Neurotoxic Mechanism of Cinnabar and Mercuric Sulfide on the Vestibulo-Ocular Reflex System of Guinea Pigs

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Cinnabar, a naturally occurring mercuric sulfide (HgS), has been combined with Chinese herbal medicine as a sedative for more than 2000 years. To date, its neurotoxic effect on the vestibulo-ocular reflex (VOR) system has not been reported. By means of a caloric test coupled with electronystagmographic recordings, the effect of commercial HgS and cinnabar on the VOR system of guinea pigs was studied. HgS or cinnabar was administered orally (1.0 g/kg) to Hartley-strain guinea pigs once daily for 7 consecutive days. A battery of electrophysiological, biochemical, and histopathological examinations were performed. The results showed that HgS induced a 60% caloric response abnormality (40% caloric hyperfunction and 20% hypofunction), whereas the abnormal responses appeared to be more severe (six out of six) in the cinnabar group. The Hg contents of whole blood and cerebellum were increased and correlated to their neurotoxic effects on the VOR system, indicating that both insoluble HgS and cinnabar could be absorbed from the gastrointestinal tract and distributed to the cerebellum. Although the vestibular labyrinth revealed no remarkable change under light microscopy, loss of Purkinje cells in the cerebellum was detected, and the enzymatic Na+/K⁺-ATPase activity of cerebellum (a higher inhibitory center of the VOR system) was significantly inhibited by HgS and cinnabar. Moreover, cerebellar nitric oxide (NO) production was increased significantly. Hence, we tentatively conclude that the increased Hg contents in the cerebellum following oral administration of HgS and cinnabar were responsible, at least in part, for the detrimental neurotoxic effect on the VOR system. Potentially, decreasing Na+/K⁺-ATPase activity and increasing NO production within the cerebellar regulatory center are postulated to mediate this VOR dysfunction caused by the mercuric compounds and cinnabar.

Key Words: caloric test; cerebellum; cinnabar; mercuric sulfide; Na+/K⁺-ATPase; vestibulo-ocular reflex.

Mercury is a toxic heavy metal, and its different chemical forms account for the various degrees of toxic effects (Horvat et al., 1999; Kato et al., 1981; Liu and Lin-Shiau, 1992; Martin-Gil et al., 1994; Schionning and Danscher, 1999). For example, methylmercury is more toxic and permeable to the blood–brain barrier than mercuric chloride (HgCl₂; Baldi, 1997), and the bioavailability of HgCl₂ (15–20%) is at least 30- to 60-fold more than the insoluble cinnabar, a naturally occurring mercuric sulfide (HgS; Schoof and Nielsen, 1997). Cinnabar has been used in combination with Chinese herbal medicine as a sedative for more than 2000 years (Yeoh et al., 1986). The estimated human therapeutic dose of cinnabar is approximately 30–80 mg/kg/day. An overdose of cinnabar in drugs such as Ba Paul San, which is administered as a sedative for infants and children, has led to occasional intoxication in the ethnic Chinese population (Tie et al., 1990). Therefore, the extent of absorption of HgS in the gastrointestinal tract, as well as the toxic effects and action mechanisms of HgS in animals and humans, must be ascertained. In this study, we assessed the neurotoxic effect of cinnabar and HgS in guinea pigs, at a dose about 10 times the human dose, on the vestibulo-ocular reflex (VOR) system. Moreover, we studied the extent of absorption as well as the tissue distribution of these mercuric compounds, and we correlated their neurotoxic effects on the VOR system.

The VOR system is an important contributor to stable vision during natural activity. It is a simple reflex arc, transmitting the velocity command from the vestibular nerve to the ocular motor neurons to ensure that eye velocity matches head velocity. Recent investigations of eye movements have revealed striking modifications to VOR gain (maximum slow-phase velocity/maximum head velocity) by vision. As a result, the eyes remain stationary with respect to the world, and images of the world are stable on the retina. The function of VOR system of the guinea pigs can be examined by the caloric test coupled with electronystagmographic (ENG) recordings (Young et al., 1991). Through the pattern of caloric nystagmus, lesions in the vestibular labyrinth, vestibular nuclei, and/or their connections to the cerebellum can be assessed (Vibert et al., 1993). For example, lesions of the brainstem typically decrease the gain of VOR or spare it. However, increased VOR gain indicates a sign of cerebellar disease that has been attributed to a loss of inhibitory effects of the archi-cerebellum on the vestibular nuclei (Balogh et al., 1979; Sharpe et al., 1981). Thus, an animal...
model has been established to test the function of the VOR system and its higher inhibitory center at the cerebellum. The histopathological study of the temporal bones and cerebellum was conducted and the colorimetric technique used to monitor the Na+/K+-ATPase activity of the cerebellum, following oral administration of mercuric compounds for 7 consecutive days. Additionally, nitric oxide analyzer (NOA) was employed to determine the nitric oxide content of the blood and cerebellum. Finally, mercury levels in whole blood and cerebellum were determined and correlated with their toxic effects on the VOR system. It is believed that this sensitive monitoring model system is useful not only to provide experimental information for the legislative regulation of these Chinese mineral drugs, but also for clinical assessment of the intoxication contents of cinnabar present in traditional Chinese medicines.

