

Protective Effects of *Hippophae rhamnoides* L. Juice on Lead-Induced Neurotoxicity in Mice

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We examined the effect of *Hippophae rhamnoides* L. (HRL) juice on lead-induced memory impairment and neuronal damage in the brains of adult mice. Kunming mice were exposed to lead acetate 10 mg/kg body weight for 20 d. Twenty percent and 40% HRL prevented the lead-induced decrease in step-through latency. In the water maze test, the swimming time was lengthened in mice treated with lead acetate, but this time was decreased in mice that received 20% and 40% HRL. The malondialdehyde (MDA) levels were increased in lead-treated mice, which were reduced by 20% and 40% HRL in dose-dependent manner. The activities of acetylcholinesterase (AChE) and monoamine oxidase-A and -B were significantly increased in the lead-treated group, which were decreased by 40% HRL but not by 20% HRL. The levels of norepinephrine, serotonin, and 5-hydroxyindole acetic acid were decreased significantly in the lead-treated mice, and the decreases were antagonized by 40% HRL, except for than in dopamine, but 20% HRL had no effect on this change. These data suggest that the different doses of the HRL juice protect against the lead acetate-induced deficits in learning and memory and changes in neurobiochemical parameters.

Key words lead; *Hippophae rhamnoides* L.; memory deficit; malondialdehyde; acetylcholinesterase; monoamine oxidase; neurotransmitter

Lead (Pb) is a highly neurotoxic agent that particularly affects the developing central nervous system. Exposure to low levels of Pb has been associated with behavioral abnormalities, learning impairment, decreased hearing, and impaired cognitive functions in humans and in experimental animals.^{1–7)} Experimental evidence suggests that cellular damage mediated by free radicals can be involved in the pathology associated with Pb intoxication. In erythrocytes from workers exposed occupationally to Pb, the activities of the antioxidant enzymes superoxide dismutase and glutathione peroxidase were significantly higher than in nonexposed workers.⁸⁾ Blood levels of malondialdehyde (MDA), a product of lipid oxidation, were strongly correlated with blood Pb concentrations in exposed workers with Pb concentrations higher than 35 $\mu\text{g}/\text{dl}$.⁹⁾ Various studies have shown that lead exposure can cause changes in catecholaminergic functions.^{10–13)} CaNa₂ EDTA is a traditional lead expellent but it is toxic to the liver and kidneys. Therefore recently there have been many studies on the use of natural agents such as vitamins and herbal drugs to expel lead.¹⁴⁾

In recent years, the clinical importance of herbal drugs has received considerable attention. As many synthetic antioxidants have been shown to have one or the other side effects,^{15,16)} there has been an upsurge of interest in the therapeutic potential of medicinal plants as antioxidants in reducing free radical-induced tissue injury.^{17–19)} Numerous plant products have been shown to have antioxidant activity, and the antioxidant vitamins, flavonoids, and polyphenolic compounds of plant origin have been extensively investigated as scavengers of free radicals and inhibitors of lipid peroxidation.^{20–23)}

Seabuckthorn (*Hippophae rhamnoides* L.; HRL) is a Euro-Asian wild, newly cultivated, edible berry with exceptionally high contents of nutrients and phytochemicals such as lipids, water- and fat-soluble vitamins, and flavonoids.^{24–26)} The ripe fruit has been reported to be a rich source of vita-

mins A, C, E, and K, carotenoids, and organic acids.^{24,27–29)} The berries have a long history of application (more than 1000 years) in Tibetan and Mongolian medicine in the treatment of various diseases. A wide spectrum of positive physiologic effects of the berries and berry products have been suggested in animal experiments and clinical investigations.^{30,31)}

However, no study has reported the effects of HRL on lead-induced neurotoxicity. The deficits in learning and memory in Pb-exposed rodents are accompanied by damage to neurons and changes in some neurotransmitters, such as the cholinergic and catecholamine neurotransmitter system, are involved.^{10–13,32–34)} Because oxidative damage is also involved in Pb toxicity, we examined parameters of oxidative stress in the brain from mice chronically exposed to the metal. In this study, we used behavioral and neurochemical experiments to determine the protective effects of HRL against the neurotoxicity induced by lead.

