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Title: Neurotoxicological effects of low-dose methylmercury and mercuric chloride in developing offspring mice

Article Type: Research Paper

Keywords: Mercurial compounds; Perinatal exposure; Neurotoxicity; Lipid peroxidation; Na+/K+-ATPase activites; Tissue Hg accumulation

Corresponding Author: Dr Shoei-Yn Lin-Shiau, PhD

Corresponding Author's Institution: National Taiwan University College of Medicine

First Author: Chun-Fa Huang, Ph.D.

Order of Authors: Chun-Fa Huang, Ph.D.; Shing-Hwa Liu, Ph.D.; Chuan-Jen Hsu, M.D., Ph.D.; Shoei-Yn Lin-Shiau, PhD

Abstract: Mercury is a well-known toxic metal and potently induces severe neurotoxicological effects, especially in infants and children. The purpose of this study was aimed at exploring the underlying mechanisms of neurotoxic effects of mercurial compounds on the different stages of developing mice. Low-doses (the probability to human exposure in mercury-contaminated areas) of MeHg (M, 0.02 mg/kg/day) and HgCl2 (H, 0.5 mg/kg/day) were administered to mice of the following groups: (1) treatment with distilled water for 7 consecutive weeks after weaning (CV); Exposure to mercurial compounds at different stages; (2) for 7 consecutive after weeks weaning (CM and CH); (3) only during perinatal and weaning stages (MV and HV); and (4) in all experimental stages (MM and HH). Results revealed the neurobehavioral defects (increased locomotor activities, motor equilibrium impairment, and auditory dysfunction) that correlated with increasing Hg accumulation in CM and CH groups. However, it revealed a decrease and an increase in locomotor activities in MV and HV groups, respectively; these became more severe in MM and HH groups than in MV and HV groups. Motor equilibrium performance in MV and HV groups remained normally, while that in MM and HH groups was decreased. The most severe auditory defects (altered auditory brainstem response, ABR test) found in MM and HH groups than those in the respective CM and CH, MV and HV, including absolute wave III delays and interwave I-III latencies, suggesting the irreversible auditory dysfunction caused by mercurial compounds. Furthermore, the alteration of LPO, Na+/K+-ATPase activities, and NOx in the brain tissues contributed to the observed neurobehavioral dysfunction and hearing impairment. These findings provide evidence that fetuses were much more susceptible to the effects of mercurial compounds with regard to inducing severely neurotoxicological injuries as that found in human beings. The signaling of ROS/Na+-K+-ATPase/NOx plays the crucial role in the underlying mechanism for mercurial compounds-induced toxic effects in offspring.

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Article Title: Neurotoxicological effects of low-dose of methylmercury and mercuric chloride on developing offspring mice

Author name: Chun-Fa Huang, Shing-Hwa Liu, Chuan-Jen Hsu, Shoei-Yn Lin-Shiau

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Signature (a scanned signature is acceptable, but each author must sign)

Chun-Fa Huang

Shing-Hwa Liu

Print name

Chun-Fa Huang

Shing-Hwa Liu

Chuan-Jen Hsu

Chuan-Jen Hsu

Shoei-Yn Lin-Shiau

Shoei-Yn Lin-Shiau

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Editor-in- Chief

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Dear Professor Kehrer:

The manuscript (TOXLET-D-10-00676) entitled "**Neurotoxicological effects of low-dose methylmercury and mercuric chloride in developing offspring mice**" was carefully revised according to your and reviewers' valuable suggestions. Please reconsider to publish this revised manuscript in *Toxicology Letters*. Further suggestions on our revised manuscript will be greatly appreciated.

With the best wishes,

Sincerely yours,

Shoei-Yn Lin-Shiau, Ph.D.
Institute of Pharmacology,
College of Medicine, National Taiwan University,
No. 1, Section 1, Jen-Ai Road, Taipei, 10043, Taiwan.
E-mail: syshiau@ntu.edu.tw
Tel: +886 2 23123456 ext: 88313
Fax: +886 2 23915297

Reply to associate editor's comments:

Q1. There are many grammatical errors. Careful proof-reading and editing of the entire manuscript, including the reference section must be conducted to improve the quality and the readability of the manuscript.

Ans. We have reduced in length, re-organized, proof-reading, and editing of this revised manuscript. We have also carefully corrected the revised manuscript with grammar and syntax by a native English speaker. If further editorial corrections are needed, we will be pleased to revise according to your suggestions.

Reply to reviewer' 1 comments:

Q1. The manuscript is unnecessarily too long, starting with the abstract. The authors can re-structure the paper and cut the numerous redundancies the excessive use of the words, "we' and our' makes for a very dull reading in a paper.

Ans. We have reduced in length and re-organized this revised manuscript. We have also corrected the mistakes, grammar, and syntax in all sections of revised manuscript. If further beneficial corrections are needed, we will be greatly appreciated and pleased to revise according to your suggestions.

Reply to reviewer' 2 comments:

Specific comments:

+++Abstract:

Q1. The authors must define the meaning of the abbreviation ABR. Here is the first time it appears in the text. Also, they must include the control group when the experimental groups are described.

Ans. We have been defined the abbreviation ABR: 'the auditory brainstem response (ABR)'(P1, L3 of the revised manuscript). Moreover, the definition of experimental groups was clearly described in P2, L7-11 of the revised manuscript.

+++Introduction:

Q1. Cheng et al., (2006a) is missing in the references' list. Only Cheng et al. (2006) is included. Saffi (1981): in the reference list is written as Shaffi.

Ans. P4, L10: 'Cheng et al., (2006a)' and 'Saffi (1981)' has been corrected to 'Cheng et al., (2006)' and 'Shaffi (1981)' in the revised manuscript.

+++Material and methods:

Q1. Page 6.- The authors must indicate which was the rationale for the selection of the experimental doses here used: 0.02 mg/kg/day of MeHg and 0.5 mg/kg/day of HgCl2. Are these doses related with the LD50 of these compounds? Are these doses

related with human exposure?

Ans.

1. The experimental doses (0.02 mg/kg/day of MeHg and 0.5 mg/kg/day of $HgCl_2$) used in this study are ‘the probability to human exposure levels in mercury-contaminated areas’ (P7, L10-11 of the revised manuscript).

2. We have explained the rationale for the selection of doses used in the section of ‘4. Discussion’ (P19, L2-16 of the revised manuscript).

Q2. Page 6.- Can the authors explain why they use 80 mg/kg of pentobarbital (ip) for newborn mice and 50 mg/kg (ip) for the mothers?

Ans.

1. Pentobarbital (I.P.) 80mg/kg for newborn mice is lethal (deep anesthesia) for sacrifice and low dose of 50mg/kg (light anesthesia) for mothers in order to draw the whole blood from an eyehole vessel.

2. In the experiment of this study, the newborn mice (at postnatal day 0) were sacrificed to measure mercury content after deep anesthesia by an intraperitoneal injection of pentobarbital (80 mg/kg). Female mice (dams) were collected the whole blood from an eyehole vessel, but not needed to sacrifice, after light anesthesia by an intraperitoneal injection of pentobarbital (50 mg/kg) (P6, L18-24 of the revised manuscript).

Q3. Page 7.- Experimental groups. The control group is not defined in this section. However, control group is shown in the figures.

Ans. We have clearly defined all experimental groups in this section (P8, L5-11 of the revised manuscript).

Q4. Page 9.- Delete Huang et al. (2008a).

Ans. This part has been deleted.

Q5. Page 9.- The authors should explain why they did not determine GSH levels or GSH/GSSG ratio in the samples.

Ans.

Lipid peroxidation (LPO) is a well-defined mechanism of cellular damage in vitro and in vivo during exposure to toxic insults or in certain disease states. Lipid peroxides are the important indicators of oxidative stress in cell membranes, which decompose to form more complex and reactive compounds such as malondialdehyde (MDA), natural bi-product of lipid peroxidation. Determining these aldehydic secondary products of lipid peroxidation are generally accepted markers of oxidative stress (Janero, 1990). Our previous studies have also demonstrated that LPO is an

important biomarker to detect mercurial compounds-induced oxidative stress damage in the central nerve system after low-dose and long-term exposure (Huang et al., 2007 and 2008).

As to the alteration of GSH contents, Wang et al.(2009) reported that MeHg exposure led to a biphasic response in the intracellular GSH content of primary astrocytes (high concentration of MeHg led to depletion of GSH, while low concentration of MeHg resulted in increasing intracellular GSH)(Wang et al., 2009).

Based on these reasons, we determined the LPO levels to understand the oxidative stress damage in the brain tissues of developing offispring mice after low-dose and long-term exposure to methyl mercury and mercuric chloride.

References:

Dalton, T. P., Chen, Y., Schneider, S. N., Nebert, D. W., and Shertzer, H. G., 2004. Genetically altered mice to evaluate glutathione homeostasis in health and disease. Free Radic Biol Med 37, 1511-1526.

Huang, C. F., Hsu, C. J., Liu, S. H., and Lin-Shiau, S. Y., 2008. Neurotoxicological mechanism of methylmercury induced by low-dose and long-term exposure in mice: oxidative stress and down-regulated Na+/K(+)-ATPase involved. Toxicol Lett 176, 188-197.

Huang, C. F., Liu, S. H., and Lin-Shiau, S. Y., 2007. Neurotoxicological effects of cinnabar (a Chinese mineral medicine, HgS) in mice. Toxicol Appl Pharmacol 224, 192-201.

Janero, D. R., 1990. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic Biol Med 9, 515-540.

Wang, L., Jiang, H., Yin, Z., Aschner, M., and Cai, J., 2009. Methylmercury toxicity and Nrf2-dependent detoxification in astrocytes. Toxicol Sci 107, 135-143.

Wu, G., Fang, Y. Z., Yang, S., Lupton, J. R., and Turner, N. D., 2004. Glutathione metabolism and its implications for health. J Nutr 134, 489-492.

Q6. Page 11.- Write 1200xg or 12,000xg, but always in the same form.

Ans. This mistake has been corrected as ‘12,000xg’ (P12, L8 of the revised manuscript).

+++Discussion:

Q1. In general terms, it seems to be too long. There are some paragraphs in which the authors simply repeat the results. The discussion should be adequately shortened, remarking the most relevant new findings of the study.

Ans. We have reduced in length and adequate statements in the revised manuscript.

Q2. Page 22.- Estevez et al. (2002). In the reference list is written "Estevez and Jordan". Check this.

Ans. P22, L15: ‘Estevez and Jordan, (2002)’ instead of ‘Estevez et al. (2002)’ sevealed in the revised manuscript (P23, L4-5).

+++References

Q1. The authors must carefully follow the instructions for authors of the journal.

Ans. We have carefully corrected and rechecked the references section according to the instructions for authors of *Toxicology Letters* (P27-P32 of the revised manuscript).

Neurotoxicological effects of low-dose methylmercury and mercuric chloride in developing offspring mice

Chun-Fa Huang[a,b], Shing-Hwa Liu[b], Chuan-Jen Hsu[d], Shoei-Yn Lin-Shiau[c,*]

[a]School of Chinese Medicine, College of Chinese Medicine, China Medical University, Taichung, Taiwan

[b]Institute of Toxicology, [c]Institute of Pharmacology, College of Medicine, National Taiwan University, Taipei, Taiwan

[d]Department of Otolaryngology, College of Medicine, National Taiwan University, Taipei, Taiwan

[*]To whom correspondence should be addressed at the Institute of Pharmacology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei 100, Taiwan.

Fax: + 886 (2) 2391-5297.

Tel.: +886 (2) 2312-3456 ext. 88313.