MATERIALS AND METHODS

**Animal preparations.** Randomly bred Hartley-strain guinea pigs weighing 200–220 g were housed at 23 ± 2°C and 55 ± 5% humidity and fed a solid diet and tap water ad libitum. The Animal Research Committee of National Taiwan University, College of Medicine, conducted the study in accordance with the guideline for the care and use of laboratory animals. Cinnabar employed herein was purchased from different traditional herb drugstores in Taipei City. HgS (indicating commercial HgS in the full text) was obtained from Ferak (Berlin, Germany). By means of inductively coupled plasma mass spectrometry (ICP-MS), Elan Model 250, Sciox, Thornhill, Ontario, Canada, Hg and Pb (lead) contents in cinnabar and HgS were determined. Animals were orally administered cinnabar or HgS or PbCl2 (lead chloride), which was suspended in saline (1.0 g/kg; nearly 12 times higher than the human therapeutic dose), or saline alone, once daily for 7 consecutive days. These animals were subjected to the caloric test on the 8th day and sacrificed 24 h after caloric testing, under deep anesthesia with pentobarbital (60 mg/kg). The whole blood, cerebellum, and temporal bones were removed. The cerebellum was sagittally cut in half for mercury analysis and enzymatic assay (Na+/K+-ATPase), or processed for histopathological study by staining with hematoxylin-eosin.

**Caloric test.** For the vestibular function test, the guinea pig was restrained in a special holder, with its four legs free. A pair of clip electrodes was attached to the vertex at the midline (Young et al., 1991). A pathological nystagmus was evaluated by the results. When ANOVA was significant, the significance between groups was assessed by means of ANOVA followed by Dunnett’s t-tests for most tests. Only discrete, relevant caloric response variables were analyzed by χ² tests, with three degrees of freedom for the trend in binomial proportions after appropriate multiplicative adjustments. It was considered to be significant when the p value was less than 0.05.

**Histopathological studies.** To correlate the caloric responses of the animals treated by various mercurial compounds, three animals from each group were processed for histopathological study. Through deep anesthesia with sodium pentobarbital, the thoracic cavity was opened and the guinea pig was perfused through the left ventricle with saline solution, which was followed by 10% neutral buffered formalin intravital fixation. After complete fixation, the animal was decapitated and the cerebellum as well as temporal bones were harvested and placed in formalin solution. Following decalcification by tri-chloroacetic acid, neutralized by Na2SO4, dehydrated by a series of alcohol solutions, finally embedded in celloidin, and serially sectioned at 20 μm. Every 200 μm, two adjacent sections were stained with hematoxylin and eosin (Young et al., 1992). Multiple full coronal sections of the cerebellum were embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin.

**Assay of Na+/K+-ATPase activity.** Membrane Na+/K+-ATPase activities were assayed as reported previously (Liu et al., 1997; Rohn et al., 1993). Briefly, the enzymatic activities were measured in triplicate in 96-covered-well microtiter plates at 37 ± 0.5°C on a shaker. Ninety μl of assay buffer (30% sucrose, pH 7.4) containing 2 μg of membrane protein was added to each well. The assay was started with the addition of 10 μl of ATP (final concentration 5 mM), producing a final reaction volume of 100 μl. The reaction was then terminated by the addition of 200 μl of malachite green plus ammonium molybdate (3:1). Pi (μM) released by substrate ATP hydrolysis was determined colorimetrically with a microplate ELISA reader (Dynatech MR 7000) at 630 nm. The specific ATPase activities were expressed as μM inorganic phosphate per milligram protein per 20 min. Values reported herein represent the mean and SE of at least three separate experiments.