MATERIALS AND METHODS

Plant Material HRL fruit were collected from the Hunchun area of Jilin province in the month of September were the plant grows wild under natural conditions. The fresh fruit were cleaned and pounded to pieces with a squeezer. The extract was filtered and the filtrate was stored at -22°C in a refrigerator. The crude extract was then diluted in sterile double-distilled water to make 20% and 40% HRL juice. The juice solutions were administered orally *via* a stomach tube to 3 groups of mice for 25 d at a dose of a dose of 1 ml/10 g body weight.

Reagent Lead acetate (PbAc) (Harbin Chemical Corporation, China) 10 mg/kg body weight was given by intraperitoneal injection (0.1 ml/10 g body weight). Norepinephrine (NE), dopamine (DA), serotonin (5-HT), and 5-hydroxyindole acetic acid (5-HIAA) were purchased from Sigma

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Chemical (St. Louis, MO, U.S.A.). The assay kits for acetylcholinesterase (AChE), monoamine oxidase (MAO), malondialdehyde (MDA), and protein quantitation were purchased from Nanjing Jiancheng Bioengineering Institute (China).

Animals The experiments were conducted on male/female Kunming mouse weighing 20–22 g housed at $25 \pm 2^\circ\text{C}$ with free access to food and water, with 10 mice per cage. Two different concentrations of HRL juice were given orally in the morning (0.1 ml/10 g body weight) and in the afternoon lead was injected at a dose of 10 mg/kg body weight i.p. for 20 d. Additional oral administration of 20% and 40% HRL juice occurred once every 2 d.

The mice were randomly divided into 5 groups (10 mice per group) and placed in separate cages during the study: group I, lead (10 mg/kg/d i.p.); group II, lead (10 mg/kg/d i.p.)+20% HRL i.g.; group III, lead (10 mg/kg/d i.p.)+40% HRL i.g.; group IV, 40% HRL i.g.; group V, control group (the same amounts of vehicle, 0.85% NaCl solution, i.p. and i.g.). The study was approved by the institute's Animal Ethical Committee and conforms to national guidelines on the care and use of laboratory animals, provided by the Yanbian Medical College Animal Center.

Passive Avoidance Test A step-through passive avoidance test apparatus (Model PACS-30, Columbus Instruments International) was used to this experiment. The shuttle box is divided into two chambers of equal size ($23.5 \times 15.5 \times 15.5$) separated by a guillotine door (6.5×4.5). The light chamber is equipped with an illuminator and mice can enter the dark chamber through the guillotine door. Mice were placed initially in the light chamber with the door open. They displayed explorative behavior and then entered the dark compartment. Upon entering the dark compartment, the door closed automatically. Training was repeated until the mice entered the dark compartment within 20 s (training trial).

The mice were given HRL juice through a stomach tube 30 min after administration of lead and after another 30 min the mice were placed in the illuminated chamber. When the mice entered the dark chamber, an electrical foot shock (1 mA) was delivered for 3 s through the grid floor and the door was closed automatically (acquisition trial). The mice were again placed in the dark chamber 24 h after the acquisition trial, and the latency time to enter the dark chamber was measured for 300 s (retention trial). If a mouse did not enter the dark chamber within the cut-off time (300 s), it was assigned a latency value of 300 s. The mice were trained for 14 d, and 24 h after the last training session the passive avoidance test was administered to determine the latency.

Water Maze Test An Lw-I water maze automatic control recorder (China Medical Academy of Science, Drug Research Institute) was used in this experiment.³⁵⁾ The water maze size is $130 \text{ cm} \times 85 \text{ cm} \times 13 \text{ cm}$, and the maze consists of a nontransparent plastic board, with a route similar to the Hebb–Williams maze, but more complicated. The swimming time of mice was recorded from starting point to terminal station. The mice were given HRL juice through a stomach tube and 30 min after the mice were trained from 2 d before the process maze task test four times per for a day, total of 8 training sessions, until they could swim to the terminal determination through the correct route. The total swimming time and the correct through rate were recorded on days 18, 21, 24 and 27.

