Chrysophanol attenuates lead exposure-induced injury to hippocampal neurons in neonatal mice

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Abstract
Previous studies have shown that chrysophanol protects against learning and memory impairments in lead-exposed adult mice. In the present study, we investigated whether chrysophanol can alleviate learning and memory dysfunction and hippocampal neuronal injury in lead-exposed neonatal mice. At the end of lactation, chrysophanol (0.1, 1.0, 10.0 mg/kg) was administered to the neonatal mice by intraperitoneal injection for 15 days. Chrysophanol significantly alleviated injury to hippocampal neurons and improved learning and memory abilities in the lead-poisoned neonatal mice. Chrysophanol also significantly decreased lead content in blood, brain, heart, spleen, liver and kidney in the lead-exposed neonatal mice. The levels of malondialdehyde in the brain, liver and kidney were significantly reduced, and superoxide dismutase and glutathione peroxidase activities were significantly increased after chrysophanol treatment. Collectively, these findings indicate that chrysophanol can significantly reduce damage to hippocampal neurons in lead-exposed neonatal mice.

Key Words: nerve regeneration; traditional Chinese medicine; chrysophanol; lead poisoning; lead; malondialdehyde; superoxide dismutase; glutathione peroxidase; neurons; neonatal mice; antioxidant; learning and memory; Morris water maze; step-down test; hippocampal neurons; ultrastructure; Medical Scientific Research Project of Health Bureau of Hebei Province; neural regeneration

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Introduction
Lead (Pb) is a common industrial toxin and environmental pollutant. A large number of studies have shown that the central nervous system is highly vulnerable to damage from external poisons during embryonic development and just before and after birth (Bellingham, 2012). Maternal exposure to low levels of lead during pregnancy and lactation can increase blood lead levels in offspring and impair nervous system development and learning and memory abilities.

Neurons and hippocampal astrocytes are major targets of lead poisoning, and lead exposure can reduce the number of mature oligodendrocytes, impair nerve conduction and even affect the survival of neurons (Chen et al., 2006; Ronchetti et al., 2006). Goyer et al. (1996) demonstrated that the developing nervous system was particularly sensitive to the toxic effects of lead, and that lead impaired the normal biochemical functioning of neurons. Hippocampal neurons are an important target of lead-induced neurotoxicity.

Chrysophanol is an active component extracted from Rhubarb. Chrysophanol can improve learning and memory abilities in Alzheimer’s disease mice, and it has anti-aging effects (Dong and Zhang, 2009; Li et al., 2009). Previous studies have shown that lead poisoning and Alzheimer’s disease have a similar pathogenesis. Chrysophanol was shown to protect against lead poisoning in adult mice (Zhang et al., 2011, 2012). In the present study, we investigated whether chrysophanol has neuroprotective effects against lead toxicity in hippocampal neurons in neonatal mice exposed to lead during pregnancy and lactation, and we investigated the underlying mechanisms of action.

Materials and Methods

Animals
Sixty adult Kunming mice, including 20 males and 40 females, weighing 22 ± 2 g, were purchased from the Experimental Animal Center of the Chinese Academy of Medical Sciences, China (license No. SCXK (Jing) 2006-0008). All animals were fed for 3 days to adapt to the environment. Then, the mice were housed in separate cages containing two females and one male for mating. The next morning, the female mice were examined for the presence of a vaginal plug, which, if present, was taken to indicate pregnancy day 0. All animal experiments in the present study were performed according to the guidelines of the Chinese Council on Animal Care and the Institutional Care Committee of Hebei North University, China.
Experimental groupings and interventions

Twenty-five pregnant mice were randomly divided into five groups: control group, model group and chrysophanol treatment groups (0.1, 1.0, 10.0 mg/kg dose groups) (Zhang et al., 2011). From pregnancy day 0 to neonatal day 21, double distilled water was given freely to control group female mice, and 0.4% lead acetate (Tianjin No. Six Chemical Reagent Factory, Tianjin, China) aqueous solution was given freely to pregnant mice in the model and chrysophanol treatment groups (Carpenter et al., 2002). On day 22 after the mice were born, the neonatal mice in each group were raised in different cages according to sex and were freely given double distilled water. The neonatal mice in the control and model groups were administered vehicle (dimethylformamide: Tween-80: normal saline = 1:1:8; Tianjin No. Six Chemical Reagent Factory) via intraperitoneal injection, once daily at 10.0 mL/kg body weight for 15 days. The neonatal mice in the chrysophanol treatment groups were administered chrysophanol (Nanjing Zelang Pharmaceutical Technology Co., Ltd., Nanjing, Jiangsu Province, China; 0.1, 1.0, 10.0 mg/kg) via intraperitoneal injection once daily at 10.0 mL/kg body weight for 15 days (Zhang et al., 2012).