E-mail: syshiau@ntu.edu.tw

Abbreviations:

MeHg, methylmercury; $HgCl_2$, mercury chloride; CV, control-vehicle; CM, control-MeHg; CH, control-$HgCl_2$; MV, MeHg-vehicle; HV, $HgCl_2$-vehicle; MM, MeHg-MeHg; HH, $HgCl_2$-$HgCl_2$; ABR, auditory brainstem response; LPO, lipid peroxidation; NO_x, nitric oxide; BBB, blood-brain barrier; ROS, reactive oxygen species; MDA, malondialdehyde; ICP-MS, Inductively Coupled Plasma Mass Spectrometry.

Abstract

Mercury is a well-known toxic metal and potently induces severe neurotoxicological effects, especially in infants and children. The purpose of this study was aimed at exploring the underlying mechanisms of neurotoxic effects of mercurial compounds on the different stages of developing mice. Low-doses (the probability to human exposure in mercury-contaminated areas) of MeHg (M, 0.02 mg/kg/day) and $HgCl_2$ (H, 0.5 mg/kg/day) were administered to mice of the following groups: (1) treatment with distilled water for 7 consecutive weeks after weaning (CV); Exposure to mercurial compounds at different stages; (2) for 7 consecutive after weeks weaning (CM and CH); (3) only during perinatal and weaning stages (MV and HV); and (4) in all experimental stages (MM and HH). Results revealed the neurobehavioral defects (increased locomotor activities, motor equilibrium impairment, and auditory dysfunction) that correlated with increasing Hg accumulation in CM and CH groups. However, it revealed a decrease and an increase in locomotor activities in MV and HV groups, respectively; these became more severe in MM and HH groups than in MV and HV groups. Motor equilibrium performance in MV and HV groups remained normally, while that in MM and HH groups was decreased. The most severe auditory defects (altered auditory brainstem response, ABR test) found in MM and HH groups than those in the respective CM and CH, MV and HV, including absolute wave III delays and interwave I-III latencies, suggesting the irreversible auditory dysfunction caused by mercurial compounds. Furthermore, the alteration of LPO, Na^+/K^+-ATPase activities, and NO_x in the brain tissues contributed to the observed neurobehavioral dysfunction and hearing impairment. These findings provide evidence that fetuses were much more susceptible to the effects of mercurial compounds with regard to inducing severely neurotoxicological injuries as that found in human beings. The signaling of ROS/Na^+-K^+-ATPase/NO_x

plays the crucial role in the underlying mechanism for mercurial compounds-induced toxic effects in offspring.

KeyWords: Mercurial compounds; Perinatal exposure; Neurotoxicity; Lipid peroxidation; Na^+/K^+-ATPase activites; Hg accumulation.

1. Introduction

Mercury is a toxic heavy metal and a widespread environmental pollutant; its different chemical forms produce varying degrees of toxic effects (Clarkson and Magos, 2006; U.S. Environmental Protection Agency (EPA) 2001). Organic methylmercury (MeHg) is more toxic and penetrates the blood-brain barrier (BBB) more easily than mercuric chloride ($HgCl_2$) (Baldi, 1997). Naturally occurring inorganic mercurial compounds or effluents from industrial pollution can be converted to MeHg by microorganisms, and the major route of human exposure to mercurial compounds is through dietary intake of mercury-contaminated fish, seafood, grain, or processed food (Clarkson et al., 2003; Cheng et al., 2006; Shaffi, 1981).

During the prenatal or neonatal period, the brain is high risk and extremely sensitive to toxicants such as MeHg (Burbacher et al., 1990; WHO, 1990). Epidemiological studies in Japan and Iraq have reported that following accidental occupational exposures, ingestion of contaminated fish or MeHg fungicide-treated grain, infant or child victims suffer acute neurotoxic effects and develop severe cerebral diseases, whereas their mothers experience only mild or smooth manifestations of this poisoning, indicating a higher risk or particular sensitivity in infants and children (Amin-Zaki et al., 1976 and 1981; Takeuchi, 1982;). Later studies indicated that fetal exposure to high-dose MeHg (0.5~11 ppm or total dose, 0.4~50 mg) via the placenta and/or breast milk during fetal and early postnatal periods causes neurological deficits and neuropathological changes in both humans and animals (Burbacher et al., 1990; Goulet et al., 2003; Sakamoto et al., 2002). Developmental exposure to low-dose MeHg was found to be a risk factor for neurobehavioral disorders, including subtle alterations of motor and cognitive behavior as well as delayed brainstem auditory-evoked-potential latencies in children of the Faroe Islands who were exposed to MeHg *in utero* or in those who consumed relatively high

amounts of MeHg-contaminated seafood (Debes et al., 2006; Grandjean et al., 1997; Murata et al., 2004). Moreover, it was reported that $HgCl_2$ is toxic to the nervous system and that, in humans, the major exposure routes are related to the specific working environment or conditions, such as mining spillage of mercuric compounds, handling of mercury salt by workers, or accidents producing intoxication by mercuric compounds (Goyer, 1995). However, the neurotoxic effects resulting from exposure to low-dose of MeHg or $HgCl_2$ during the prenatal period are not well established. Therefore, it raised important issue to study the neurotoxicological effects and potential mechanisms of exposure to low-dose of mercurial compounds in animal model during perinatal or developmental period, which was representing the human probable exposure dose in mercury-contaminated areas.

Reactive oxygen species (ROS) can induce undesirable biological reactions, including cell death or tissue injury within the central and peripheral nervous systems. It has been indicated that an increased ROS production caused by mercury-induced toxic effects can cause the altered brain or other cellular functions, eventually resulting in cell death and pathophysiological injury and, that event is accompanied with a decrease in antioxidant enzymatic activities (Inoue et al., 2004; Sarafian, 1999; Valko et al., 2005). Recently, ROS has also been suggested as an additional mechanism by which mercury exerts initial neurotoxic effects, accompanied by altered Na^+/K^+-ATPase activities in mammals (Huang et al., 2008a; Rodrigo et al., 2007). Moreover, some studies reported that acute or chronic mercury intoxication is characterized by an inhibition of neuronal Na^+/K^+-ATPase activities (Chuu et al., 2007; Verma et al., 1983). Inactivation of Na^+/K^+-ATPase activities can lead to partial membrane depolarization allowing excessive Ca^{2+} entry into neurons and resulting in toxic events similar to excitotoxicity, and has been implicated in pathological and physiological abnormalities and/or neurodegenerative diseases (Xie and Cai, 2003; Yu,

2003). Thus, this enzyme is very sensitive to oxidizing agents or heavy metal toxicities. Furthermore, mercurial compounds can bind and significantly suppress the enzymatic activities, causing cellular or organic dysfunction (Anner and Moosmayer, 1992; Rajanna et al., 1990). Recently, Lehotsky et al. (1999) and Rodrigo et al. (2007) have reported that ROS-induced damages were associated with the inhibition of Na^+/K^+-ATPase activities, and the antioxidants might prevent the inhibition of this enzyme activity.

Taken together, in this study, we attempted to elucidate the neurotoxicological effects induced by exposure to low-dose mercurial compounds (0.02 mg/kg/day MeHg [M groups] and 0.5mg/kg/day $HgCl_2$ [H groups]) in offspring mice (which were exposed after weaning, or only in perinatal and weaning stages, or in all stages studied) and to verify the working hypothesis that mercurial compounds-induced neurotoxicity was derived from induction of oxidative stress, alteration of Na^+/K^+-ATPase activities, and NO levels. Therefore, we designed experiments to monitor various neurofunctional parameters, including ABR test, spontaneous locomotor activities and motor equilibrium performance, to evaluate the biochemical changes and the Hg contents of the brain tissues after exposure to mercurial compounds. The results expected to shed some light on differential susceptibilities to various neurotoxicological effects and change in biochemical signals derived from prenatal and weaning exposure versus postnatal exposure to MeHg and $HgCl_2$.

2. Materials and methods:

2.1. Animal preparation

Randomly bred, male and female ICR mice were obtained from the Animal Center of the College of Medicine, National Taiwan University. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC), and the care and use of laboratory animals were conducted in accordance with the guidelines of the Animal Research Committee of College of Medicine, National Taiwan University and previous described (Huang et al., 2008b). The adult male and female ICR mice (4~5 weeks old, 22-25g) were randomly assigned to three initial dosing groups and then were orally gavaged distilled water, 0.02 mg/kg/day MeHg or 0.5mg/kg/day $HgCl_2$ (the probability to human exposure levels in mercury-contaminated areas) for 4 consecutive weeks before mating, and then two females were placed per cage with one male breeder for mating. Gestational day 0 (GD 0) was confirmed by the presence of a vaginal plug in the morning. At that time, the female mice with vaginal plugs (dams) were placed into individual cage from GD0 to postnatal day (PND) 21 (weaning) and maintained exposure to mercurial compounds. Thus, the exclusive route of offspring exposure to mercurial compounds was through maternal milk (Franco et al., 2006). At postnatal day (PND) 0, the offspring newborn mice (pups) were recorded the number in the litter, and randomly selected from different litters (three or four per litter) and sacrificed after deep anesthesia by an intraperitoneal injection of pentobarbital (80 mg/kg). Meanwhile, the whole blood samples of mothers were collected to eppendorf tube from an eyehole vessel, but not needed to sacrifice, after light anesthesia by an intraperitoneal injection of pentobarbital (50 mg/kg). These samples were analyzed Hg contents. At PND 21, male offspring (pups) within the original dose group assignment (distilled water, 0.02 mg/kg/day MeHg, or 0.5mg/kg/day $HgCl_2$) were randomly separated into two groups (seven per cage, total

numbers (*n*) = 12-15/group) and then orally gavaged with distilled water or original designed dose of mercurial compound for 7 consecutive weeks, respectively. Figure 1 illustrates the time course and groups of administration of mercurial compounds to offspring (0.02 mg/kg/day MeHg or 0.5mg/kg/day $HgCl_2$) and distilled water exposure during maternal gestation and weaning or following weaning (pups were: (1) orally gavaged with distilled water for 7 consecutive weeks after weaning (control-vehicle (CV) group); (2) exposed to mercurial compounds for 7 consecutive weeks after weaning (control-MeHg (CM) and control-$HgCl_2$ (CH) groups), (3) exposed to mercurial compounds during perinatal and weaning stages (MeHg- vehicle (MV) and $HgCl_2$-vehicle (HV) groups); or (4) exposed to mercurial compounds in all experimental stages (MeHg-MeHg (MM) and $HgCl_2$-$HgCl_2$ (HH) groups)). Body weight, behavioral and ABR tests were measured after administration with mercurial compounds for 7 consecutive weeks. All experimental animals were sacrificed by decapitation under pentobarbital anesthesia (80 mg/kg, i.p.) after administration with the vehicle control or mercurial compounds. The whole blood samples were collected from the peripheral vessels and various tissues were quickly removed to liquid nitrogen and stored at -80 ℃ until use.

2.2. Spontaneous locomotor activities

In the spontaneous locomotor activities tests, the experimental mice were performed during the day (9:00 a.m.-18:00 p.m.). The mice were individually placed in an open field and performed in a separated room with no interference noise or human activity. A large colorless rectangular box with a metallic grid floor was used (70-cm wide, 90-cm long and 60-cm high). The photobeam activity monitors (Tru Scan coulbourn intruments) was use and real-time for detecting track-type plots.

Overall pulses were recorded in an electromechanical counter as a gross measure of activity. Typical application of X-Y activity recording (floor plane activity) sensory ring drops over the cage and rests on ring support. Movement was detected by 16 ×16 infrared photobeam detectors and transducers set 1.5 cm above the floor of the apparatus and measured by a PC. Mice was allowed to move freely for 5 min but data were not scored, and then the number of squares crossed and the plots of tracking were counted during a period of 30 min for all experiments and quantification of data was by TruScan99 software.