**Nitric oxide (NO) determination.** The cerebella were weighed and homogenized in the homogenate buffer (0.32 M sucrose-histidine buffer, pH = 7.4) and then centrifuged at 4°C for 20 min at 10,000 × g. For total protein denatured, 70% ethanol was added to the sample (the homogenate 1:1) overnight, then all samples were centrifuged at 4°C for 20 min at 12,000 × g. The supernatants of cerebellar cortex were collected and assayed for NOx by the NO/ozone chemiluminescence (NO-Analyzer 280A Sievers; McKee et al., 1994). Briefly, oxidation products (NO2- and NO3-) of NO were measured using a reaction vessel that contained a reducing system (0.1 M vanadium chloride, Aldrich Co., U.S.). Detection of NO was then completed by its reaction with oxygen, which led to the emission of red light (NO + O3→NO2 + O2; NO2*→NO2 + hv). Standard curves were made prior to concentration (1, 5, 10, 15, and 20 μM NO), which were from freshly prepared solutions of NaNO2 (10 μl) in distilled water.

**Determination of mercury accumulation.** Various tissues (500 μl of blood, 300 mg of cerebellum) were placed in a wide-mouth polyethylene scintillation vial, and 3 ml of a 3:1 mixture of concentrated nitric acid:70% perchloric acids was added along with 50 mg of vanadium pentoxide. The vials were capped and allowed to stand overnight at room temperature. Following predigestion, the capped vials were heated for 4 h on a shaking water bath at 68 ± 0.5°C, then uncapped and heated continually for 3 h. After cooling, five drops of 30% hydrogen peroxide were added, and the vials were again capped and allowed to stand overnight to complete the digestion. Suitable dilutions were made from the digested material, and the total mercury content was determined with a solution of 2% SnCl2 in 0.05 M H2SO4. The reduction with SnCl2 and total tissue mercury content analysis by cold-vapor atomic absorption spectrophotometry were performed with automated equipment (Hitachi, Model Z-8200 and HFS-2 Hydride formation system). The reducing agents and apparatus were the same as reported (Iverson et al., 1974) except that 1.0 ml of diluted digest was placed in the modified Erlemeyer flask. Then 4.0 ml of the reduced mixture was added to the closed system through a flexible plastic tube sealed into the ball-joint cap and connected to a syringe type automatic pipette.

**Statistical analysis.** Analysis of variance (ANOVA and χ²) was used to evaluate the results. When ANOVA was significant, the significance between groups was assessed by means of ANOVA followed by Dunnett’s t-tests for most tests. Only discrete, relevant caloric response variables were analyzed by χ² tests, with three degrees of freedom for the trend in binomial proportions after appropriate multiplicative adjustments. It was considered to be significant when the p value was less than 0.05.
RESULTS

Effects of mercuric compounds on general health status of guinea pigs. Although 12 animals were treated with cinnabar from different herb drugstores, only six animals were treated with the batch of cinnabar that contained mercury (855.1 mg/g), and certain amounts of Pb (10.8 mg/g) were used for the VOR experiments. Twenty animals were treated with HgS, consisting of a mercury content of 862.1 mg/g, and the background level of Pb (0.09 mg/g). All guinea pigs revealed good-health status during the administration period, but those of either the HgS group or the cinnabar group appeared rather quiet and less active during caloric testing. The increases in mean body weights in each group after the 7-day administration were: 211 ± 8 g (mean ± SD) up to 237 ± 15 g for the control group, 208 ± 6 g to 240 ± 16 g for the HgS group, and 206 ± 5 g to 236 ± 10 g for the cinnabar group, respectively. The differences among them were statistically non-significant (p > 0.05, t-test).

Effects of mercuric compounds on caloric response. Throughout the experiment, neither spontaneous nystagmus nor positional nystagmus was noted in guinea pigs of each group. Following administration for 7 consecutive days, HgS exerted a moderate deleterious effect on the caloric test (60% abnormality, including 40% hyperfunction and 20% hypofunction; Figs. 1B and 1C). However, the neurotoxic effects of cinnabar in six animals definitely provoked a higher percentage of abnormal caloric responses, including hyperfunction in four (67%) and hypofunction in two (33%; Figs. 1D and 1E). Comparison of the abnormal caloric responses between the control group (0%) and the HgS group (60%) or cinnabar group (100%) revealed a significant difference (p < 0.05, χ² test; Table 1). Eight animals treated with PbCl₂ alone also disclosed 100% abnormality, including caloric hyperfunction in seven (88%) and caloric hypofunction in one (12%). Table 1 lists the corresponding duration and slow phase velocity of caloric nystagmus derived from guinea pigs in each group.