Behavioral assessment

Step-down test

Step-down test training was started 30 minutes after 8 days of administration of chrysophanol, and the test was performed 24 hours after the final administration to evaluate the learning and memory ability of lead-exposed neonatal mice. The step-down test is a one-time stimulus avoidance response test. The apparatus is a rectangular reflective box, in which the bottom is covered with a copper grid (2-mm spacing) electrified at 36 V. There is a platform in the box, 4.5 cm in height and 4.5 cm in diameter. During the experiment, the mice were put on the copper grid and the current was switched on. Mice received an electric shock. The normal reaction of the mice is to avoid the shock and jump on to the platform, but most mice would jump off the platform even after repeatedly receiving an electric shock. The training lasted for 3 minutes, and the time that each mouse remained on the platform for the first time before jumping off was recorded as the latency, and the number of shocks within 3 minutes was recorded as the number of errors. After 24 hours, the test was repeated as the memory test, and the latency and number of errors were recorded as memory test scores.

Morris water maze test

The Morris water maze test was performed 30 minutes after the 10-day administration of chrysophanol. The Morris water maze consisted of a cylindrical pool (diameter, 120 cm; height, 50 cm) and a removable cylindrical transparent glass platform (diameter, 12 cm; height, 24 cm). The pool was divided into 4 quadrants, and the platform was arranged on the first quadrant. Every mouse was trained to find the platform for 4 consecutive days, and trained four times every day. Each time, the mouse was put into the pool from different quadrants for training for 120 seconds. The latency and swimming distance in finding the platform were recorded. If the mouse did not find the platform within 120 seconds, the latency was recorded as 120 seconds. Then, the mouse was replaced on the platform for 20 seconds, and the next training was performed after 120 seconds of rest. On day 5 of training, the platform was removed, and the number of crossings of the platform location within 120 seconds (crossing number) was recorded.

Harvesting of tissues and serum sample collection

Within 1 hour after the 15-day administration of chrysophanol, the neonatal mice were decapitated rapidly, 1.0 mL blood from the neck was removed into a test tube containing heparin, and the brain (divided into two parts at the midline), liver (two parts), both kidneys, heart and spleen were quickly removed on ice. All the tissues were cleaned with normal saline, dried with filter paper, accurately weighed and preserved at −80°C (Thermo Fisher, Waltham, MA, USA).

Assay for lead content in blood, brain, heart, liver, spleen and kidney

Within 1 hour after the 15-day administration of chrysophanol, lead levels in blood, brain, heart, liver, spleen and kidney were analyzed. 1.0 mL blood was placed in a plastic centrifuge tube with 10% nitric acid (Tianjin Yaohua Chemical Reagent Co., Ltd., Tianjin, China). The right brain, heart, liver, spleen and right kidney were separately placed in plastic centrifuge tubes with 10% nitric acid. Lead content was determined by graphite furnace atomic absorption spectrometry (Shimadzu Corporation, Japan).

Determination of malondialdehyde (MDA) content and glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities

Within 1 hour after the 15-day administration of chrysophanol, the brain, liver and kidney samples were collected and the levels of MDA and GSH-Px and SOD activities were determined. Normal saline at 4°C was added to the left brain, liver and left kidney samples at a ratio of 1:9 (w/v). The tissue homogenate was prepared with a homogenizer, and the supernatant was then centrifuged at 3,500 r/min for 15 minutes and then collected. SOD, MDA and GSH-Px were determined with the corresponding kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu Province, China). Protein content of tissues was determined using the Coomassie brilliant blue method. The content of MDA and the activities of SOD and GSH-Px per gram protein were calculated.