Determination of Biochemical Parameters The mice were injected intraperitoneally with lead and administered HRL juice orally for 20 d, with additional administration of HRL juice every other day during that period. On day 31, the mice were killed by cervical dislocation. The cerebrum of each mouse was removed, weighed, and homogenized and the AChE activity, MDA level, and MAO-A and -B levels were determined in the homogenates following the manufacturer's instructions provided with the assay kits.

Catecholamine Neurotransmitter Measurements Amines were estimated using the fluorimetric method.^{36–40)} The method of lead intoxication was the same as described above. On day 31, the mice were killed by cervical dislocation. The brain tissue was weighed, homogenized with cold η -butyl alcohol at a 1:10 volume, η -butyl alcohol up to 4 ml was added, shaken well for 5 min, and centrifuged at $3000 \times g$ for 5 min. To the supernatant was added η -heptane 5.0 ml and 0.1 mol/l HCl 5 ml, the mixture was vortexed for 5 min and then recentrifuged at $3000 \times g$ for 5 min. The water phase contained 5-HT, NE, and DA, and the organic phase contained 5-HIAA, a 5-HT metabolite.

NE and DA Assays: To 1 ml of the water phase was added 1/15 mol/l of phosphate saline buffer 1.7 ml (pH 7.2), and then iodine reagent 0.1 ml was added and allowed to stand for exactly 2 min, after which 0.5 ml of alkaline sodium sulfite solution was added. After 2 min, 0.6 ml of 6 mol/l glacial acetic acid was added. The mixture was boiled for 20 min, then cooling, and the NE fluorescence was read at 385/475 nm, the DA fluorescence at 322/370 nm. The accuracy and precision of the assay were checked by running, in parallel, different concentrations of external and internal standards and blanks with tissue samples. The standard curves were linear over the range of concentrations obtained from the tissue samples.

5-HT Assay: To 0.5 ml of the water phase was added 0.5% cysteine 0.1 ml and 0.004% *o*-benzene dicarboxylic acid 3 ml. The mixture was boiled for 10 min, cooled, and the absorbance was measured at 475/350 nm to detect 5-HT fluorescence.

5-HIAA Assay: To 0.5 ml of the organic phase was added 0.5 mol/l phosphate saline buffer 1.5 ml (pH 7.0), and then the mixture was shaken well for 10 min and centrifuged at $3000 \times g$ for 5 min. A 1 ml aliquot of the water phase containing 5-HIAA was removed and 1% cysteine 0.1 ml, 0.1% *o*-benzene dicarboxylic acid 0.1 ml, and 0.02% NaIO_4 0.1 ml were added. The mixture was boiled for 5 min, allowed to cool, and then the absorbance was read at 475/350 nm to detect 5-HIAA fluorescence.

Statistical Analysis Statistical significance was determined using Student's *t*-test, the one-way ANOVA test, and nonparametric test, and $p < 0.05$ was considered to indicate statistical significance. SPSS for Windows (version 9.0.0) was used for statistical analysis.

RESULTS

Effects of HRL Juice on Lead-Induced Learning and Memory Impairment In the group of mice that received injections of lead 10 mg/kg body weight for 15 d, the latency period in the step-through test was markedly shorter compared with the other groups. These effects were attenuated in

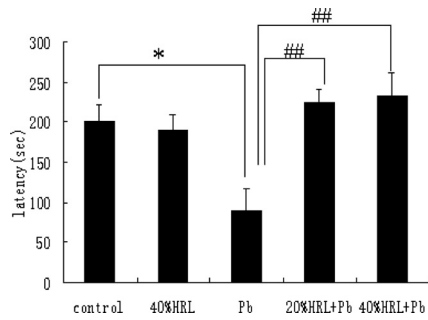


Fig. 1. Effects of HRL on Lead-Induced Memory Disturbance of Mice in the Passive Avoidance Test

Data represent mean±S.E. from 10 mice. **p*<0.05, significantly different from the control group, ##*p*<0.01 significantly different from the Pb only-treated group in the nonparametric test.

