

Letter to the Editor



Protective Effect of Mulberry Extract against Pb-induced Learning and Memory Deficits in Mice*

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Lead (Pb) is ubiquitous in the environment, and low-level Pb exposure can cause neurotoxicity and irreversible damage to children's cognition, learning and memory ability. Nutritional intervention is an effective method to prevent Pb poisoning. Mulberry is rich in anthocyanins, possessing protective effects for nerves. This study investigated the neuroprotective effects of mulberry extract (ME) against Pb-induced learning and memory deficits in mice. The results showed that the learning and memory abilities of mice, assessed using the Morris test, improved significantly after treatment with ME at a dose of 100 mg/kg body weight. The level of Pb in the brains of mice in the three ME intervention groups decreased significantly, while NO production and anti-oxidant enzymes were significantly restored. It is suggested that ME inhibits Pb-induced neurotoxicity by reversing Pb-induced alterations in the aspect of neurotoxic effects and improving learning and memory.

Lead (Pb), as the most abundant heavy metal in the earth's crust, represents a highly neurotoxic agent that affects the developing central nervous system. Exposure to low levels of Pb has been associated with behavioral abnormalities, learning impairment, decreased hearing, neuromuscular weakness, and impaired cognitive functions in humans and experimental animals^[1]. Nutritional intervention is an effective method to prevent Pb poisoning. A recent study has shown that puerarin^[2], curcuminoids, smilax glabra extracts^[3] possess potent protective effects against Pb-induced nerve damage. Anthocyanins represent a class of important antioxidants because they are common in human foods. Recently, many studies on the function and *in vitro* antioxidant activity of anthocyanins have been performed^[4]. Mulberries, a

kind of fruit, have been used as a traditional Chinese medicine for dizziness and blurred vision. Our previous studies^[5] have shown that mulberries are rich in anthocyanins. In addition, after *in vitro* gastro-intestinal digestion, the digest of mulberries possesses a high antioxidant capacity. Thus, the mulberry fruit may protect against brain damage and memory impairment in vascular dementia^[6].

The use of nutritional intervention for the prevention of Pb poisoning has drawn wide attention from many fields of study. However, the protective effects of mulberry extract (ME) against Pb-induced injuries in mice have not yet been reported. Therefore, the aim of the present study was to examine the potential of ME to protect against heavy metal-induced neurotoxicity and determine the feasibility of nutritional intervention from natural food resources for the prevention of lead poisoning.

The mulberry anthocyanins were extracted by aqueous two-phase extraction and the extracted anthocyanins were identified by HPLC-ESI-MS/MS analysis.

The mice were randomly divided into eight groups (ten mice per group), which were exposed to Pb by applying 500 µg/L Pb acetate for 3 w. Mice were gavaged with ME solution individually or in combination with DMSA.

The brain, liver, and kidneys were removed, rinsed with cold saline water and weighed and were used along with the blood samples for the biochemical variable and elemental analysis. The biochemical parameters and enzymes were determined by commercial kits, and analyzed by UV (TU-1800, Beijing). The metal concentrations in blood and tissue samples were measured using Vista-MPX Simultaneous ICP-OES (Varian, Inc. USA).

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Data were expressed as mean±SD values. *P* values less than 0.05 was considered statistically significant.

The effects of Pb exposure and ME treatment on spatial learning and memory in the Morris water maze were compared. Figure 1 shows the effects of ME and DMSA used individually or in combination and Zn intervention in Pb-induced mice, including (a) the escape latency in the place navigation test, (b) the escape latency without the platform in the target quadrant.

The effects of Pb exposure and the use of ME and DMSA individually or in combination on spatial learning and memory in the Morris water maze were compared. Treatment with HDM (group V, 100 mg/kg) significantly reduced the escape latency in Pb-induced mice ($P<0.05$ or $P<0.01$) and was equally effective as DMSA, indicating that ME improved the

learning ability of Pb-induced mice. Previous study has shown that anthocyanins have the ability to improve memory of rats in Morris water maze, and that of mice in the inhibitory avoidance task^[7]. In line with this view, the Cyanidin-3-O-glucopyranoside (Cy-3G) has recently been identified as a potent neuroprotective phytochemical since this compound protects against cytotoxicity in neurocytes and also can reduce cerebral ischemia damage and age-related neuronal deficits. Thus, the administration of ME and DMSA individually or in combination alleviated the impairment induced by Pb and increased exploration, attention, and ability to learning.