Observation of hippocampal ultrastructure

Within 1 hour after the 15-day administration of chrysophanol, another five neonatal mice from each group were used to observe hippocampal ultrastructure. Perfusion was performed according to the methods of Bacq and Ghirretti (1950) and McCarey et al. (1973). Then, the brains were removed rapidly and immersed in 2.5% glutaraldehyde solution. After immersing for 20 minutes, hippocampal tissues were...
taken out with forceps and cut into 1-mm³ pieces, which were placed in 2.5% glutaraldehyde at 4°C overnight (Shen et al., 2009). The next day, the samples were cleaned with 6 changes of 0.01 mol/L PBS to remove the glutaraldehyde. The tissues were then fixed with 1% osmic acid solution for 2 hours and cleaned with 5 changes of 0.01 mol/L PBS.
Figure 4 Effects of chrysophanol on biochemical markers in tissues in lead-poisoned neonatal mice. Group 1: Control ($n=12$); group 2: model ($n=13$); group 3: 0.1 mg/kg chrysophanol ($n=14$); group 4: 1.0 mg/kg chrysophanol ($n=13$); group 5: 10.0 mg/kg chrysophanol ($n=14$). Data are expressed as mean ± SD. *$P<0.01$, vs. control group; #$P<0.05$, ##$P<0.01$, vs. model group (one-way analysis of variance, Student-Newman-Keuls test). MDA: Malondialdehyde; GSH-Px: glutathione peroxidase; SOD: superoxide dismutase.

Figure 5 Effects of chrysophanol on hippocampal neurons in lead-poisoned neonatal mice (electron microscope, × 20,000, conventional uranium-lead dyeing). (A) In the control group, mitochondria, rough-surfaced endoplasmic reticulum and free ribosomes (arrows) can be seen. (B) In the model group, some of the cristae appeared cracked or missing, and most of the mitochondrial membrane (arrows) was fused or missing. (C) In the 0.1 mg/kg chrysophanol group, the rough endoplasmic reticulum arrows appeared mildly fused and degranulated. (D) In the 1.0 mg/kg chrysophanol group, a part of the mitochondrial membrane (arrows) was slightly fused, and the rough endoplasmic reticulum appeared mildly degranulated. (E) In the 10.0 mg/kg chrysophanol group, secondary lysosomes (arrows) were visible, and a small portion of the mitochondrial membrane was fused.

Figure 6 Effects of chrysophanol on hippocampal capillary endothelial cells in lead-poisoned neonatal mice (conventional uranium-lead dyeing). (A) In the control group, normal mitochondria and rough endoplasmic reticulum (arrow) were visible in the cytoplasm (× 20,000). (B) In the model group, most mitochondrial cristae and a small part of the membrane (arrows) were fused and blurred at the edge of the cells, and a few mitochondrial cristae were cracked or not visible (× 20,000). (C) In the 0.1 mg/kg chrysophanol group, the rough endoplasmic reticulum was clearly visible, the microvilli were normal, pinocytotic vesicles (arrow) were fewer than normal, and there was no edema or blood (× 20,000). (D) In the 1.0 mg/kg chrysophanol group, the endothelial cell nuclei and intravascular red cells (arrow) were clearly visible, a small part of the mitochondrial cristae was fused, and microvilli and pinocytotic vesicles were fewer (× 4,000). (E) In the 10.0 mg/kg chrysophanol group, the nucleus, mitochondria, vesicles and microvilli (arrows) had a normal morphology and were clearly visible (× 12,000).
and then subjected to acetone gradient dehydration (Koga et al., 2012). After dehydration, the samples were immersed in penetrant (acetone and epoxy resin EPON 812 mixed in a ratio of 1:1 at 37°C) for 24 hours, and then embedded. 24 hours later, the embedded polymer was hardened and positioned accurately. An LKBNoVa II ultramicrotome (LKB Instrument, Stockholm, Sweden) was used to cut sections at a thickness of 40–50 nm, and conventional uranium-lead dyeing was performed according to previously described methods (Zheng et al., 2013). The slices were observed and photographed with a JEM100CX II transmission electron microscope (JEOL Corporation, Tokyo, Japan).

Statistical analysis
Data were processed with SPSS 13.0 statistical software (SPSS, Chicago, IL, USA). All experimental data were expressed as mean ± SD, and comparisons between group were performed with one-way analysis of variance followed by Student-Newman-Keuls multiple comparison test. \( P < 0.05 \) was considered statistically significant.