2.3. Motor Equilibrium Performance

Motor coordination performance, a more complex motor skill task and required an intact cerebellar function and motor coordination, is a useful marker for monitoring MeHg-induced neurotoxicity (Sakamoto et al., 2002). In this test, an accelerating rotating rod treadmill (Ugo Basile; Stoelting Co., Chicago, IL) was using to examine the effect of mercurial compounds on experimental mice. The rotating rod was set in motion at a constant speed (60rpm) and the mice were placed into individual sections of rotating rod. Each time an animal fell, it was noted whether the fall had occurred when it sat still or when it walked. The animal's performance score in seconds was recorded when the mice were unable to stay on the rotating rod, tripped a plate and stopped the timer. Three successive trials separated by a 10 minutes pause were performed.

2.4. Recording of auditory brainstem response (ABR)

ABR was monitored after administration with mercurial compounds at 7 weeks. The ABR recording was based on that described by Huang et al. (2008b). Briefly, experimental mice were deeply anesthetized with an intraperitoneal injection of

pentobarbital (50 mg/kg), kept the body temperature by an electric blanket, and record the brainstem evoked response in a sound attenuated room. Subcutaneous needle electrodes with active electrodes placed in the vertex and ipsilateral retro-auricular region and a ground electrode on the neck of the animal recorded the click-evoked ABR by an auditory evoked potential system (Nicolet, Spirit, Madison, WI, USA). Mice were presented with a stimulus intensity series, which was initiated at 110 dB sound pressure level (SPL) and reaches a minimum of -5 dB SPL. The intensity of the stimulus was varied in a 5-dB stepwise decrement. Click stimuli were calibrated with a calibrated B&K precision sound level meter (duration 100 μs, stimulation rate 57.7/s, and frequency from 0 to 150 Hz). ABR threshold was defined as the lowest intensity capable of eliciting replicable and detectable waveforms. The absolute wave and interwave latencies of ABR waveforms were also recorded at a 105 dB SPL signal intensity. ABR was evoked by clicks in this study because the click-elicited ABR is a simplified and effective electrophysiological test to examine the hearing loss induced by mercurial compounds and its hearing thresholds would correlate with the enzyme activities of brainstem in the experimental mice.

2.5. Determination of lipid peroxidation in the brain tissues

The amount of MDA production, a substance produced during lipid peroxidation, was estimated according to the manufacturer's instruction (Calbiochem commercially available colorimetric assay kit) and with minor modifications. Briefly, the brain tissues (cerebral cortex, cerebellar cortex and brainstem) were weighed and homogenized separately in ice-cold 20 mM Tris-HCl buffer (pH-7.4, 100 mg tissue/ ml buffer), then homogenized samples were assayed immediately. The samples were added 3.25 volumes of diluted R1 reagent (10.3 mM *N*-methyl-2-phenylindole in acetonitrile), followed by gentle vortex mixing. After addition of 0.75 volumes of

37% HCl, the mixtures were incubated at 45°C for 60 min. After cooling, the absorbance of the clear supernatant was read at 586 nm. The linearity of the standard curve was confirmed with 0, 5, 10, 20 and 40 μM MDA standard (1, 1, 3, 3-Tetramethoxypropane). The protein concentration was determined by the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). LPO level was estimated from the standard curve and expressed as nanomoles (nmol) MDA per milligram protein.

2.6. Na^+/K^+-ATPase activities of brain tissues

The brain tissues of mice were acquired and analyzed for the Na^+/K^+-ATPase after 7 weeks subsequent low-dose of mercurial compounds fed. Membrane Na^+/K^+-ATPase activities were assayed as described previously (Huang et al., 2008a). The method allowed for quantification of two distinct Na^+/K^+-ATPase and Mg^{2+}-ATPase activities in the same sample. The enzymatic activities were measured in triplicate in covered 96 well microliter plates at 37±0.5℃ on a shaker. Thirty microliters of assay buffer (118 mM NaCl, 1.67 mM KCl, 1.2 mM $MgCl_2$, 12.3 mM $NaHCO_3$, 11 mM glucose, 0.5 mM EGTA, PH:7.4) containing 2 μg of membrance protein was added to each well. The Na^+/K^+-ATPase activity were determined by subtracting the ouabain (1.25 mM) insensitive Mg^{2+}-ATPase activity from the overall $Na^+/K^+/Mg^{2+}$-ATPase and the assay was started with the addition of 10 μl of ATP (final concentration 5 mM) making the final reaction volume of 100 μl. The reaction was terminated after pre-incubation at 37 ± 0.5℃ by the addition of 200 μl of malachite green (MG) plus ammonium molybdate (AM) (3:1). The inorganic phosphate (Pi) released from the substrate ATP was colorimetrically assayed by a microplate ELISA reader (Dynatech MR7000, Ashford, Middesex, UK) at 630 nm.

The absorbance values obtained were converted to activity values by linear regression using a standard curve of sodium monobasic phosphate that included in the assay procedure. The specific ATPase activities were expressed as Pi μmole (micromoles inorganic phosphate) released per mg protein per hr.

2.7. Measurement of nitric oxide (NO_x) levels

The quantitative nitric oxide (NO_x) assay was based on that described by Huang et al., (2008b). Briefly, the whole blood samples were completely denatured total protein by added 95% ethanol into the eppendorf at 4℃ overnight (12-16 hrs). Next day, all samples were centrifuged at 4℃ for 20 min at 12,000×g. The brain tissues used herein were weighed and homogenized separately in 0.32M sucrose-histidine buffer (pH 7.4, 100 mg tissue/ml buffer) and avoided incomplete denatured total protein by adding final concentration of 0.2N perchloric acid ($HClO_4$). After vortex violently, all samples were centrifuged at 4 ◦C for 20 min at 12,000×*g*. The supernatants of these samples were collected and assayed by the NO/ozone chemiluminescence (NO Analyzer 280A SIEVERS) for quantitative NO_x levels which measured the oxidation products (NO_2^- and NO_3^-) of NO using a reaction vessel containing a reducing system (0.1 M vanadium chloride, Aldrich Co., Germany). Detection of NO_x is then completed by its reaction with ozone, which leads to the emission of red light ($NO + O_3 \rightarrow NO_2^* + O_2$; $NO_2^* \rightarrow NO_2 + hv$). Standard curves were made prior to concentration (1, 5, 10 15 and 20 μM NO), which were freshly prepared solutions of $NaNO_2$ in distilled water.

2.8. Determination of mercury contents

To determine the Hg concentrations, various tissues (300 mg of whole blood,

cerebral cortex, cerebellar cortex, brainstem or the offspring newborn mice) were placed in a 15 mL polyethylene tube, and 0.5~1 mL of a 3:1 mixture of hydrochloric acid (35%) and nitric acid (70%) was added. The tubes were capped and allowed to stand overnight at 50 degree oven. After cooling, suitable dilution buffer (0.3% nitric acid and 0.1% Triton X-100 in distilled water) was added to the digested material, and the total mercury content was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The detection limit for mercury was ~0.1 ppb (μg/L).

2.9. Statistical analysis

The results in the text are given as mean ± standard errors. The significance of difference was evaluated by the paired Student's *t* test. When more than one group was compared with one control, significance was evaluated according to one-way analysis of variance (ANOVA) followed by Duncans's post hoc test to identify group differences. The P value less than 0.05 was considered to be significant. The statistical package SPSS, version 11.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

3. Results

3.1. Changes in litter number and mercury contents at postnatal day (PND) 1

The means of the number of offspring per litter significantly decreased (12.1 ± 0.7 and 12.3 ± 0.6 in MeHg and $HgCl_2$ treatment, respectively) compared with vehicle control (14.4 ± 0.4) (Figure 2). The body weights of offspring in groups exposed to mercurial compounds at PND 1 (1.4 ± 0.03, and 1.5 ± 0.02 g in MeHg and $HgCl_2$ treatment, respectively) were significantly lower than those of age-matched control (1.6 ± 0.02 g). Moreover, the Hg contents of maternal (dam) whole blood were reached up to 142.9 ± 5.8 and 69.8 ± 8.8 ppb in MeHg- and $HgCl_2$-exposed group, respectively, which were significantly higher than that of control dams (2.0 ± 0.3 ppb) (Table 1). The Hg contents of whole body of pups in mercurial compounds exposed groups at PND 1 were also found to be markedly higher than that in age-matched control (Table 1).

3.2. Mercurial compounds-induced body weight changes, neurobehavioral abnormalities, and auditory dysfunction in offspring mice

3.2.1. Alteration of body weight in offspring

As shown in Figure 3, both CM and CH mice developed normally, with body weight gain comparable to CV mice. However, body weight decreased significantly by 19.3% and 12.4% in offspring treated with MeHg and $HgCl_2$, respectively, during perinatal and weaning stages (MV and HV groups); it further reduced after continuous exposure for 7 consecutive weeks (MM and HH groups). The decrease after exposure to MeHg (by 32%) was much more pronounced than that after exposure to $HgCl_2$ (by

14.9 %).

3.2.2. Abnormalities of spontaneous locomotor activities and motor equilibrium performance induced by mercurial compounds

It has been reported that spontaneous locomotor activities to adopt as a useful method for detecting central function of neurotransmission or motor functional changes on MeHg-induced neurotoxicity (Huang et al., 2008a). To investigate neurotoxicity induced by exposure to low-dose mercurial compounds in offspring, we examined their influence on spontaneous locomotor activities in offspring. As shown in Figure 4A, CM and CH groups (treated with mercurial compounds beginning from weaning to 7 consecutive weeks) revealed hyperactivity in quantitative ambulatory distances ($^{\&}p < 0.05$ as compared with CV age-matched control). However, significant decrease in MV group, but increase in HV group (exposure to mercurial compounds in perinatal and weaning stages) of ambulatory distances were observed ($^{\#}p < 0.05$ as compared with the CV group), which were more severe by continuing exposure for further 7 consecutive weeks (MM or HH group) ($^{*}p < 0.05$ as compared with MV or HV group). Stereotype-1 episodes in MeHg exposed (Figure 4B) were increased in CM group, but slightly decreased in MV group, and prominently decreased in MM groups. Conversely, $HgCl_2$ had an opposite effect, decreasing stereotype-1 episodes in CH group, increasing them in HV group, and further increasing them in HH group.

With regard to the effects of mercurial compounds on motor equilibrium performance, it was found that retention times on the rotating rod (60 rpm) were mostly decreased (CM, CH, MM and HH groups) and that unaffected in mice exposed only in perinatal and weaning stages (MV and HV groups; Figure 4C).

3.2.3. Auditory dysfunction induced by mercurial compounds

To understand whether the exposure to low-dose mercurial compounds (MeHg-0.02 mg/kg or $HgCl_2$-0.5 mg/kg) might induce ototoxicity in offspring, the hearing thresholds were determined by measuring auditory brainstem response (ABR). As shown in Figure 5A, the means of hearing thresholds were significantly elevated in all offspring exposed to mercurial compounds, especially in MM and HH groups.

The absolute wave and interwave latencies of ABR were found to be significantly delayed by treatment with mercurial compounds (Figure 5A and 5B). Compared with the age-matched control (CV group), the mean values of the absolute latency of wave I were unaffected; those of wave III were increased in the MV, MM, HV, and HH groups; and those of wave V were increased in all exposed groups, especially in MM and HH groups. In addition, the interwave latencies were also significantly increased; those of interwave I-III increased in MV, MM, HV, and HH groups. Meanwhile, those of interwave I-V and III-V were increased in all exposed groups, especially in MM and HH groups.