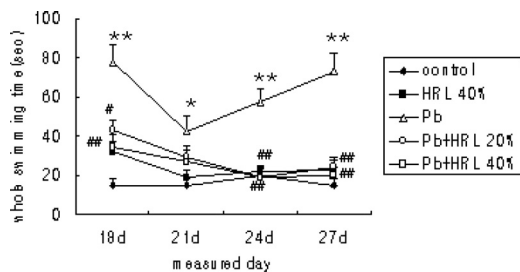


Fig. 2. Effects of Repeated Administrations of HRL on Spatial Memory Deficits Induced by Lead in the Water Maze Test

Data represent mean±S.E. from 10 mice. **p*<0.05, ***p*<0.01 significantly different from the control group; #*p*<0.05, ##*p*<0.01 significantly different from the Pb only-treated group in one-way ANOVA.

the groups administered 20% and 40% HRL juice, although HRL alone did not significantly affect the learning and memory function (Fig. 1). In the water-maze test conducted on days 18, 21, 24, and 27, the swimming time was markedly longer and the correct through rate (data not shown) was significantly decreased in the lead-treated group. The effects of lead were attenuated in the groups that received 20% and 40% HRL juice (Fig. 2).

MDA Levels The MDA level was significantly increased in the lead-treated group compared with the control group, and the increase was prevented by 20% and 40% HRL juice in a dose-dependent manner (Fig. 3). The 40% HRL juice alone decreased the MDA value significantly (Fig. 3), in agreement with several previous reports.^{31,41–43} This result indicates that 20% and 40% HRL juice can prevent the oxidative injury induced by lead.

AchE and MAO-A and -B Activity First, we examined the activity of AchE in homogenized cerebrum extracts. The activity of AchE was significantly increased after injection of lead acetate, which was prevented by 40% HRL, and 40% HRL juice alone decreased AchE activity (Fig. 4). Second, the activity of MAO-A and -B was determined in another set of brain tissue extracts. Both the activities of MAO-A and -B were increased in lead-exposed mice, and 40% HRL juice prevented this change by lead acetate, although the 20% HRL juice did not. The 40% HRL juice alone significantly decreased the activity of both MAO-A and -B (Fig. 5).

Catecholamine Neurotransmitter Levels The levels of NE, 5-HT, and 5-HIAA from whole brain homogenates were significantly decreased in the lead acetate-treated group, and

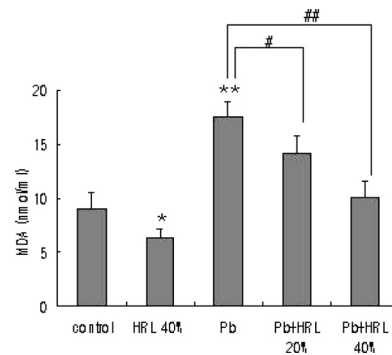


Fig. 3. Effects of HRL and/or Pb on Brain MDA Levels in Mice

Data represent mean±S.E. (*n*=5). **p*<0.05, ***p*<0.01 significantly different from the control group; #*p*<0.05, ##*p*<0.01 significantly different from the Pb only-treated group in one-way ANOVA.

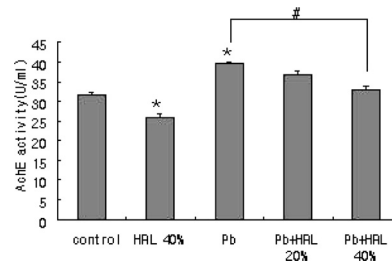


Fig. 4. AchE Activity in Lead- and HRL-Treated Mice Brains

Each value represents mean±S.E. (*n*=5). At least two experiments were carried out in triplicate. **p*<0.05 significantly different from the vehicle control group; #*p*<0.05 significantly different from the Pb only-treated group in one-way ANOVA.

