of ACh could be hydrolyzed by AChE after releasing to the clearance of synapse. AChE is one of the highest efficient enzymes in the body. It could stop the process of producing ACh. NO is a new kind of neurotransmitter(Kim, 2002; Golpon, 2003). It could transfer signal in the nerve centre and play an important role in immunity (Ohkuma, 2001; Arivazhagan, 2002). 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, 2000). Researches showed that mercury can influence various neurotransmitters and oxidative stress of rats. However, previous works were focused on laboratory experiments with high exposure dose and long exposure time with a lack of low exposure dose and short exposure time for sensitive bio-index. In order to study the molecular mechanism of injury in rat organs induced by MMC, and the relationship between neurotransmitter and oxidative damage in the toxicity process of rat injury by methylmercury, two kinds of neurotransmitter, Acetylcholine (ACh) and NO were discussed, and MDA, GSH, GSH-Px and SOD were chose to detect the ROS-injury.

1 Materials and methods

1.1 Animals and procedures

The US EPA Reference Dose for methylmercury is 0.1 μg/kg body weight per day. The reference dose is an estimate of daily exposure to the human population that is likely to be without an appreciable risk of adverse effects during a lifetime. In previous studies(Himi, 1996; Ikeda, 1999), it had been reported that in the methylmercury-treated(10 mg/(kg·d) for 9 d) cerebellum, Purkinje cells were positive both for NADPH-diaphorase and for neuronal NOS, Calcium-dependent NOS activity was increased to 160% of the controls. Concentrations of mercury that show acute and subacute toxicity in meals have been already determined. The LC_{50}(lethal concentration, 50%) value for rat exposed to mercury under static test was determined to be 57 mg/kg.

Thus, mercury levels below lethal concentrations were chose for the following experiments.

Sprague-Dawley rats(purchased from Shanghai Animal Experimental Center of China Science Institute, 200 ± 20 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. The rats were divided into three groups: (1) control group(injected with physiological saline of 0.9%); (2) 5 mg/(kg·d) MMC(injected with MMC according to weight); (3) 10 mg/(kg·d) MMC(injected with MMC according to weight). Each group has 7 rats(the males were 4, the females were 3). Rats need weight at 9:00 am every other day. And marked the day before feed as the zero days. The exposure time is 7 d.

1.2　Blood and organs collection

In the day 7 after administration of MMC, all the animals were deeply anaesthetized with 10% ketamine clorhydrate(0.5 ml/100g weight). Blood was divided into 2 parts. Serum was separated by centrifugation at 3000 r/min for 20 min from one part. The other part was added anticoagulant(natrium oxalate, analytical grade, purchased from Shanghai Reagent Company) quickly. The ratio between anticoagulant and blood was 1 ml:1 mg. The chest and skull were opened and then removed organs including brain, liver and kidney. Store these organs in – 20 ℃ refrigerator and make homogenate in 6 h.

1.3　Neurotransmitter

The activity of AChE and the ACh content was determined using regular method (Srivatsan, 1999). NO amount was determined by enzymatic reduction assay, and total 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 manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China), followed by spectrophotometric analysis.

1.4　Oxidative damage
1.4.1　GSH and MDA

The level of lipid peroxides was determined by spectrophotometry of the pink-colored product of the thiobarbituric acid-reactive substances complex, as described in the literature(Géret, 2002; Roméo, 1997). GSH content was measured by the modified Beutler method (Cookson, 1996; Zalups, 1999). Homogenate was precipitated by 10% TCA, centrifuged and the supernatant was collected. The supernatant was mixed with 0.3 mol sodium phosphate buffer (pH 8.0) and 5 ml 0.04% DTNB and incubated for 5 min at room temperature. The absorbance of the samples was read against the blank at 412 nm and the GSH concentration was calculated from the standard curve.

1.4.2　Antioxidant enzymic activities

SOD activity was assayed by the inhibition of pyrogallol autoxidation at 25 ℃, and was followed kinetically at 420 nm (Shinyashiki, 1996; Berntssen, 2003). One unit of SOD activity was defined as the amount of enzyme that caused 50% inhibition of pyrogallol autoxidation. The activity of GSH-Px was determined by 5,5'-dithionbis(2-nitrobenzoic acid)(DTNB) photometric method(Bakan, 2003). Briefly, 400 μl homogenate was mixed with 400 μl 1.0 mmol/L GSH, and incubated at 37 ℃ for 5 min followed by the addition of

1.25 mmol/L H_2O_2 200 μl. After incubating at 37 ℃ for 3 min, 4 ml 5% trichloroacetic acid was added. After centrifugation, the supernatant was collected and mixed with 2.5 ml disodium hydrogen phosphate, 0.12 ml 4 mol/L NaOH and 500 μl DTNB. The absorbance of the sample was recorded against the blank at 422 nm using a spectrophotometer (Unico UV-260, Unico, China). To correct spontaneous reactions in the absence of enzyme, blanks were run without sample and then subtracted from the assay values. Absorbance changes were measured using a Unico UV-2102 PCS spectrophotometer (Unico Corp., China) and analysed with Unico Application Software(Unico Corp., China). All enzyme activities are expressed per mg protein. The protein content was measured by method of Lowry *et al*. (Thirunavukkarasu, 2003) with bovine serum albumin as the standard.