3.3. Effects of mercurial compounds on lipid peroxidation (LPO) levels and Na^+/K^+-ATPase activities in brain tissues of offspring mice

To investigate the neurotoxic effects of exposure to low-dose mercurial compounds on the ROS generation in offspring mice, lipid peroxidation (LPO) levels were measured in various regions of the brain. LPO levels were significantly increased in the cerebral cortex, cerebellar cortex, and brainstem of all exposed groups. However, the increase in LPO levels appeared to be less in MV and HV groups (Figure 6).

To further examine whether enzymatic activities of Na^+/K^+-ATPase in the brain regions were also influenced by mercurial compounds, we analyzed Na^+/K^+-ATPase activities in the cerebral cortex, cerebellar cortex and brainstem. As shown in Figure 7,

Na^+/K^+-ATPase activities in the cerebral cortex was significantly increased in all exposed groups; however, the extent of increase in MeHg-treated groups was more than that in $HgCl_2$-treated groups. In the cerebellar cortex, Na^+/K^+-ATPase activities was markedly decreased in the CM and CH groups, but were increased in the other treated groups, especially in both MM and HH groups. Moreover, in the brainstem, MeHg treatment caused the decrease of enzymatic activities in CM and MM groups, but the increase in MV group. Treatment with $HgCl_2$ appeared the opposite effects; it increased Na^+/K^+-ATPase activities in CH and HV, but only slightly decreased in HH group.

3.4. Influence of mercurial compounds on nitric oxide (NO_x) levels in the whole blood and brain tissues of offspring mice

To further investigate whether the involvement of nitric oxide in mercurial compounds-induced neurotoxicity in offspring mice, the nitric oxide (NO_x: nitrate plus nitrite) levels of whole blood and brain tissues were analyzed by NO/ozone chemiluminescence. As shown in Figure 8A, it was found that NO_x levels in the whole blood were significantly increased in CM and MV groups, but were markedly decreased in MM group. However, $HgCl_2$ treatment caused a decreased NO_x levels in CH group but increased levels in HV and HH groups.

Moreover, NO_x levels in the cerebral cortex were markedly increased in CM, MV, and MM groups, especially that in MM group was higher than those in CM and MV groups. By contrast, NO_x levels were significantly decreased in all $HgCl_2$-treated groups (CH, HV, and HH); especially those in HV and HH groups. In the cerebellar cortex, a significant increase in NO_x levels was found in MV and MM groups, but not in CM group; it was particularly high in MM group. Remarkably decreased NO_x levels were found in all $HgCl_2$-treated groups (CH, HV, and HH); that in HH group

was significantly higher than that in CH or HV group (P<0.05). Meanwhile, NO_x levels of the brainstem in mercurial compounds-treated groups were had significantly decreased, except that in MM group was significantly increased (Figure 8B).

3.5. Exposure to mercurial compounds caused significant mercury accumulation in the brain tissues of offspring mice

To examine whether mercury could be absorbed by the gastrointestinal (G-I) tract, passed through the BBB, and then accumulated in the brain regions, we analyzed the Hg content of whole blood, cerebral cortex, cerebellar cortex, and brainstem using ICP-MS. As shown in Figure 9, Hg contents in the whole blood were significantly increased in CM, CH, MM, and HH groups, but were non-significantly altered in the MV and HV groups. It was also greatly increased in Hg contents in the cerebral cortex, cerebellar cortex, and brainstem after mercurial compounds exposure, even those in MV (12.2 ± 0.5, 28.4 ± 0.4, 19.9 ± 1.0 ppb) and HV (16.4 ± 0.8, 27.1 ± 0.2, 23.9 ± 2.9 ppb) groups were sill significantly increased to a lesser extent after a period of cessation to mercurial compounds exposure for 7 weeks (Figure 9).

4. Discussion

Many studies have documented the neurotoxic effects induce by high-dose MeHg in experimental animals during fetal or postnatal development, including walking capability and hind-limb dysfunction, learning memory and rotarod impairment, severe movement or postural disorders, and cerebellar Purkinje and granule cells disruption (Choi et al., 1981; Goulet et al., 2003; Sakamoto et al., 2002; Watanabe et al., 1999). In addition, $HgCl_2$-exposure can be excreted into maternal milk, results in higher mercuric concentrations, and causes the neurotoxicological pathologies (Franco et al., 2007; Goyer, 1995). Despite increasing studies have shown that MeHg can induce the neurotoxicity in both humans and animals, the precise mechanism of MeHg- and/or $HgCl_2$-induced neurotoxicological effects during the perinatal or developmental periods is not well understood, especially in cases of low-dose exposure. Thus, the mercury doses used in this study were concerning the low levels by comparable to the probable exposure dose in human via the ingestion of mercury-contaminated fish containing up to 3 ppm or 438.8 ng/g of mercury (Akagi et al., 2000; Grandjean et al., 1992; Kjellström et al., 1986). Here, the results showed that exposure to low-dose mercurial compounds (0.02 mg/kg/day MeHg or 0.5mg/kg/day $HgCl_2$) in offspring had the following effects: (1) caused the abnormality of ambulatory distances of locomotor activities in all groups as compared with control (CV) group (Figure 4A); (2) stereotype-1 episodes of locomotor activities were significantly altered in MeHg- or $HgCl_2$-treated groups, except that MV and HV groups remained unaffected. (Figure 4B); (3) dysfunction of motor equilibrium performance was found in the mercurial compounds-treated CM and CH groups, but

not in MV and HV groups (Figure 4C); and (4) in the MM and HH groups, motor equilibrium performance dysfunction was correlated with the increased brain LPO levels, which was not deteriorated to the extent of LPO levels in CM and CH mice. These findings apparently implicate that the offspring exposure to MeHg and $HgCl_2$ in prenatal stages have developed adaptation to further exposure.

In victims of mercury-contaminated areas such as Minamata of Japan and Iraq, severe neuropathologic deteriorations were correlated with high Hg levels in the brain (above 12 ppm), with the threshold for detrimental clinical effects in the fetus estimated at 1 ppm (Amin-Zaki et al., 1976; Choi et al., 1978; Takeuchi et al., 1999). In this study, effects of low-dose mercurial compounds exposure on the alteration of Na^+/K^+-ATPase activities and increase in LPO levels were observed (Figures 6 and 7) when mercury concentrations in the brain regions were below 300 ng/g (0.3ppm, Figure 9). This concentration of mercury is lower than or equal to levels detected in autopsied brain regions from victims of the mercury-contaminated areas. Moreover, our results showed that higher mercury levels were also detected in pups at PND 1 (Table 1), and then decreased to approximately 20-30 ng/g in MV and HV groups (Figure 9), which was accompanied by the neurobehavioral abnormalities (Figures 4), such as auditory dysfunction (Figure 5), and biochemical changes (Figures 6, 7, and 8). These findings were similar to that of a study by Goulet et al. (2003) in which levels of mercury in the brain at PND 0 were higher (approximately 8 ppm) and then decreased to below 1 ppm at PND 42 owing to exposure to high-dose MeHg in offspring during fetal and early postnatal development. Based on these findings, it indicates that: (1) perinatal exposure to low-dose MeHg or $HgCl_2$ causes irreversible neurotoxic effects; (2) further exposure to mercurial compounds can induce more severe neurotoxicological damages; and (3) although different responses in neurobehavioral effects and biochemical alterations were observed between CM, CH

groups and MM, HH groups, and the detailed mechanisms of these effects deserved for further investigation.

It has been reported that exposure to high-dose mercurial compounds could cause auditory dysfunction in humans within mercury-contaminated areas and in experimental animals. It is noticeable that increases in hearing thresholds, prolongation of interwave latency, and high Hg accumulation of in the blood and/or brainstem were found in children and adolescents in the mercury-contaminated areas (Counter et al., 1998; Murata et al., 1999 and 2004; Uchino et al., 1995) and also in experimental animals after short-term exposure to high-dose mercurial compounds (Wu et al., 1985; Chuu et al., 2001). These phenomena might be due to the fact that mercurial compounds can be absorbed by G-I tract and pass through the BBB subsequently accumulating in the brainstem and cause dysfunction of the brainstem auditory pathway. The delay of wave III latency and prolonged I-III interwave latency of the brainstem auditory-evoked-potentials indicated that some neurotoxic effects resulting from intrauterine MeHg exposure were irreversible and appeared to serve as a marker of prenatal MeHg toxicity from maternal ingestion of mercury-contaminated seafood (Murata et al., 1999 and 2004). However, the possible mechanisms by which mercurial compounds affect the auditory system remain poorly understood, especially with regard to long-term, low-dose exposure. The present work revealed that the hearing thresholds of ABRs were significantly increased in CM and CH groups and associated with abnormal prolonged wave (V) and interwave (I-V and III-V) latencies of ABR. Elevated hearing thresholds were also found in MV and HV groups, which exhibited prominent abnormal prolonged wave (III and V) and interwave (I-III, I-V, and III-V) latencies of ABRs (the dysfunction of the central auditory system in the brainstem), and those in MM and HH groups were more severely affected (Figure 5). Furthermore, these auditory dysfunctions were accompanied with significant

biochemical alterations (increase of LPO, and alteration of Na^+/K^+-ATPase activities and NO_x levels) and mercury accumulation in the whole blood and/or brainstem of all mercurial compounds exposure (CM, CH, MM, and HH) groups, even mercury levels in the brainstem of MV and HV groups decreased to about 20~30 ng/g, which were still significantly higher than those in the control (Figure 9). Therefore, these findings indicate that exposure to low-dose mercurial compounds (the probable concentrations for human exposure) in the perinatal stages can induce irreversible ototoxicity; further exposure results in more severely toxic effects in the later developmental stages. Moreover, the neurobiochemical studies of the brainstem tissues indicated that mercurial compounds-induced ototoxicity may be mediated by oxidative stress damage, and altered Na^+/K^+-ATPase activities and NO_x levels in the brainstem (main organs of the central auditory system). These findings provide useful information regarding the previous observations in humans where gestational and developmental MeHg exposure induced motor dysfunction and delayed brainstem auditory-evoked-potential latencies in children and adolescents from the Faroe Islands (Grandjean et al., 1997; Murata et al., 1999 and 2004).