Histopathological studies. Morphological study of the temporal bones in guinea pigs treated with either HgS or cinnabar revealed no marked change in the contour of the semicircular canal by light microscopy. Intact hair cells of the vestibular labyrinth were also observed (Fig. 2). By contrast, the cerebellar lesion of guinea pigs was shown. In the control group, the Purkinje cells of the cerebellum were large flask-shaped cells with extensively branched dendrites extending into the outer molecular layer, and its numbers reached 64 ± 4 cells per folium field. A large nucleus with its nucleoli was evident in each cell body. The cells of the molecular layer were widely spaced; in contrast, the cells of the granular layer were closely packed (Fig. 3A). In guinea pigs treated with HgS (1.0 g/kg/day for 7 consecutive days), the number of Purkinje cells decreased significantly (45 ± 1 cells per folium field, p < 0.05, as compared to the control group; t-test), and the shape of Purkinje cells was also distorted (Fig. 3B). In guinea pigs treated with cinnabar (1.0 g/kg/day for 7 consecutive days), a diffuse loss of Purkinje cells was detected.
Assay of cerebellar Na⁺/K⁺-ATPase activities. The cerebellar Na⁺/K⁺-ATPase activity was considered crucial to regulate the normal caloric response of the VOR system of guinea pigs. Figure 4 demonstrates that the Na⁺/K⁺-ATPase activity of the cerebellar cortex of the guinea pigs treated with HgS (3.94 ± 0.47 Pi μmol/mg protein/20 min) or cinnabar (2.47 ± 0.26 Pi μmol/mg protein/20 min) for 7 consecutive days, decreased significantly as compared with control vehicle (6.91 ± 0.79 Pi μmol/mg protein/20 min; p < 0.05, t-test).

Effect of mercuric compounds on nitric oxide production of cerebellar cortex. Nitric oxide (NO) is oxidized easily to nitrite (NO₂⁻) and nitrate (NO₃⁻) by oxygen, and most measurement techniques of NO-release involve indirect chemical detection of oxidation products. NO levels were discovered as remarkable and produced a discernible increase in cerebellum following daily administration of HgS (172.9 ± 19.3 nmol/g) or cinnabar (243.0 ± 22.7 nmol/g) for 7 consecutive days, as compared with that of the control vehicle (88.4 ± 17.6 nmol/g; Fig. 5).

Determination of Hg contents in various tissues. Figure 6 reveals that mercury could largely accumulate in the blood and cerebellum of guinea pigs following daily oral administration of either HgS or cinnabar (1.0 g/kg) for 7 consecutive days. In fact, mercury content can attain 350 ± 28.9 ppb and 230 ± 27.8 ppb in blood and cerebellum, respectively, after a high dose of HgS. Compared to the control group (24 ± 2.8 ppb and 22 ± 2.9 ppb, respectively), administration with cinnabar (1.0 g/kg) significantly increased mercury contents in whole blood and cerebellum (840 ± 56.4 ppb and 400 ± 43.2 ppb, respectively), correlated to the greater loss of Purkinje cells in the histopathological study (Fig. 3C).

### DISCUSSION

Mercuric sulfide, or its counterpart cinnabar that occurs in nature, has long been associated with traditional Chinese medicine. Due to the reputed insolubility of this compound, it was assumed that it would not be absorbed significantly from the gastrointestinal tract following oral administration, and it is generally considered to be less toxic in vivo (Sin and Teh, 1992). However, several reports suggest that HgS can be absorbed and accumulated in the kidney and liver following oral administration of cinnabar to mice (Yeoh et al., 1986) or humans (Gabica et al., 1975). Whether these mercury compounds can deposit in brain tissue has not been elucidated. In this study, guinea pigs fed either HgS or cinnabar orally, (38 ± 5 cells per folium field, p < 0.05, as compared to either control or HgS groups; t-test; Fig. 3C).
confirmed that the mercurials could be absorbed and distributed to the blood and cerebellum, resulting in the increased or decreased reaction in the VOR system.

The VOR system consists of a direct excitatory projection from the lateral semicircular canal to the medial vestibular nucleus, then to the ipsilateral medial rectus subnucleus through the ascending tract of Deiters (McCrea et al., 1987). Another direct excitatory pathway projects into the contralateral abducens nucleus. The latter contains both motor neurons to the lateral rectus muscle and interneurons that go via the medial longitudinal fasciculus to the medial rectus motor neurons. If lesions occur on the vestibular labyrinth or brainstem, absent or decreased VOR gain (caloric hypofunction) will ensue, whereas increased VOR gain (caloric hyperfunction) attributes to central disinhibition due to lesions in the cerebellum. In this study, guinea pigs treated with either cinnabar or...
HgS in a similar dose (1.0 g/kg) resulted in caloric hyperfunction or hypofunction, and possible mechanisms of these results were explored.