1.5　Statistical analysis

All results were expressed as means ± *SD*. Statistical analysis was performed using SPSSv. 11.0 (SPSS). All significance testing took place at 0.05 level.

2　Results and discussion

2.1　Changes of rat's weight after exposure to MMC

The changes of weight after exposure to MMC are presented in Fig.1. We can see that the weight of control group increased normally while that of the 5 mg/kg group increased slowly. And the weight of 5 mg/kg group maintained in the evening of exposure time(5—7 d). The weight of 10 mg/kg group increased initially and decreased later, then reached its maximum level at the day 4 and began to decrease at the day 8, its weight was the same as that of the day 1. The weight of exposure group was much less than that of the control group. Methylmercury could restrain the growth of rat obviously and had relationship with the exposure time. During the experiment, we also found some abnormal behavior such as emaciation, bluntness in exposure groups. This might because of that: (1) MMC restrained the appetite of rat; (2) MMC decreased the circulation of cortiso (Hontela, 1999); (3) MMC restrained the function of thyroid gland(Friedmann, 1996).
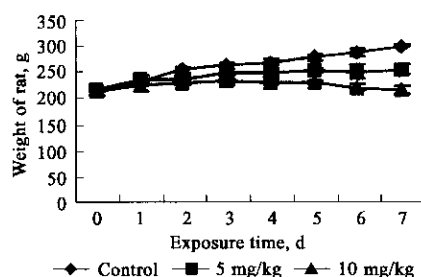

Fig.1　Changes of rat weight after exposure to methylmercury
n = 7 rats in each group

2.2　Total mercury in rat's brain after exposure to MMC

Mercury analysis was carried out by an AMA254 solid/ liquid mercury analyzer (Milestone, Italy) with an absolute detection limit of 0.01 ng. The content of total mercury in rat's brain increased sharply and had significant difference

compared with the control group ($P < 0.01$)(Table 1). This might be caused by the high fat-soluble of MMC so that it could break through the blood-brain barrier easily.

Table 1 The mercury content in brain after MMC exposure for 7 d

Group	Control	5 mg/kg	10 mg/kg
Content, μg/g	0.03 ± 0.05	$9.66 \pm 0.49^{**}$	$21.22 \pm 2.90^{**}$

Notes: ** Significant difference($P < 0.01$); compared with the control group

2.3 Changes of neurotransmitter after exposure to MMC

Table 2 shows that the content of ACh in different organs decreased after exposure to MMC for 7 d and had relationship with the exposure dose. After exposure to MMC for 7 d, the activities of AChE decreased clearly ($P < 0.01$) and had relationship with the exposure dose, too. The differences in ACh and the activity of AChE should be associated with the accumulation of mercury in rats. From the result of changes of ACh, we could see that in liver and kidney, the content of ACh decreased more sharply. This was because of that Hg was mainly accumulated in liver and kidney. And with extending the exposure time, the level of Hg in liver and kidney increased continuously and then the activities of ACh decreased. Previous studies showed that lower mercury concentration could facilitate the release of ACh, while higher mercury concentrations might induce inhibition of ACh release. Some data showed(Rajanna,1997) that MMC could decrease the content and the convert ratio of ACh in cortex, cerebellum, hippocampi, corpus striatum, interbrain-midbrain and pontine. Some studies (Golpon, 2003) also indicated that MMC could release ACh automatically and could restrain the high affinity of choline. MMC could exhaust ACh continually in nerve cell and decreased the level of ACh at last. Mercury acts at the neuromuscular junction and the synaptic cleft, producing a weakened postsynaptic potentiation. The significant decrease of AChE activity in rats might be resulted from a conformational change by the binding of Hg to a sulfhydryl group on the cholinesterase. Another means could be the destruction of anticholinergic cells.