Accumulated evidence has revealed that acute or chronic exposure to mercurial compounds, which specifically bind to Na^+/K^+-ATPase, significantly altered Na^+/K^+-ATPase enzymatic activities, especially in the brain regions, and were associated with neurotoxic impairments in animal models (Rajanna et al., 1990; Chuu et al., 2001). Na^+/K^+-ATPase is an ion-exchange enzyme that is essential for the generation and maintenance of sodium (Na^+) and potassium (K^+) gradient homeostasis between intra- and extra-cellular milieus and also responsible for functions of specialized organs such as the nervous system (Rakowski et al., 1989). Many studies have reported that various insults, such as mercury toxicities and chemical, induce neurological disorders or hearing loss *in vivo* and those disorders correspond to

changes in Na^+/K^+-ATPase activities of the brain regions and cochlear lateral wall (Huang et al., 2008a and 2008b; Cheng et al., 2005). Kourie et al. (1998) and Rodrigo et al. (2007) have also demonstrated that induction of oxidative stress, which causes LPO production, is closely paralleled with the inhibition of Na^+/K^+-ATPase activities, indicating ion transport ATPase is targeted by ROS damage. Furthermore, NO_x is an important signaling molecule that not only mediates several physiological functions but also regulates many pathological processes (Moncada and Higgs, 1991; Estevez and Jordan, 2002). Regulation of Na^+/K^+-ATPase activities by NO_x in various tissues has been reported, and increased or reduced NO_x production due to mercury exposure may induce neurotoxicity (Cheng et al., 2005; Chuu et al., 2007; Huang et al., 2008a). Nevertheless, the important roles of ROS, Na^+/K^+-ATPase, and NO_x in low-dose mercurial compounds-induced neurotoxicity remain unclear, especially with regard to perinatal or developmental (childhood) exposure. In this study, the results showed that LPO levels in all exposed groups significantly increased, with a less extent in the MV and HV groups (Figure 6). Alterations of Na^+/K^+-ATPase activities and NO_x levels in the whole blood and/or brain tissues were also found in the CM and CH groupsn and more markedly changes in groups MM and HH. (Figures 7 and 8). Those biochemical parameters altered by exposure to MeHg/$HgCl_2$ differed between CM, CH groups and MM, HH groups and were accompanied with significant neurobehavioral abnormalities and auditory dysfunction. Therefore, these findings imply that low-dose exposure to mercurial compounds can induce ROS generation and alter Na^+/K^+-ATPase activities as well as NO_x levels in brain regions, and that these actions maybe responsible for mercury inducing nervous and auditory system dysfunction during perinatal and/or developmental stages exposure. These results confirmed the contention that changes in Na^+/K^+-ATPase activities can be a biochemical indicator for chemical-induced neuronal activity or subclinical disease (Czaplinski et al., 2005;

Nicolini et al., 2004; Palecz et al., 2005). Based on these reasons and our findings, we believe that Na^+/K^+-ATPase activities serve as an important and useful biochemical marker in low-dose mercurial compounds-induced neurotoxicity.

5. Conclusion:

In conclusion, our findings demonstrated that fetuses are highly susceptible to mercurial compounds, which is evidenced by a strong toxicological basis for displaying the irreversibility of neurotoxicity and biochemical alterations. The signaling of ROS/Na^+-K^+-ATPase/NO_x plays the crucial role in the underlying mechanism for mercurial compounds-induced neurotoxicological effects in offspring, which may infer to the human exposure in mercury-contaminated areas. Moreover, this study also provides evidence for the first time that Na^+/K^+-ATPase activities in the brain can be regarded as an important and useful biomarker for evaluating neurotoxicity induced by perinatal exposure to low-dose mercurial compounds.

Conflict of interest statement:

All authors declare that there are no conflicts of interest in this study.

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Figure legends

Figure 1. Time course of oral administration of vehicle control or mercurial compounds to offspring (0.02 mg/kg/day MeHg or 0.5mg/kg/day $HgCl_2$) during maternal gestation and weaning or after weaning.

Figure 2. Decrease of litter number induced by mercurial compounds. The mercurial compounds (0.02 mg/kg/day MeHg or 0.5mg/kg/day $HgCl_2$) were orally administrated to the dams during gestation period. The litter numbers of newborn pup were recorded at postnatal day (PND) 1 as described in Materials and Methods. All data are presented as mean ± S.E. (n = 12-15/group). $^{\#}p < 0.05$ as compared with control vehicle (CV) group.

Figure 3. Effects of mercurial compounds on body weight gain of offspring mice. After weaning, the offspring mice were randomly selected as representatives of their respective litters (n = 12-15/group), and body weights were determined in various experimental groups as described in Figure 1. Data are presented as mean ± S.E. $^{\#}$:$p <$ 0.05 as compared with CV group; $^{*}p < 0.05$ as compared with MV group.

Figure 4. Effects of mercurial compounds on locomotor activities and motor equilibrium performance of offspring mice. Experimental mice were treated with mercurial compounds (0.02 mg/kg/day MeHg or 0.5mg/kg/day $HgCl_2$) or distilled water as described in Figure 1. Spontaneous total movement and motor equilibrium performance (retention times on rotating rod, 60 rpm) were recorded and analyzed as

described in the Materials and Methods. The locomotor activities (ambulatory distance (A), stereotypy-1 episodes (B) and equilibrium retention times on rotating rod (C) of all groups were recorded as described in Materials and Methods. Data are presented as mean ± S.E. (n = 12-15/group). $^{\#}$:$p < 0.05$ as compared with CV group; $^{*}p < 0.05$ as compared with MV or HV group; $^{\$}$:$p < 0.05$ as compared with CM or CH group, respectively.

Figure 5. Chronological increase of hearing thresholds and changes of the absolute and the interwave latencies of ABRs waveforms in offspring mice treated with mercurial compounds. Mice were orally gavaged with either mercurial compounds or distilled water as described in Figure 1. The hearing thresholds (A), absolute wave (I, III, and V)(B) and the interwave latencies (I-III, I-V and III-V)(C) of ABRs waveforms were recorded as described in Materials and Methods. Data are presented as mean ± S.E. (n = 12-15/group). $^{\#}$:$p < 0.05$ as compared with CV group and $^{*}p < 0.05$ as compared with MV or HV group; $^{\$}$:$p < 0.05$ as compared with CM or CH group, respectively.

Figure 6. Lipid peroxidation (LPO) levels of brain tissues of offspring mice treated with mercurial compounds. Experimental mice were orally gavaged with mercurial compounds (0.02 mg/kg/day MeHg or 0.5mg/kg/day $HgCl_2$) as described in Figure 1. LPO levels of cerebral cortex, cerebellar cortex and brainstem were determined as described in Materials and Methods. All data are presented as mean ± S.E. (n = 12-15/group). $^{\#}$:$p < 0.05$ as compared with CV group; $^{*}p < 0.05$ as compared with MV or HV group, respectively.

Figure 7. Changes of Na^+/K^+-ATPase activities in brain tissues of offspring mice

treated with mercurial compounds. Experimental mice were orally administrated with distilled water or mercurial compounds (0.02 mg/kg/day MeHg or 0.5mg/kg/day $HgCl_2$) as described in Figure 1. Na^+/K^+-ATPase activities of cerebral cortex, crerbellar cortex and brainstem were determined as described in Materials and Methods. All data are presented as mean ± S.E. (n = 12-15/group). $^{\#}$:$p < 0.05$ as compared with CV group; $^{*}p < 0.05$ as compared with MV or HV group; $^{\$}$:$p < 0.05$ as compared with CM or CH group, respectively.

Figure 8. Nitric oxide (NO_x) levels of whole blood and bran tissues of offspring mice treated with mercurial compounds. Experimental mice were orally gavaged with mercurial compounds (0.02 mg/kg/day MeHg or 0.5mg/kg/day $HgCl_2$) as described in Figure 1. Whole blood (A) and brain tissues (B) were acquired, de-proteinized and determined of NO_x levels (NO^-_2 plus NO^-_3) as described in Materials and Methods. All data are presented as mean ± S.E. (n = 12-15/group). $^{\#}$:$p < 0.05$ as compared with CV group; $^{*}p < 0.05$ as compared with MV or HV group; $^{\$}$:$p < 0.05$ as compared with CM or CH group, respectively.

Figure 9. Determinations of mercury contents in whole blood and brain tissues of offspring mice treated with mercurial compounds. Hg contents of various tissues (whole blood, cerebral cortex, cerebellar cortex and brainstem) of offspring mice exposed to distilled water or mercurial compounds (0.02 mg/kg/day MeHg or 0.5mg/kg/day $HgCl_2$) as described in Figure 1 were determined as described in Materials and Methods. All data are presented as mean ± S.E. (n = 12-15/group). $^{\#}$:$p < 0.05$ as compared with CV group; $^{*}p < 0.05$ as compared with MV or HV group; $^{\$}$:$p < 0.05$ as compared with CM or CH group, respectively.

Table 1.

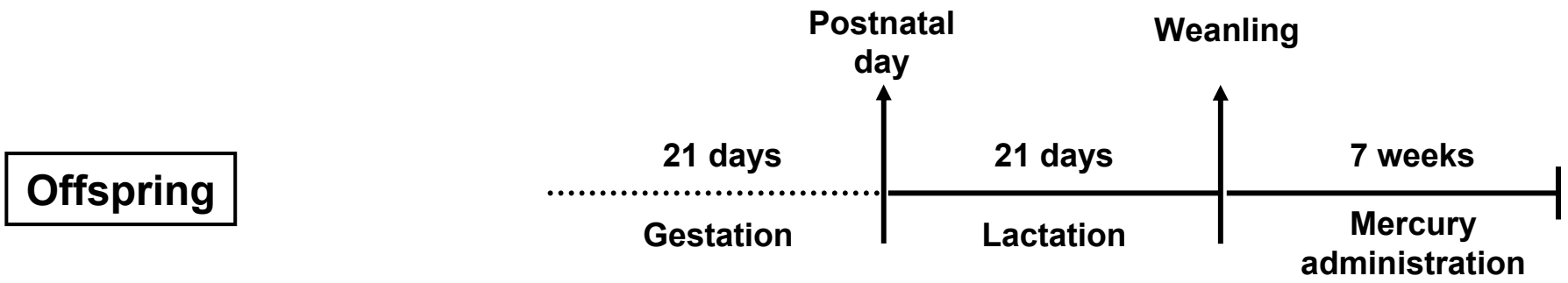
Hg contents of dam's blood and offspring (whole body at PND 1) mice after administration with low-dose of mercurial compounds.

	Dam's (whole blood)	Pups (whole body)
Control (Distilled water)	2.0 ± 0.32	1.8 ± 0.06
MeHg-0.02 mg/kg/day	142.9 ± 5.8*	47.6 ± 1.14*
$HgCl_2$-0.5 mg/kg/day	69.8 ± 8.8*	28.2 ± 0.95*

1. At postneonatal day (PND) 1, the offspring mice (pups) were randomly selected as representatives of their respective litters (four or five per litter).
2. Hg content was expressed as nanogram/gram of wet-weight and presented as mean ± S.E. *:$p<0.05$ as compared with control group by one-way ANOVA.

Figure(s)

Figure 1.