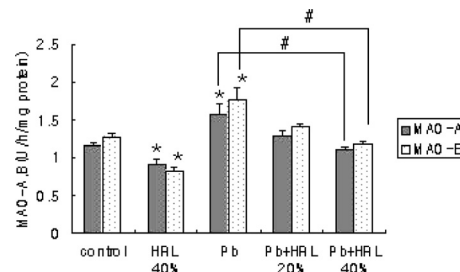


Fig. 5. MAO-A, B Activity in Lead- and/or HRL-Treated Mice Cerebral Tissue

Each value represents mean±S.E. (*n*=5). At least two experiments were carried out in triplicate. **p*<0.05 significantly different from the vehicle control group; #*p*<0.05 significantly different from the Pb only-treated group in one-way ANOVA.

only the level of DA showed no statistical difference between the control and lead acetate groups. The 40% HRL juice can antagonized the changes in 5-HT and 5-HIAA levels induced by lead, but not those in NE or DA. The 20% HRL did not prevent the change in any bioamine level in the lead and HRL coadministration group. The 40% HRL juice alone only significantly increased the level of 5-HT, and the value of other monoamines also increased but did not reach statistical significance (Fig. 6).

DISCUSSION

To our knowledge, prevention of lead-induced neurotoxic injury by HRL juice is reported here for the first time. In the present work, we investigated the effects of HRL on lead-induced learning and memory impairment and oxidative stress

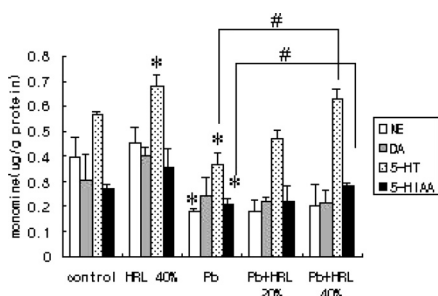


Fig. 6. Effects of HRL and/or Pb on Catecholamine Neurotransmitters in Cerebral Tissue in Mice

Each value represents mean \pm S.E. ($n=5$). At least two experiments were carried out in triplicate. * $p<0.05$ significantly different from the vehicle control group; # $p<0.05$ significantly different from the Pb only-treated group in one-way ANOVA.

and changes in some biochemical parameters.

Exposure to low levels of inorganic lead during early development has been implicated in lasting behavioral abnormalities and cognitive deficits in children and experimental animals.⁴⁴⁻⁴⁶ Error frequencies and total time in the water maze were significantly increased in the groups exposed to lead.⁴⁷ We performed two behavioral tests, the step-through test and water-maze test, in which the learning and memory deficits induced by lead were prevented by different doses of HRL.

To elucidate the cognition-enhancing and neuroprotective mechanisms of HRL, we investigated the effects of HRL on oxidative stress and the changes in cholinergic and monoamine neurotransmitters induced by lead. Free radicals play an important role in lead neurotoxicity, since lead exerts its neurotoxicity through oxidative stress.⁴⁸ Pb²⁺-induced disruption of the prooxidant/antioxidant balance in the brain could induce injury *via* oxidative damage to critical biomolecules.^{4,8,9,49-51} Pb²⁺ increases the rate of lipid oxidation in brain membranes.⁵² Increased oxidative stress reflected as a higher MDA level in lead-treated mice was prevented by HRL.

The antioxidant activity of HRL juice has been shown in *in vitro*, cell culture, and animal studies. Different fractions of HRL fruits inhibit 2,2-azobis (2,4-dimethylvaleronitrile) and ascorbate-iron-induced lipid peroxidation *in vitro*.³⁵ HRL juice, as well as vitamin E, decreases the MDA content in hyperlipidemic rabbit serum-cultured smooth muscle cells.⁴¹ Nicotine-induced increases in the erythrocyte MDA level were prevented by HRL extract.⁴³

The levels of Ach, a key neurotransmitter for cognitive function, were quantified after all behavioral tests were completed. The deficits in performance on tests of spatial learning and short-term memory in Pb-exposed rodents are consistent with dysfunction of the hippocampus and its cholinergic innervation.³²⁻³⁴ The involvement of the cholinergic system has been implicated by the observations that early lead exposure results in a significant reduction in high-affinity choline uptake in mouse forebrain synaptosomes⁵³ and a depressed acetylcholine turnover in the hippocampus of suckling rats,¹⁰ although the underlying mechanism(s) of these effects has not been resolved. We found that the activity of AchE was increased by lead, and Ach neurotransmission was decreased, which were attenuated by 40% HRL. This result is consistent with results from the behavior tests.