After exposure to MMC for 7 d, the activities of NOS and the content of NO in different organs increased obviously ($P < 0.01$) and had relationship with the exposure level (Table 2). Changes in NO and NOS might indirectly indicate the effects of mercury on normal physiological functions (endocrine, immunity, reproduction, etc.) of organisms. Some reports(Géret, 2002) showed that a great deal of NO could participate in the toxicity of MDA and could generate cell toxicity by combining with superoxide. Researches (Sang, 2002) also tell us that MMC could increase the activities of NOS and the content of NO in liver and kidney. Our experiment shows that the exposure of MMC did increase the level of NOS and NO in body. Activation of N-methyl-D-aspartate (NMDA) receptors had been shown in several studies to stimulate nNOS activity, through a calcium-calmodulin dependent mechanism(Stuehr, 1999). NO can augment release of both glutamate (Bogdanov, 1997) and dopamine in the striatum, perhaps through local interactions with synapses, because the spines of medium spiny neurons receive inputs from glutamatergic, dopaminergic, and nNOS

neurons(Morello, 1997). The increased release of dopamine and glutamate might be dependent on activation of NMDA receptors (Segovia, 1998). We propose that glutamate released in the rat in response to MMC administration activates NMDA receptors, which trigger a feed forward mechanism of stimulation of nNOS activity, which produces NO, which further induces release of glutamate and dopamine.

2.4 Changes of oxidative damage after exposure to MMC

The content of MDA in different organs of 5 mg/kg group and 10 mg/kg group increased obviously and had significant difference compared with control group ($P < 0.01$) after exposure to MMC for 7 d(Table 3). High expression of MDA was regarded as an indicator of lipid peroxidation. An increase in MDA production was probably a consequent result of augmented activity of ROS. Lipid peroxidative damage, determined as MDA production, showed that MDA production increased with Hg concentration increasing. In the same way, as time exposure increases the lipid peroxidative damage was higher. The production of reactive oxygen species and oxidative tissue damage might be associated with metal toxicity and DNA damage. Tsuzuki et al. reported that both the cytotoxicity and the DNA breakage induced by Cd and Hg may be associated with active oxygen species inside cells, because H_2O_2-resistant Chinese hamster ovary cells were cross-resistant to the damaged caused by $CdCl_2$ and $HgCl_2$ (Tsuzuki, 1994).

Table 2 Changes of some neurotransmitter after exposure of MMC for 7 d

		AChE, μmol/ (ml·min)	ACh, μg/ml	NOS, U/ml	NO, μmol/L
Control	Brain	15.7 ± 2.7	179.2 ± 21.6	7.4 ± 1.5	8.0 ± 3.9
	Serum	18.6 ± 2.4	232.6 ± 19.7	23.1 ± 1.7	10.4 ± 4.2
	Liver	11.3 ± 1.5	165.2 ± 13.4	20.9 ± 2.1	15.3 ± 6.3
	Kidney	5.4 ± 0.9	112.6 ± 10.3	11.5 ± 1.1	8.1 ± 3.5
5 mg/(kg·d)	Brain	11.4 ± 2.3	147.5 ± 12.5	13.9 ± 0.9	14.5 ± 3.7
	Serum	14.4 ± 1.8	193.8 ± 14.1	34.6 ± 2.7	14.7 ± 4.1
	Liver	5.7 ± 0.7	139.2 ± 11.7	28.7 ± 2.4	20.2 ± 3.2
	Kidney	3.4 ± 0.5	99.8 ± 12.1	15 ± 1.2	13.7 ± 2.6
10 mg/(kg·d)	Brain	9.1 ± 1.2	123.4 ± 13.4	15.6 ± 2.0	16.8 ± 1.9
	Serum	13.3 ± 3.2	160.3 ± 15.7	39.4 ± 2.4	17.5 ± 2.1
	Liver	3.2 ± 0.5	110.7 ± 9.4	34.0 ± 3.5	22.2 ± 1.5
	Kidney	2.8 ± 0.3	82.6 ± 16.5	18.0 ± 0.7	15.7 ± 2.2

Table 3 shows that, after exposure of MMC for 7 d, the activities of SOD and GSH-Px in different organs of 5 mg/kg group and 10 mg/kg groups decreased obviously and have significant difference compared with control group ($P < 0.01$). the SOD and GSH-Px are active scavengers of free radicals. GSH-Px catalyzes the reduction of lipid and hydrogen peroxides to less harmful hydroxides. Superoxide dismutase (SOD) is an enzyme that has anti-inflammatory capacity because of its ability to scavenge the superoxide free-radical, but its biological activity in rats and humans is poorly understood. They are both involved in protecting against potential cell injury and neuropathological conditions. Differences in anti-inflammatory activity with different SOD sources have been reported before(Garcia-Gonzalez, 1999). 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. In the present study both SOD and GSH-Px decreased after methylmercury exposure. This

indicates an adaptive response of the redox-defence system in rats, as opposed to a general break-down of the redox defence system in the rat after methylmercury exposures.