Groups

CV: Control-Vehicle	—	—	—
CM: Control-MeHg	—	—	+
CH: Control-$HgCl_2$	—	—	+
MV: MeHg-Vehicle	+	+	—
MM: MeHg-MeHg	+	+	+
HV: $HgCl_2$-Vehicle	+	+	—
HH: $HgCl_2$-$HgCl_2$	+	+	+

Figure. 2

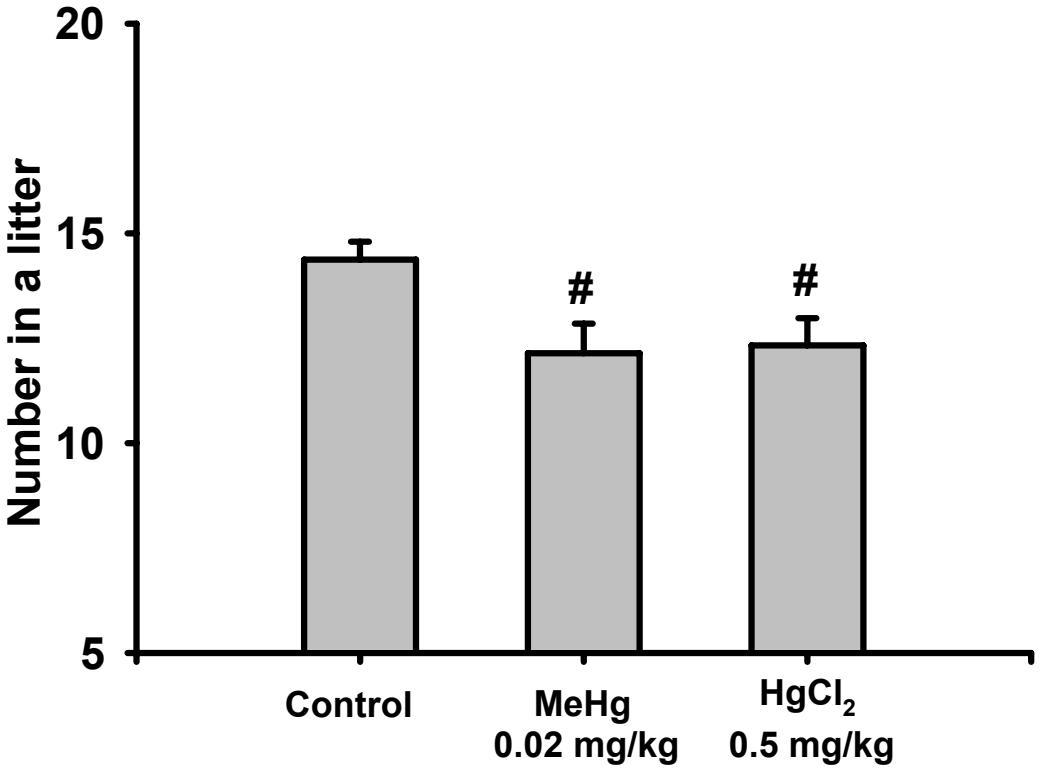

20
15
10
5
Number in a litter
#
#
Control
MeHg
0.02 mg/kg
HgCl2
0.5 mg/kg

Figure. 3

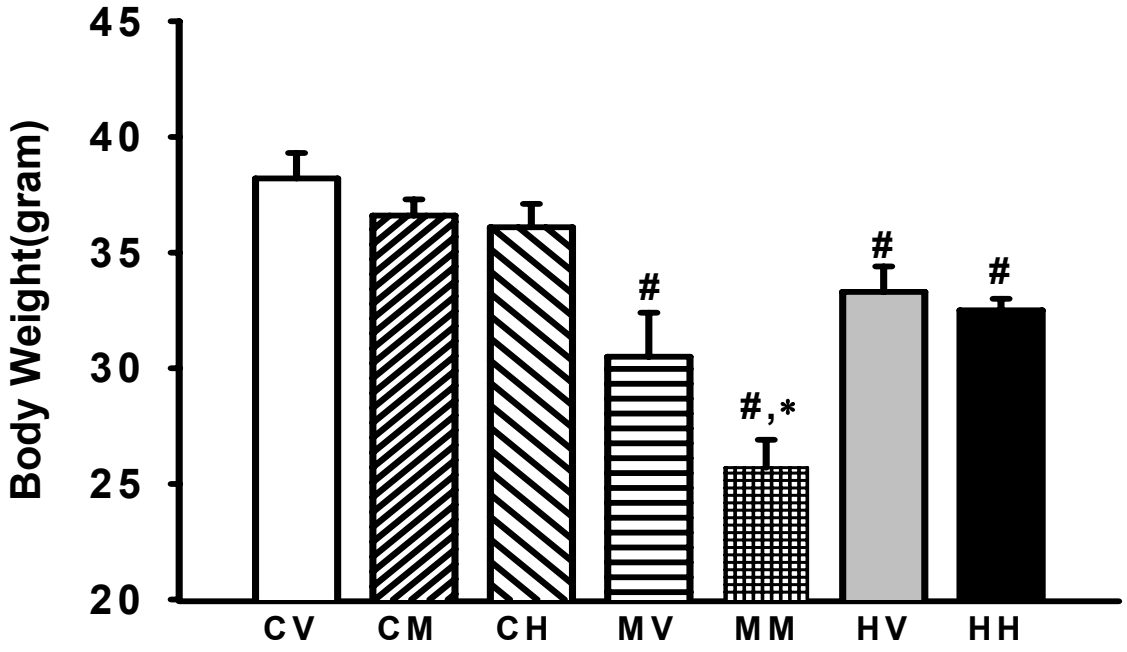


Figure. 4

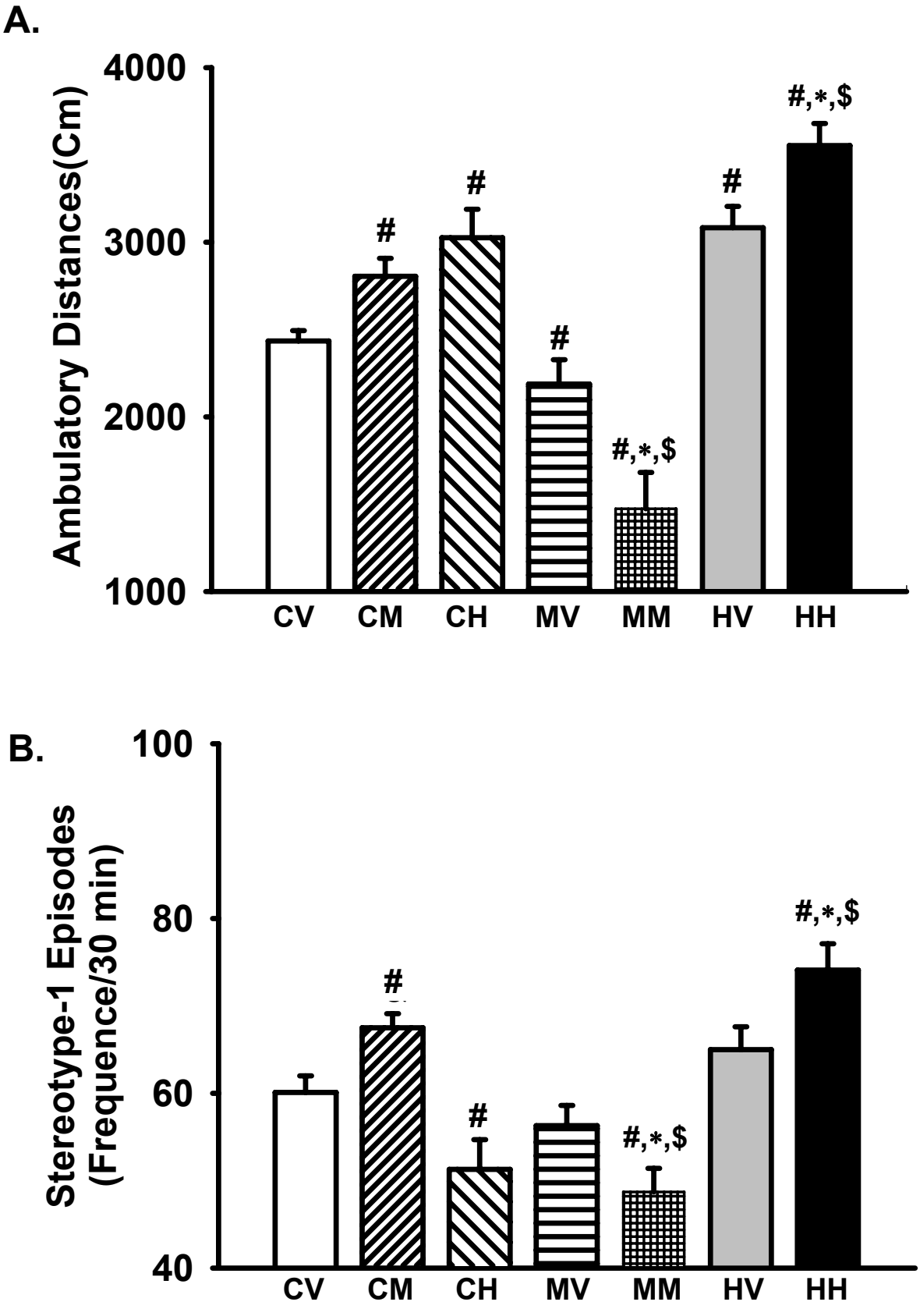

A.
Ambulatory Distances(Cm)
4000
3000
2000
1000
#
#
#
#
#,*,$
#,*,$
CV
CM
CH
MV
MM
HV
HH
B.
Stereotype-1 Episodes
(Frequence/30 min)
100
80
60
40
#
#
#,*,$
#,*,$
CV
CM
CH
MV
MM
HV
HH