First, vestibulotoxicity by mercurial compound was considered. It has been reported that intoxication of mercuric chloride in the inner ears of guinea pigs may damage labyrinthine blood vessels by causing swelling of the endothelial cells, mitochondrial disintegration, and sometimes protrusion of endothelial cell cytoplasm herniating into the blood vessel lumen. Chronic mercuric chloride intoxication resulted in distorted endothelial cells with an increase in cytoplasmic density (Anniko and Sarkady, 1977). Second, mercury deposits typically occurred in the cerebelli of methylmercuric chloride, mercury deposits in the cerebellar cortex were restricted to Purkinje cells and Golgi cells. Similarly, the number of Purkinje cells of the cinnabar group also had greater loss than those of the HgS group. The reason for a more severe cerebellar dysfunction caused by cinnabar is not known. We suspect that the existence of other elements such as lead in the cinnabar may enhance the solubility, absorption, and detrimental effect of cinnabar on the VOR system. Actually, PbCl₂ by itself is also toxic to the VOR system (Table 1).

Among various tissues, the cerebellar Purkinje cells were the most obvious staining targets for mercury accumulation (Warfvinge et al., 1992). In this study, it was also demonstrated that distortion or obvious loss in the Purkinje cell layer occurred following HgS or cinnabar treatment. Moller-Madsen (1991) reported that after a 20-day (20 mg/day) treatment of methylmercuric chloride, mercury deposits in the cerebellar cortex were restricted to Purkinje cells and Golgi cells. Similarly, in the spinal cord, the majority of mercury was located in the motor neurons, which inhibited VOR relay cells mediated through floccular Purkinje cells (Doya, 2000; Ito et al., 1970; Snyder, 1999). In addition, nodulo-uvular Purkinje cells inhibit vestibular nuclear cells that are projecting into the cerebellar cortex (Precht et al., 1976). Additionally, degeneration of Purkinje cells (Figs. 3B and 3C) may cause central disinhibition of the VOR system (Figs. 1B and 1D) or abolish VOR adaptation (Figs. 1C and 1E), resulting in abnormal caloric response (Robinson et al., 1976).

Membrane Na⁺/K⁺-ATPase activity is essential to maintaining the Na⁺- and K⁺-gradient across the neuronal membrane. Notably, the inhibition of this enzymatic activity can produce membrane depolarization, leading to the suppression of neuronal transmission (Albrecht and Hilgier, 1994; Anner et al., 1992; Balestrino et al., 1999). This study indicated that Na⁺/K⁺-ATPase activity of the cerebellum in the cinnabar group decreased more than that in the HgS group. In previous reports, the cerebellum was proved to be one of the most vulnerable regions, as it contains the highest deposition of mercury (Moller-Madsen, 1991; Warfvinge et al., 1992). Thus, it is proposed that HgS and cinnabar inhibit the cerebellar Na⁺/K⁺-ATPase, which may be responsible for inducing dysfunction of the VOR system of guinea pigs.
Nitric oxide (NO) was found to act as an essential factor in cerebellar functional modulation (Avrova et al., 1999). The regulation of Na\(^+\)/K\(^+\)-ATPase activity by NO and cyclic guanosine monophosphate (cGMP) in various tissues including brain has been proposed (Lin et al., 1999). However, its detailed role in the VOR system remains unknown. Figures 4 and 5 demonstrate that HgS and cinnabar reduced Na\(^+\)/K\(^+\)-ATPase activity and increased cerebellar NO production in guinea pigs. Although numerous action mechanisms have been suggested for methylmercury compounds including effects on neurotransmitter release, block of voltage-gated cationic channels, increase in intracellular calcium, and mitochondrial function (Atchison and Hare, 1994), these effects probably induced the cascade of neurotoxic effect of mercury. Hence, the decreased Na\(^+\)/K\(^+\)-ATPase activity and the increased nitric oxide level in the cerebellum are suspected to induce dysfunction of the VOR system in guinea pigs treated with HgS or cinnabar.

In conclusion, we have demonstrated for the first report that the insoluble form of HgS and cinnabar can be absorbed from the gastrointestinal tract and distributed into the blood and cerebellum. The loss of Purkinje cells as well as the mercureic contents in the cerebellum correlates with the severity of abnormal caloric responses. The decreased Na\(^+\)/K\(^+\)-ATPase activity and the increased NO production in cerebellar tissue are proposed, at least in part, to be responsible for the dysfunction of the VOR system caused by HgS and cinnabar. The implication of these findings is that cinnabar, a naturally occurring HgS, which is used as a sedative in Chinese medicines, should be recognized as a potentially harmful substance.

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