Results
Quantitative analysis of experimental animals
Twenty-five pregnant female mice were randomly divided into solvent, model, and chrysophanol 0.1, 1.0 and 10.0 mg/kg treatment groups, with five pregnant mice in each group, delivering 17, 18, 19, 18 and 19 pups, respectively. Five neonatal mice with lead poisoning in each group were used to observe hippocampal ultrastructure. The remaining neonatal mice were used for the step-down test on day 8 after administration and for the water maze test on day 10. On day 15, the mice were killed by decapitation, and lead levels in blood, brain, heart, liver, spleen and kidney were measured, and biochemical markers in brain, liver and kidney were assessed. There were 12, 13, 14, 13 and 14 neonatal mice, respectively, in the solvent, model, and chrysophanol 0.1, 1.0 and 10.0 mg/kg treatment groups for the various tests. A total of 91 neonatal mice were used for quantitative analysis without any loss.

Effects of chrysophanol on learning and memory abilities in lead-poisoned neonatal mice in the step-down test
Step-down training was started within 30 minutes after the 8th day of chrysophanol treatment, and the step-down test was performed 24 hours after administration to assess the learning and memory abilities of lead-exposed neonatal mice. As shown in Figure 1, latency in the model group was significantly shorter than in the control group \( (P < 0.01) \), in both the learning training and memory tests, while the number of errors in the model group was significantly higher than in the control group \( (P < 0.01) \). This shows that the modeling method used in the current study successfully induced lead poisoning and significantly decreased learning and memory abilities in the lead-poisoned neonatal mice. These findings are consistent with a previous report (Zhang et al., 2011). Compared with the model group, the latency was not significantly different, but the number of errors was significantly reduced in the chrysophanol treatment group (0.1 mg/kg, \( P < 0.05 \)). Compared with the model group, the number of errors in the chrysophanol treatment groups (1.0 and 10.0 mg/kg) was significantly decreased and latency was significantly increased \( (P < 0.01) \).

Effects of chrysophanol on learning and memory abilities in lead-poisoned neonatal mice in the Morris water maze test
The Morris water maze test was performed 30 minutes after the 10-day administration. As shown in Figure 2, as the number of training days increased, the latency and swimming distance in finding the platform decreased. On days 3 and 4, the latency and swimming distance of mice in the model group were significantly longer compared with the control group \( (P < 0.01) \). The latency and swimming distance of mice in the 0.1 mg/kg chrysophanol treatment group were significantly shortened on day 4 compared with the model group \( (P < 0.05) \). The latency and swimming distance in the 1.0 and 10.0 mg/kg chrysophanol treatment groups were significantly shortened on days 3 and 4 compared with the model group \( (P < 0.05, P < 0.01) \). Compared with the control group, the number of crossings in the model group was significantly reduced \( (P < 0.01) \). Chrysophanol (0.1, 1.0, 10.0 mg/kg) increased the number of platform crossings in lead-poisoned neonatal mice \( (P < 0.05, P < 0.01) \).

Effects of chrysophanol on lead content in blood and organ tissues in lead-poisoned neonatal mice
Within 1 hour after the 15-day administration of chrysophanol, organ tissue and serum samples were collected and the lead content was determined. As shown in Figure 3, lead content in blood and tissues of mice in the model group were significantly higher than in the control group \( (P < 0.01) \). Compared with the model group, lead levels in blood and tissues in the chrysophanol treatment groups were decreased to varying degrees \( (P < 0.05, P < 0.01) \). Chrysophanol (1.0, 10.0 mg/kg) significantly reduced lead content in blood and tissues \( (P < 0.01) \).