Figure. 4

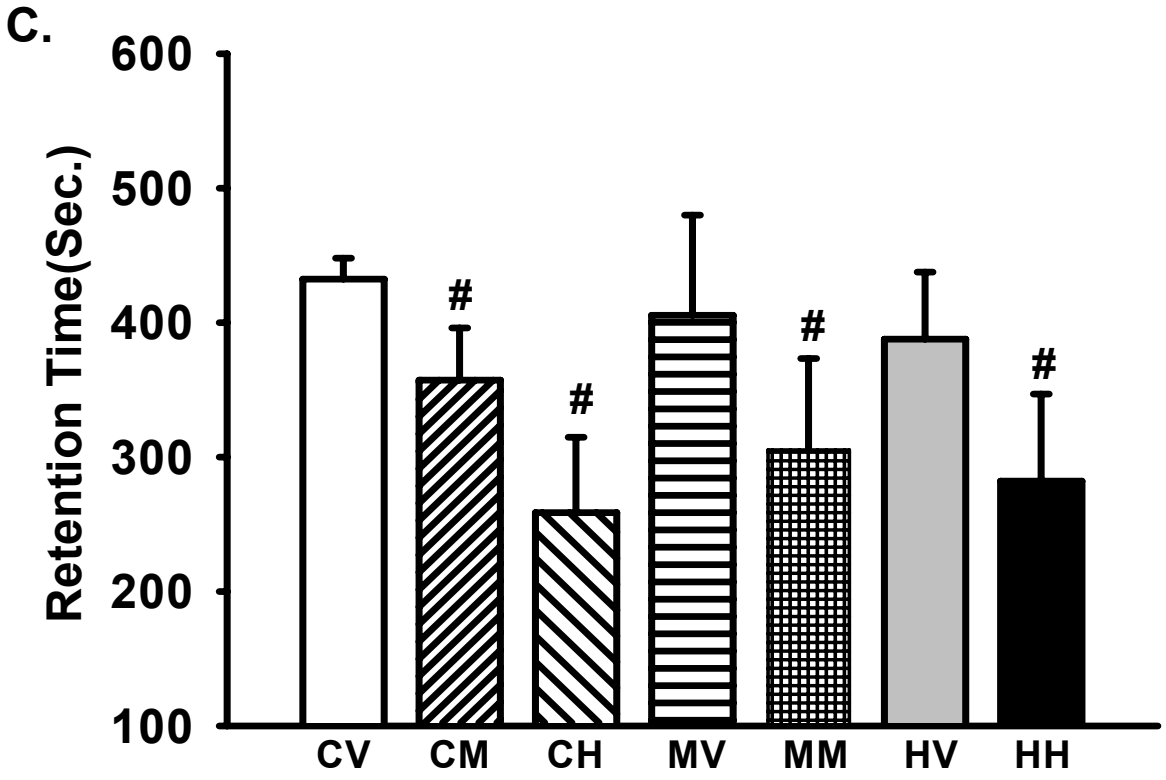


Figure. 5

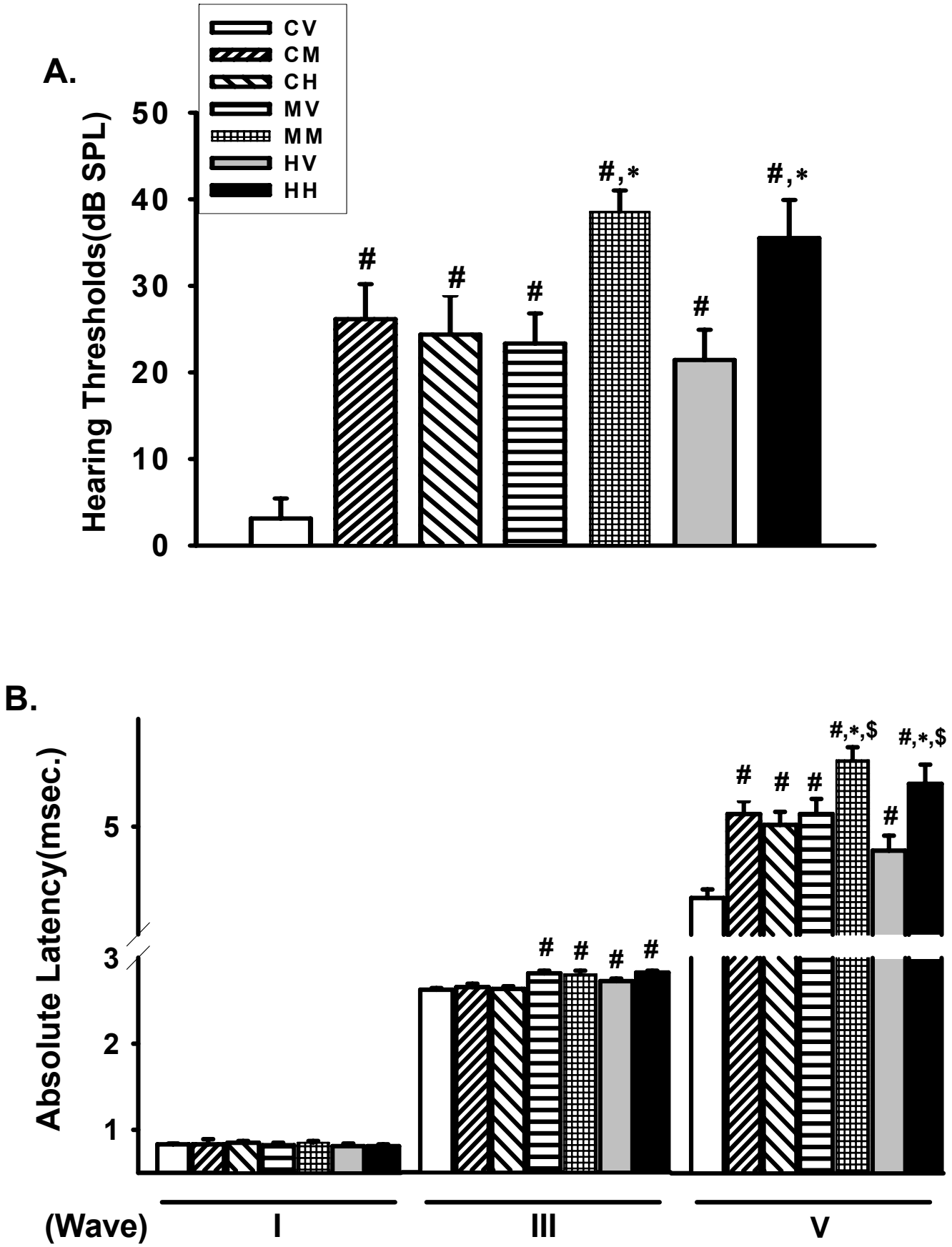

A.
CV
CM
CH
MV
MM
HV
HH
Hearing Thresholds(dB SPL)
0
10
20
30
40
50
#
#
#
#,*
#
#,*
B.
Absolute Latency(msec.)
1
2
3
5
#
#
#
#
#
#
#
#
#,*,$
#,*,$
(Wave)
I
III
V

Figure. 5

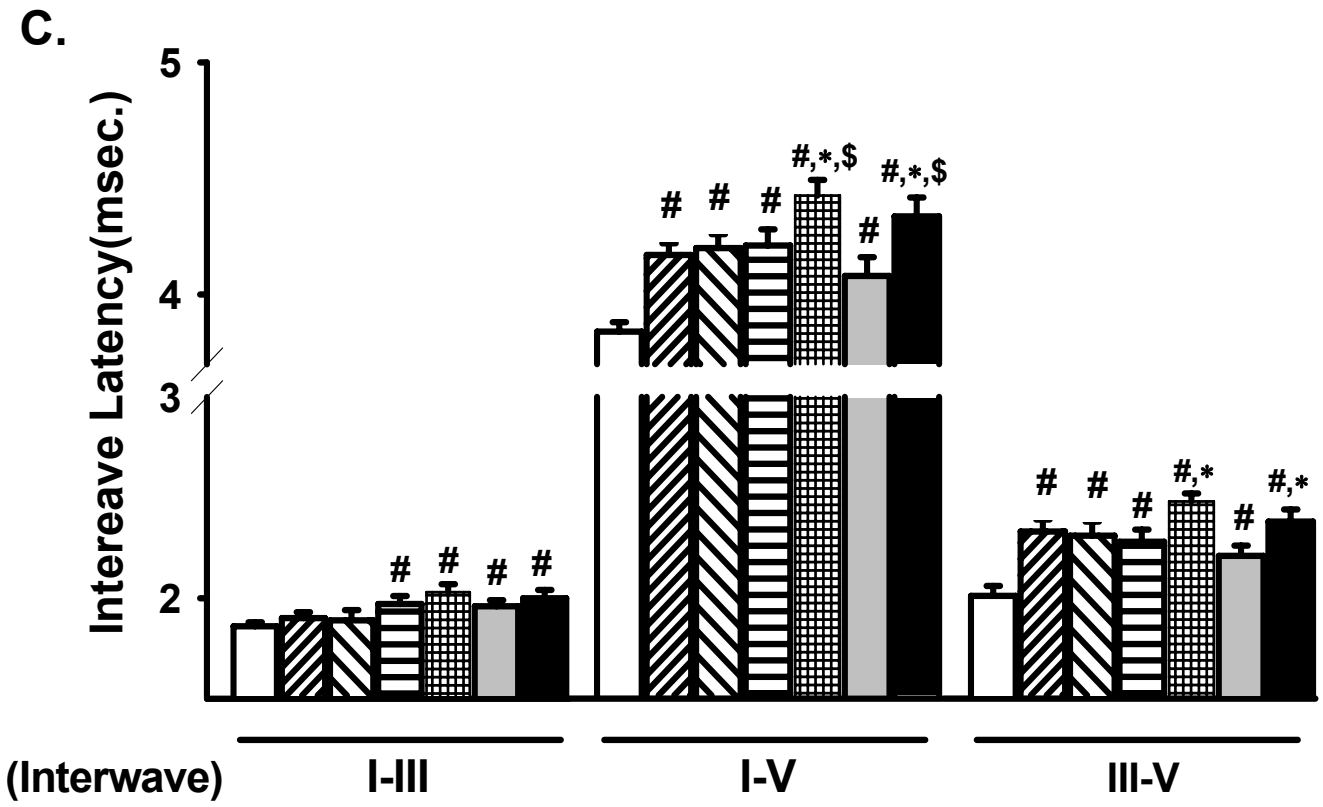


Figure. 6

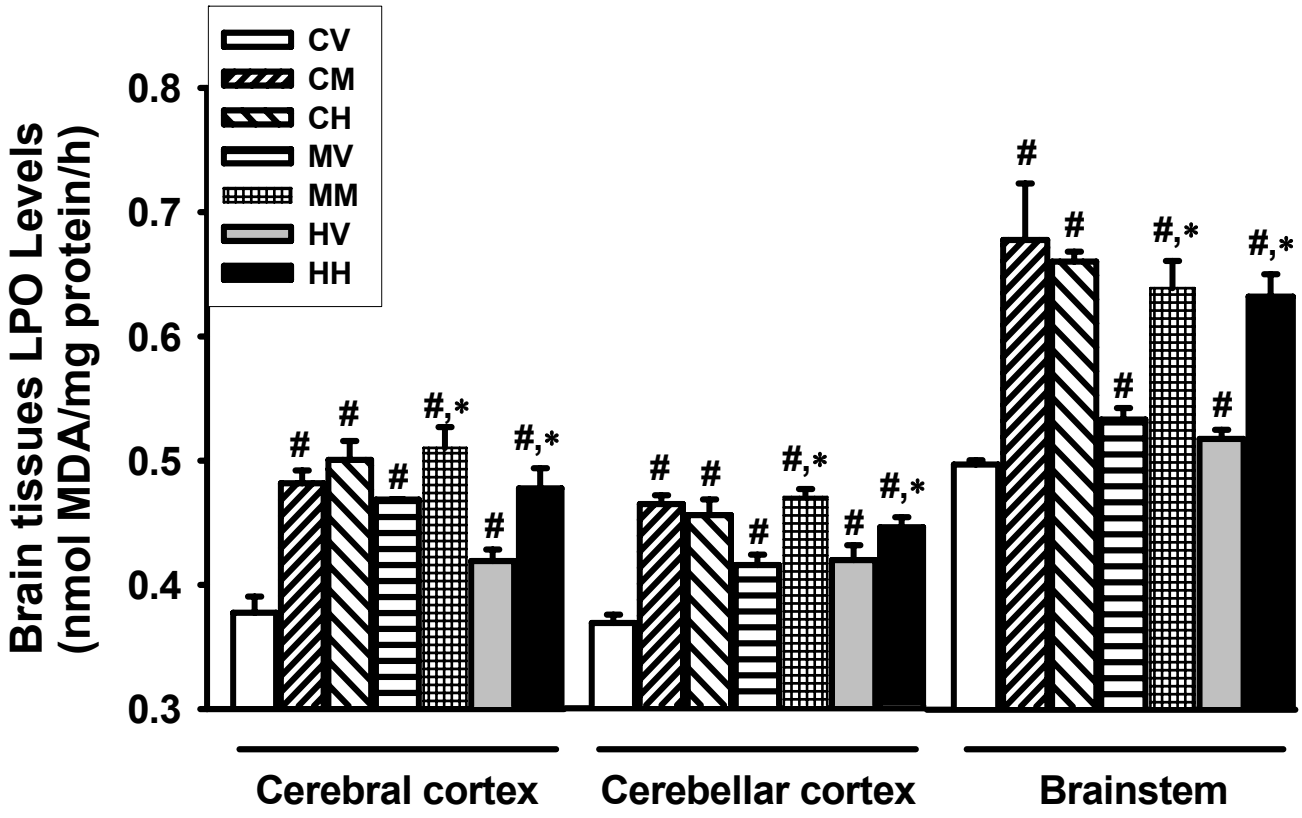

CV
CM
CH
MV
MM
HV
HH
Brain tissues LPO Levels
(nmol MDA/mg protein/h)
0.8
0.7
0.6
0.5
0.4
0.3
#
#,*
Cerebral cortex
Cerebellar cortex
Brainstem