The Pb and Ca contents of the blood and soft tissues of mice in the eight groups are listed in Figure 2. Mice in the treatment groups had a lower Pb content in the blood and soft tissue than in the Pb exposed group, especially in the brain ($P<0.05$ or

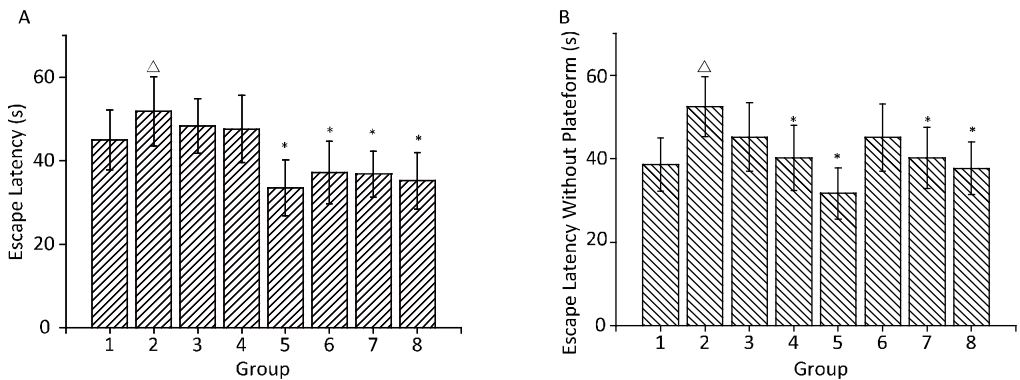


Figure 1. Effect of ME on the Morris test. (A) The escape latency in the place navigation test; (B) The escape latency without platform in the target quadrant. Values given are the mean±SEM ($n=10$). $\Delta\Delta P<0.01$, $\Delta P<0.05$ as compared to normal control group; $**P<0.01$, $*P<0.05$ as compared to Pb group. 1. Group I: Normal; 2. Group II: Pb; 3. Group III : LDM; 4. Group IV: MDM; 5. Group V: HDM; 6. Group VI: ME+DMSA; 7. Group VII: Zn; 8. Group VIII: DMSA.

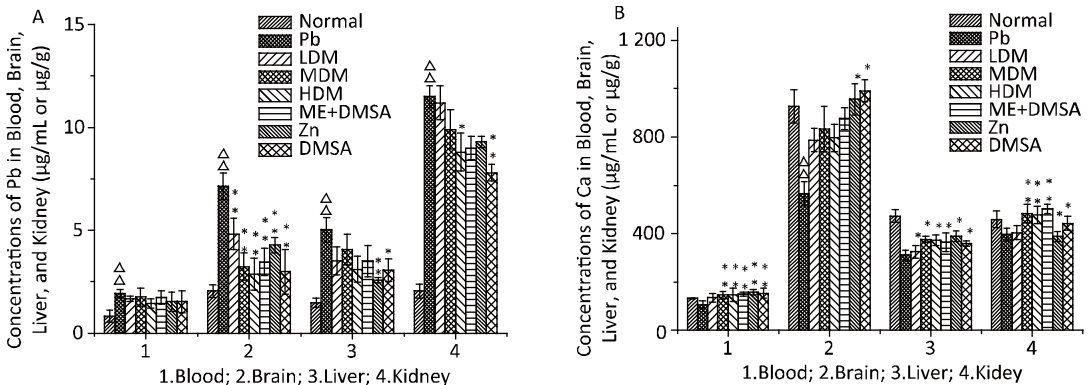


Figure 2. Effect of ME, DMSA or their combination and Zn treatment on Pb and Ca contents in blood, brain, liver and kidney of mice. The tissues were tumbled dry by electric heating air blowing drier. Values are mean±SEM, $n=10$. $\Delta\Delta P<0.01$, $\Delta P<0.05$ as compared to normal control group; $**P<0.01$, $*P<0.05$ as compared to Pb group.