We also found that 40% HRL juice inhibited AchE activity, and it is thought that Ach neurotransmission in the synaptic cleft might be increased. HRL juice can maintain acetylcholine levels in the brain and improve cognitive ability.

Various studies have shown that lead exposure can cause changes in catecholaminergic functions.¹⁰⁻¹³

Chronic exposure to low amounts of inorganic lead results in alterations in the function of biogenic amine neuronal systems. After exposure to lead at 43.1 mg/dl for 90 d, no changes were observed in the concentration of NE, DA, DA metabolites in any brain region. No changes in 5-HT content and turnover were observed in any brain region, but 5-HIAA levels were decreased in six of the nine brain regions examined.⁵⁴

Another report compared brain regions in middle-aged Fisher 344 rats with those of young adult controls. In the prefrontal cortex and septum, 3,4-dihydroxyphenylglycol (MHPG) and the MHPG/NE ratio were decreased. In the septum, the DA and dihydroxyphenylacetic acid (DOPAC) levels were increased, but not the DOPAC/DA ratio. The hippocampus and thalamus showed an increase in 5-HIAA, demonstrating that selected neurotransmitter systems in the brain are altered at an early stage of senescence.⁵⁵ The present study found that levels of NE, 5-HT and 5-HIAA were decreased significantly in the lead-treated group, and this change was antagonized by 40% HRL; only the DA value showed no significant change in Pb-treated animals. The activity of MAO-A and -B, monoamine neurotransmitter digestion enzymes, is increased by lead. The 5-HIAA/5-HT ratio was also increased, which was attenuated by 40% HRL.

Lead-induced oxidative stress, decreased Ach level, and inactivation of catecholaminergic neurotransmitters can cause to impairment of learning and memory. Lead intoxication can cause deficiency of vitamin C *in vivo*, and lead can accelerate the oxidation of vitamin C, causing it to lose its bioactivity.⁵⁶ HRL juice contains high concentrations of vitamin C,³⁰ and the value of vitamin C is found in most vegetables.⁵⁷ HRL juice contains vitamin C 948.80 mg/100 g HRL.⁵⁸ Vitamin C and EDTA have similar chelating activity,⁵⁶ and decrease absorption of Pb and Pb accumulated in tissue. Goyer and Cherian, in an animal experiment, demonstrated that vitamin C has better Pb repellent activity compared with EDTA.⁵⁶ HRL juice contains vitamins B₁, B₂, and B₆,⁵⁷ and vitamin B₁ can accelerate lead excretion.⁵⁹ This indicates that it is possible to decrease the absorption and accumulation of lead *in vivo*.

Free radicals play an important role in lead neurotoxicity, which is exerted through oxidative stress.⁴⁸ Flash HRL fruit contains SOD 2746 U/g, double the amount in ginseng.⁶⁰ HRL juice contains the most vitamin E of all vegetables and fruit, and thus the antioxidative activity of HRL is very potent. It seems that alterations of antioxidant enzyme activities in response to lead-induced lipid peroxidation and also antioxidant supplements such as vitamin E and HRL are complicated.

Nutritive conditions also affect the sensitivity to lead. HRL juice contains many nutrients that can improve immune function and increase resistance to lead, such as vitamins C and E, carotenes (α , β , δ), riboflavin, folic acid, sugar, glycerides of palmitic, stearic, and oleic acids, polyphenols, and some essential amino acids.^{30,61} These rich nutrient through sev-

eral protective mechanisms prevent the neurotoxicity induced by lead.

In the future, we need to determine the concentration of lead in blood and urine, and the lead levels in organs like the brain, kidney, liver *etc.* could provide further evidence that herbal drugs such as HRL could be used as lead repellents.

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