Figure. 7

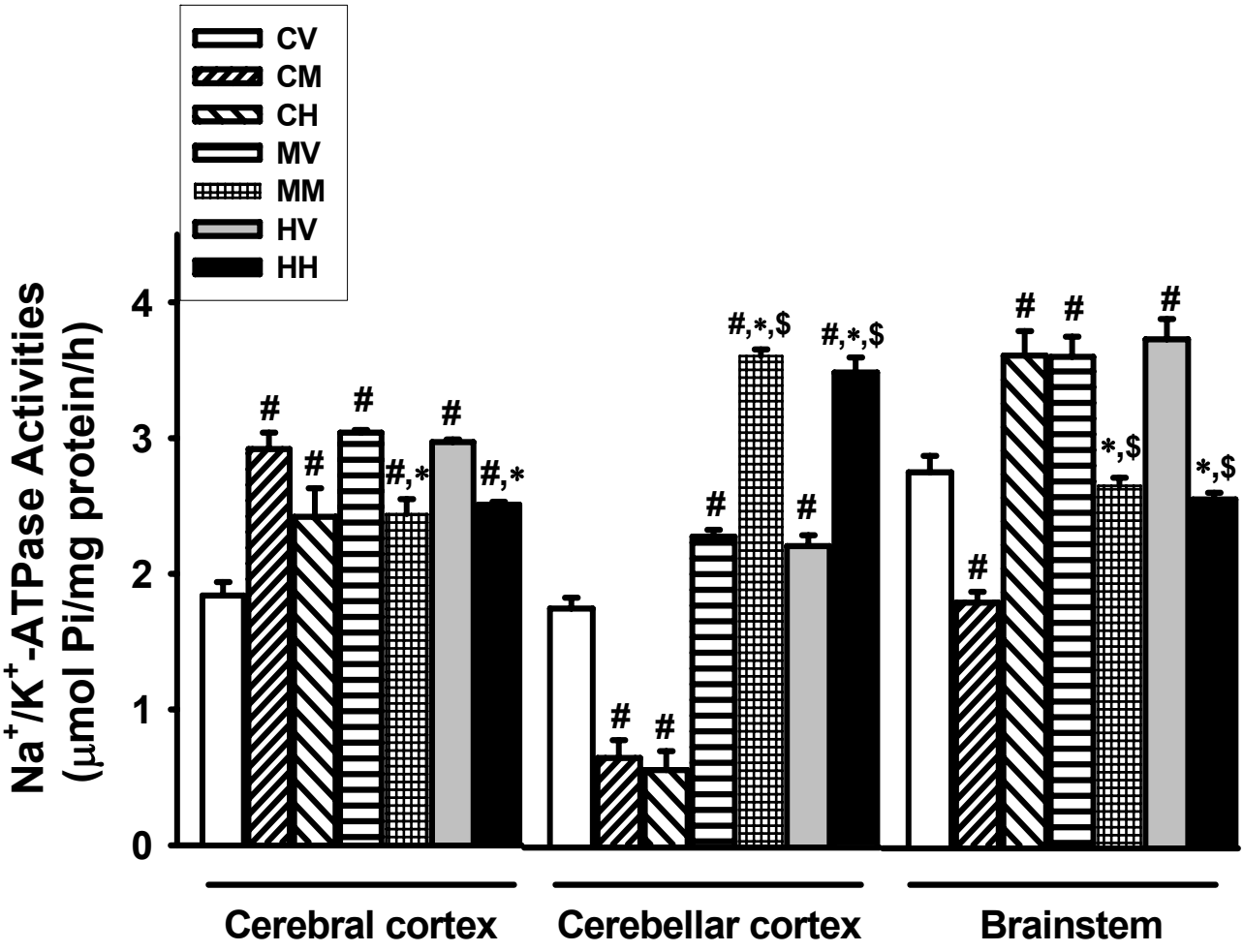

CV
CM
CH
MV
MM
HV
HH
Na+/K+-ATPase Activities
(μmol Pi/mg protein/h)
0
1
2
3
4
#
#,*
#,*,$
*,$
Cerebral cortex
Cerebellar cortex
Brainstem

Figure. 8

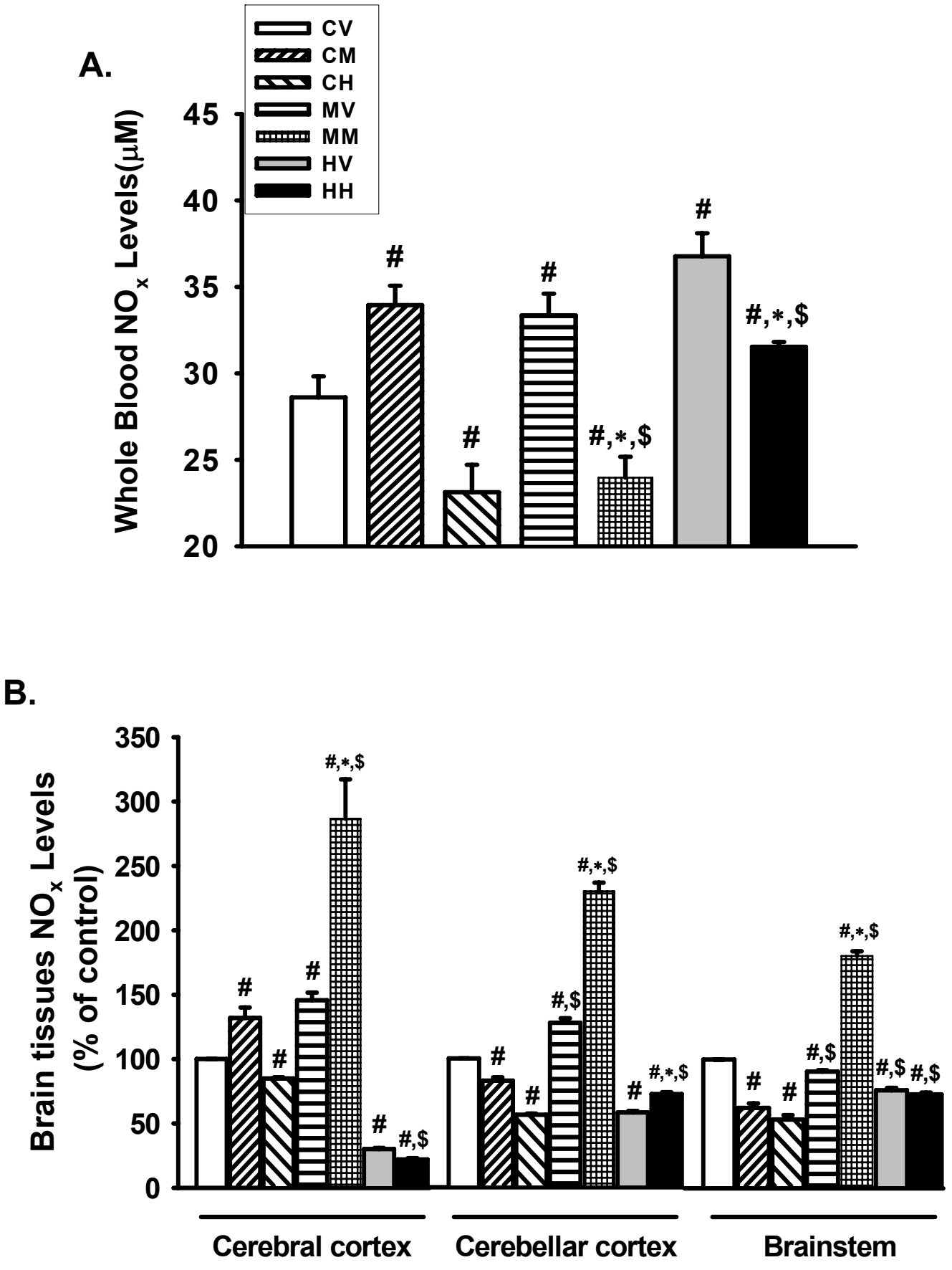
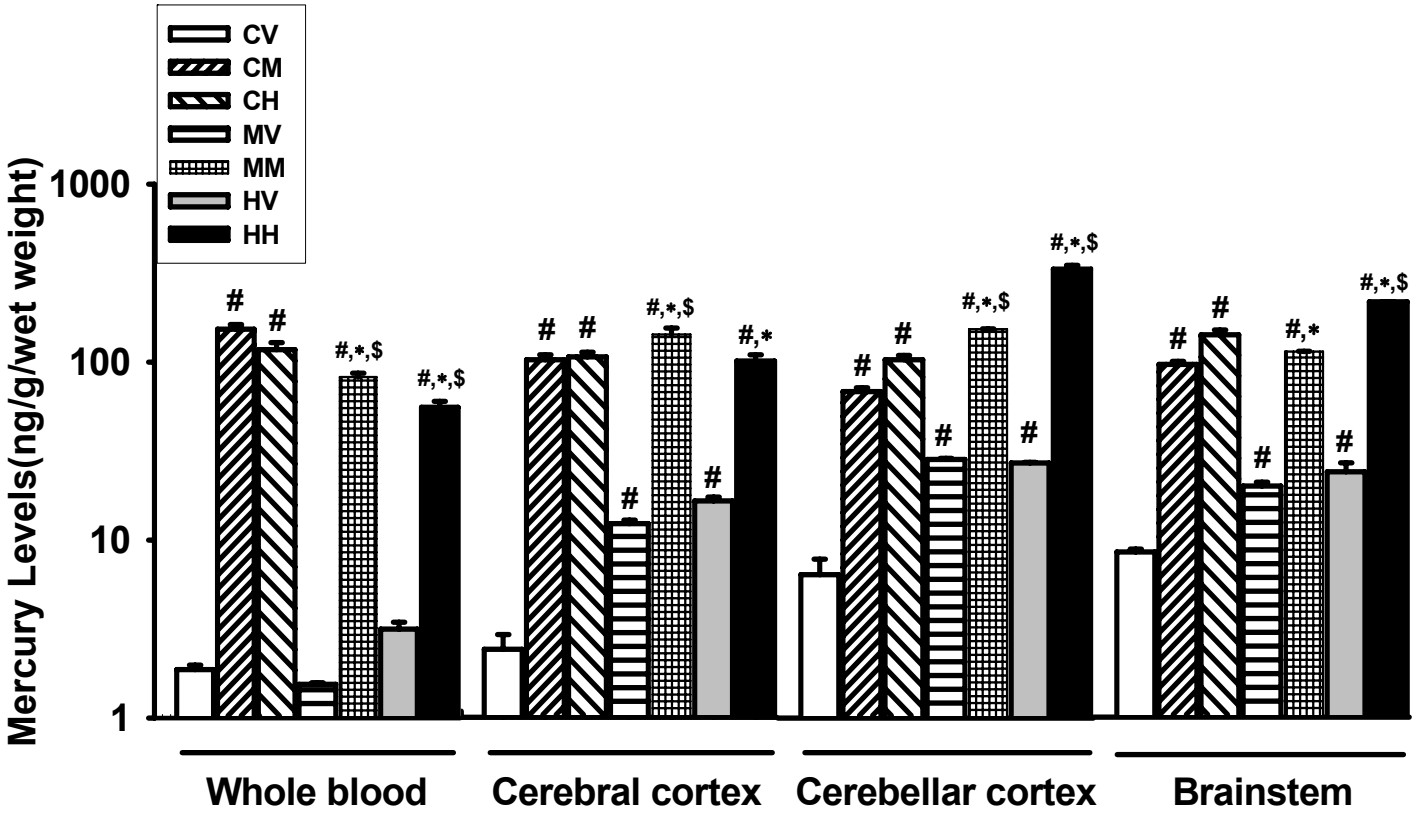

A.
CV
CM
CH
MV
MM
HV
HH
Whole Blood NOx Levels(μM)
45
40
35
30
25
20
#
#
#
#
#,*,$
#
#,*,$
B.
Brain tissues NOx Levels
(% of control)
350
300
250
200
150
100
50
0
#
#
#
#,*,$
#
#,$
#
#,*,$
#,$
#,*,$
#
#,*,$
#
#
#,$
#,*,$
#,$
#,$
Cerebral cortex
Cerebellar cortex
Brainstem

Figure. 9


CV
CM
CH
MV
MM
HV
HH
Mercury Levels(ng/g/wet weight)
1000
100
10
1
#
#,*,$
#,*
Whole blood
Cerebral cortex
Cerebellar cortex
Brainstem