$P < 0.01$). The Ca content is $873.61 \pm 30.00 \mu\text{g/g}$ in the ME sample. Significant differences in the Ca content were observed in the blood and soft tissues ($P < 0.05$ or $P < 0.01$). After treatment with ME individually or in combination with DMSA, the content of Ca increased in the blood and soft tissues ($P < 0.05$ or $P < 0.01$) of mice in the MDM and HDM groups ($P < 0.05$ or $P < 0.01$).

Ca is essential for cellular membrane integrity and metabolism and is a central part of over 300 enzymes and proteins. However, Pb is thought to exert toxic effects by disrupting Ca dependent mechanisms. Pb may compete with Ca for the same binding sites on proteins belonging to a large family of ion binding proteins, and Pb often substitutes for Ca in such proteins^[8]. Pb may displace calcium at its binding sites and induce the production of reactive oxygen species (ROS). As a result, Ca is freed from the protein and is excreted with urine, which may explain the observed increase in lipid peroxidation. Combined administration of ME and DMSA and ME alone scavenged free radicals. Thus, ME may bind to Pb and prevent this heavy metal from inducing free radical generation and enhanced cell viability.

Sensitive biochemical variables changed significantly after treatment with ME and DMSA, either individually or in combination. The level of SOD and MDA in brain is shown in Figure 3. As illustrated in the figures, the co-incubation of primary brain neurons with Pb significantly ($P < 0.05$ or $P < 0.01$) increased MDA levels. Co-incubation of brains with ME significantly reduced lipid peroxidation in a concentration-dependent manner ($P < 0.05$ or $P < 0.01$), but the degree of variation was not significant compared to the normal control. However, ME failed to enhance neuron viability at lower concentrations (25 and 50 mg/kg), similar to the ME+DMSA group. cNOS and TNOS expression were markedly increased in the brains of Pb-treated mice compared to that of the normal control group ($P < 0.05$ or $P < 0.01$).

Pb is a redox inactive metal. However, it has prooxidative activity and reduces cell antioxidant defenses, such as antioxidant enzymes and glutathione. Pb may generate free radicals, which results in the elevation of lipid peroxidation^[9], mainly via depletion of the cellular antioxidant pool. Pb has a high affinity for sulfhydryl groups or metal cofactors in antioxidant enzymes and molecules, which reduces the activities of antioxidant enzymes, such as SOD. Our studies demonstrated that SOD significantly