Effects of chrysophanol on biochemical markers in tissues of lead-poisoned neonatal mice
Within 1 hour after the 15-day administration of chrysophanol, tissue biochemical markers in the lead-poisoned neonatal mice were analyzed. As shown in Figure 4, activities of SOD and GSH-Px in the brain, liver and kidney in the model group were significantly decreased compared with the control group \( (P < 0.01) \). Compared with the model group, chrysophanol (0.1, 1.0, 10.0 mg/kg) significantly increased the activities of SOD and GSH-Px in the liver, kidney and brain of neonatal mice \( (P < 0.01) \). Compared with the control group, levels of MDA in the kidney, brain and liver in the model group were significantly increased \( (P < 0.01) \). Compared with the model group, chrysophanol (0.1 mg/kg) significantly reduced MDA content in brain and liver in lead-poisoned neonatal mice \( (P < 0.05, P < 0.01) \), but it had no significant effect on MDA content in kidney.
Compared with the model group, chrysophanol (1.0, 10.0 mg/kg) significantly reduced MDA content in brain, liver and kidney of lead-poisoned neonatal mice \((P < 0.01)\).

**Effects of chrysophanol on the morphology of hippocampal neurons and capillary microvascular endothelial cells in lead-poisoned neonatal mice**

**Effects of chrysophanol on hippocampal neurons**

Within 1 hour after the 15-day administration of chrysophanol, hippocampal tissue was harvested from five lead-poisoned neonatal mice from each group to observe hippocampal ultrastructure. The results are shown in Figure 5. In hippocampal neurons in the control group, the nuclei were large, round and had prominent nucleoli and a clear nuclear membrane. There were abundant mitochondria, rough-surfaced endoplasmic reticulum and free ribosomes in hippocampal neurons in the control group (Figure 5A). In hippocampal neurons in the model group, cytoplasmic edema was severe. The number of organelles was decreased significantly, and most mitochondrial cristae were fused and even blurred. Portions of the cristae appeared cracked and some had disappeared, and most of the mitochondrial membrane was fused or missing (Figure 5B). These findings indicate that lead induces hippocampal neuronal damage in neonatal mice, and that it is highly neurotoxic. In hippocampal neurons in the 0.1 mg/kg chrysophanol treatment group, cytoplasmic edema was mild, a few mitochondrial cristae appeared cracked, and a small portion of the mitochondrial membrane was fused. The rough endoplasmic reticulum was slightly fused and degranulated (Figure 5C). In hippocampal neurons in the 1.0 mg/kg chrysophanol treatment group, most mitochondria tended to be normal, but a part of the mitochondrial membrane was slightly fused, and the rough endoplasmic reticulum was mildly degranulated (Figure 5D). In hippocampal neurons in the 10.0 mg/kg chrysophanol treatment group, secondary lysosomes were visible, a small portion of the mitochondrial membrane was fused, a few mitochondrial cristae were slightly cracked, and a part of the rough endoplasmic reticulum was mildly expanded, with some fused granules and degranulation (Figure 5E). These results show that chrysophanol at different doses reduces hippocampal damage in lead-poisoned neonatal mice, indicating that the medicine is neuroprotective.

**Effects of chrysophanol on hippocampal capillary endothelial cells**

The ultrastructure of hippocampal capillary endothelial cells was also observed under the electron microscope. In hippocampal capillary endothelial cells of the control group, normal mitochondria and rough endoplasmic reticulum were visible in the cytoplasm (Figure 6A). In these cells in the model group, most mitochondrial cristae and a small part of the membrane were fused and blurred near the edge of the cells, and a few mitochondrial cristae appeared cracked or missing (Figure 6B). In hippocampal capillary endothelial cells in the 0.1 mg/kg chrysophanol treatment group, mitochondrial cristae were nearly absent, degranulation of the rough endoplasmic reticulum was obvious, the microvilli were normal, pinocytic vesicles were fewer than normal, and there was no edema or blood (Figure 6C). In the 1.0 mg/kg chrysophanol treatment group, the endothelial cell nuclei and intravascular red cells were clearly visible, a small part of the mitochondrial cristae was fused, and microvilli and pinocytic vesicles were fewer (Figure 6D). In hippocampal capillary endothelial cells in the 10.0 mg/kg chrysophanol treatment group, the nucleus, mitochondria, vesicles and microvilli were clearly visible (Figure 6E), and cells tended to be normal. These results show that chrysophanol protects against capillary endothelial cell damage in lead-poisoned neonatal mice, which further validates the neuroprotective effects of the medicine.