Table 3   Levels of MDA, SOD, GSH and GSH-dependent enzymes in the organs of rats

		MDA, nmol/ml	SOD, U/ml	GSH-Px, U/mgprot	GSH, Umol/L
Brain	Control	2.12 ± 0.18	112.07 ± 9.12	26.24 ± 2.09	10.28 ± 1.68
	5 mg/(kg·d)	2.48 ± 0.18**	91.19 ± 11.31**	20.13 ± 3.26''	7.19 ± 1.97*
	10 mg/(kg·d)	2.70 ± 0.32''	82.61 ± 11.22**	15.53 ± 2.87**	6.53 ± 1.78**
Liver	Control	3.96 ± 0.49	727.63 ± 28.66	27.47 ± 3.62	22.73 ± 4.51
	5 mg/(kg·d)	6.39 ± 0.76**	647.29 ± 59.37**	21.31 ± 0.75**	16.08 ± 4.92'
	10 mg/(kg·d)	7.03 ± 1.02**	603.45 ± 66.11**	18.16 ± 4.22**	11.26 ± 3.72''
Kidney	Control	3.22 ± 0.14	625.95 ± 41.47	19.45 ± 1.42	22.20 ± 1.84
	5 mg/(kg·d)	3.71 ± 0.19**	505.54 ± 85.22**	16.35 ± 2.04''	15.90 ± 4.23*
	10 mg/(kg·d)	4.18 ± 0.31''	435.26 ± 85.75''	14.48 ± 1.90**	18.82 ± 4.00**
Serum	Control	7.22 ± 0.61	39.41 ± 5.97	175.70 ± 3.95	
	5 mg/(kg·d)	8.18 ± 0.28**	29.81 ± 2.86''	138.57 ± 5.50**	
	10 mg/(kg·d)	10.15 ± 1.75**	25.56 ± 2.86''	100.96 ± 27.63''	

Notes: ** $P < 0.01$, * $P < 0.05$, compared to control group; $n = 7$ rats in each group

Table 3 shows that the content of GSH in different organs of 5 mg/kg group had difference compared with control group($P < 0.05$) and that of 10 mg/kg group had significant difference compared with control group ($P < 0.01$) after exposure to MMC for 7 d. And the content of GSH decreased obviously. This was because Hg could bind to GSH forming a GSH-Hg conjugate. As a result, the total GSH measured by the enzymatic method will decrease. In Channa punctatus, short-term subacute mercury exposure induced an enhancement of GSH, while longer exposure resulted in a decrease of the thiol(Rana, 1995). Moreover, other metals, such as cadmium, copper, and zinc, as well as other organic contaminants(chlorothalonil, aromatic hydrocarbons, PAH, and PCB) cause an increase of GSH level in fish, providing an additional protection against the cytotoxic effects of biologically reactive molecules (Paris-Palacios, 2000). Mercury and copper seem to stimulate the production of GSH also in mammalian source; these metals caused a decline of GSH content in cell line, thereby stimulating the synthesis of additional thiol (Hultberg, 1999). On the contrary, the decrease of GSH level induced by metals could be due either to a direct binding of metals to GSH through its SH group (rising the metal-SG complexes) or to an enhanced oxidation of this thiol.

The experiments found after exposure for 7 d, the content of GSH in different organs all decreased sharply, and the activities of SOD and GSH-Px also decreased sharply while the content of MDA increased obviously(Table 3). This told us that the ability of free radical elimination in rat's body decreased obviously, the damage of free radical enhanced. As a result, the ROS-injury begins. During the experiment we found pathological changes like liver tumefaction. And among these organs discussed above; liver had the highest content or activities of different neurotransmitter. This showed that liver tumefaction is related to the detoxification of liver. After exposure by high content of MMC, the function of detoxification of liver was damaged badly. The experiment showed that after exposure of MMC, the SOD content of different organs decreased while that of the MDA increased. The ratio between MDA and SOD

increased and then caused an unbalance between free radical and free radical clean system. As a result, the tissue was more easily to be damaged. The damage of neurotransmitter may cause the chaos of free radical. This may because of that ACh is one of the most important neurotransmitter in the nerve centre and participated widely in the activities in the body. When ACh was damaged, it might cause abnormal metabolizability of oxygen in the tissue and increased the content of O_2^- . And the chaos of free radical may also cause more damage to neurotransmitter vice versa.

3   Conclusions

After exposure to MMC for 7 d, the content of GSH in different organs all decreased sharply, and the activities of SOD and GSH-Px also decreased sharply while the content of MDA increased obviously. It indicated that the ability of free radical elimination in rat's body decreased obviously, the damage of free radical enhanced. The ratio between MDA and SOD increased and then caused an unbalance between free radical and free radical clean system. It could be concluded that MMC did effect the change of neurotransmitter and free radical. They participated in the toxicity process of injury by MMC. The damage of neurotransmitter may cause the chaos of free radical and the chaos of free radical may also do more damage to neurotransmitter vice versa.

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