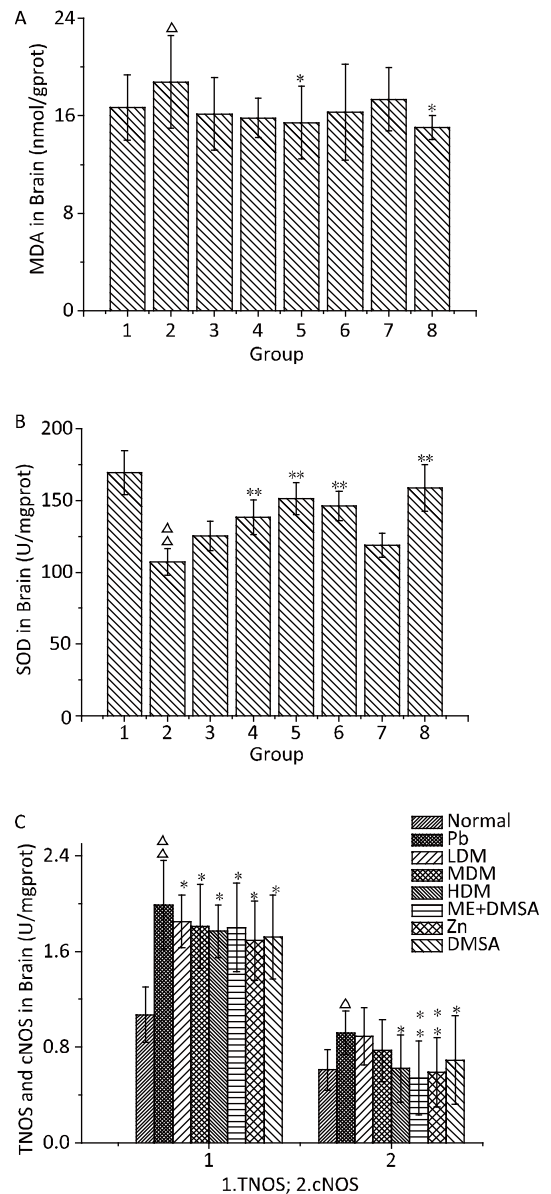


Figure 3. Effect of Pb alone or Pb and ME, DMSA or their combination and Zn treatment on lead-induced nerve damage in brain neurons. (A) The MDA levels in brain of Pb-induced mice; (B) The SOD levels in brain of Pb-induced mice; (C) The vitality of NOS in brain of Pb-induced mice. Each bar represents the mean \pm SEM ($n=10$). $\Delta\Delta P < 0.01$, $\Delta P < 0.05$ as compared to normal control group; $** P < 0.01$, $* P < 0.05$ as compared to Pb group. In A, B, 1. Group I: Normal; 2. Group II: Pb; 3. Group III: LDM; 4. Group IV: MDM; 5. Group V: HDM; 6. Group VI: ME+DMSA; 7. Group VII: Zn; 8. Group VIII: DMSA.

decreased in the brains of Pb-induced mice, while the MDA level in the brain increased after Pb exposure, which was indicative of increased ROS activity to enhance lipid peroxidation. Taking our data together, ME can act as a free radical scavenger and inhibit ROS generation. In the present study, ME inhibited ROS generation in Pb-induced mice, which may be due to its powerful antioxidant and free radical scavenging activities.

Nitric oxide (NO) is an unstable molecule that plays key roles in morphogenesis and synaptic plasticity and acts as a messenger molecule or neurotransmitter in the brain^[10]. Our results showed that elevated NOS levels occurred in brains due to Pb exposure, indicating that Pb-induced neurotoxicity may involve changes in NO production. Our findings suggest that ME restored the expression levels of NOS and NO production in the brains of Pb-induced mice, which indicated that ME protected mice by balancing NO/NOS production.

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REFERENCES

1. Flora SJS, Saxena FG, and Mehta A. Reversal of lead- induced neuronal apoptosis by chelation treatment in rats: Role of reactive oxygen species and intracellular Ca²⁺. *J Pharmacol Exp Ther*, 2007; 322, 108-16.
2. Xia DZ, Yu XF, Liao SP, et al. Protective effect of Smilax glabra extract against lead-induced oxidative stress in rats. *J Ethnopharmacol*, 2010; 130, 414-20.
3. Liu CM, Zheng GH, Ming QL, et al. Protective effect of puerarin on lead-induced mouse cognitive impairment via altering activities of acetyl cholinesterase, monoamine oxidase and nitric oxide synthase. *Environ Toxicol Phar*, 2013; 35, 502-10.
4. Kong JM, Chia LS, Goh NK, et al. Analysis and biological activities of anthocyanins. *Phytochemistry*, 2003; 64, 923-33.
5. Wu XY, Liang LH, Zou Y, et al. Aqueous two-phase extraction, identification and antioxidant activity of anthocyanins from mulberry (*Morus atropurpurea* Roxb). *Food Chem*, 2011; 129, 443-53.
6. Kaewkaen P, Tong-Un T, Wattanathorn J, et al. Mulberry Fruit Extract Protects against Memory Impairment and Hippocampal Damage in Animal Model of Vascular Dementia. *Evid-Based Compl Alt*, 2012.
7. Barros D, Amaral OB, Izquierdo I, et al. Behavioral and genoprotective effects of Vaccinium berries intake in mice. *Pharmacol Biochem Be*, 2006; 84, 229-34.
8. Silbergeld EK, Waalkes M, and Rice JM. Lead as a carcinogen: Experimental evidence and mechanisms of action. *Am J Ind Med*, 2000; 38, 316-23.
9. Tulsawani R and Bhattacharya R. Effect of alpha-ketoglutarate on cyanide-induced biochemical alterations in rat brain and liver. *Biomed Environ Sci*, 2006; 19, 61-6.
10. Chen SM, Swilley S, Bell R, et al. Lead induced alterations in nitrite and nitrate levels in different regions of the rat brain. *Comp Biochem and Physiol C-Pharmacol Toxicol*, 2000; 125, 315-23.