**Discussion**

Lead is a toxic heavy metal widely present in working and living environments, and can damage multiple systems and organs in the body, especially the nervous and circulatory systems. Nelson and Espy (2009) found that preschool children with low-level lead exposure took longer to learn associations than preschool children with very low levels of lead exposure, and this difference could not be attributed to increased distractibility or perseverative responding (Chen et al., 1999). Children are more sensitive to lead than adults. The issue of lead pollution and its effects on child health and mental development have been tackled by many domestic and foreign scholars. A study by Faust and Brown (1987) showed that blood lead content in children is negatively associated with intelligence. Hippocampal neurons are an important target of lead neurotoxicity (Wilkins et al., 2003). The study of Gullarte et al. (1998) found that lead levels in blood and brain of neonatal mice whose mother was exposed to low-level lead during pregnancy and lactation were significantly higher than in normal control mice.

In the current study, we established a model of lead exposure during pregnancy and lactation in neonatal mice and explored the protective effects of chrysophanol on learning and memory abilities in these animals. In this experiment, chrysophanol was administered for 15 days to the pups starting 22 days postnatally. The Morris water maze and step-down tests were performed to evaluate the cognitive ability of lead-poisoned neonatal mice. We found that chrysophanol alleviated the learning and memory impairment caused by lead poisoning. The 10.0 mg/kg dose of chrysophanol was most effective, consistent with preliminary results (Zhang et al., 2012). Thus, this dose of the drug might be therapeutically effective in a clinical setting.

Under normal conditions, GSH-Px, SOD, CAT and GSH and other antioxidants can effectively remove free radicals and maintain cellular oxidant/antioxidant balance. MDA is a decomposition product of lipid peroxidation, and its levels reflect lipid peroxidation in body tissues (Cormard et al., 2005). The present study found that MDA content was increased and activities of GSH-Px and SOD were decreased significantly in brain, liver and kidney of lead-poisoned neonatal mice, consistent with previous reports (Shalan et al., 2005). The rapid increase in MDA suggests that oxidative/
antioxidant balance was disrupted, resulting in higher lipid peroxidation. The decrease in activities of GSH-Px and SOD suggests that high levels of lead had accumulated in the tissues, inhibiting the activities of the antioxidases (Sandhir et al., 1994). Tissue MDA levels were decreased and the activities of GSH-Px and SOD were significantly increased after chrysophanol was given to lead-poisoned neonatal mice, suggesting that, in addition to promoting lead excretion from the body, chrysophanol can significantly decrease lipid peroxidation and increase antioxidative enzyme activities in mice brain, liver and kidney. These results are consistent with the results of a previous study (Zhang et al., 2011).

The nervous system is very sensitive to the toxic effects of lead. In brain cells, lead is mainly deposited in the mitochondria, resulting in mitochondrial damage, including swelling and disruption of the cristae. The hippocampus is an important structure involved in learning and memory formation, and is also the main target of lead toxicity in the brain (Carpenter et al., 2002). Therefore, we focused on the ultrastructure of hippocampal neurons and endothelial cells in lead-poisoned neonatal mice to evaluate the neuroprotective effects of chrysophanol. We found that exposure to low levels of lead during pregnancy and lactation can lead to severe cytoplasmic edema in hippocampal neurons. Furthermore, the number of organelles was decreased, mitochondria and rough endoplasmic reticulum showed severe morphological changes, and the capillary endothelial cells also showed substantial damage, including membrane fusion. After administration of chrysophanol (0.1, 1.0, 10.0 mg/kg) via intraperitoneal injection for 15 days, these pathological changes were attenuated, including a reduction in mitochondrial membrane fusion. In addition, endoplasmic reticulum degranulation was alleviated, most mitochondria tended to be normal, and some secondary lysosomes could be visible. There was an obvious dose-effect relationship, with the 10.0 mg/kg dose of chrysophanol having the best protective effect on cellular ultrastructure.

In conclusion, chrysophanol improved learning and memory abilities and protected against hippocampal neuronal injury induced by lead by increasing antioxidant capacity. Concomitantly, lead content in blood and tissues was reduced significantly. Our findings suggest that chrysophanol may have significant therapeutic potential in the prevention and treatment of lead-induced neurotoxicity. In future studies, we will investigate in-depth the mechanisms underlying the neuroprotective effects of chrysophanol against lead neurotoxicity, and we will further examine the dose-response relationship to provide additional insight into the therapeutic potential of the medicine.

References


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