Supplementary Material

Harmine enhances type H vessel formation and prevents bone loss in ovariectomized mice

Jie Huang[1,4*], Hao Yin[1,4*], Shan-Shan Rao[1,6], Ping-Li Xie[7], Xu Cao[8], Tai Rao[9], Shu-Ying Liu[10], Zhen-Xing Wang[1], Jia Cao[1], Yin Hu[1,4], Yan Zhang[1], Juan Luo[1], Yi-Juan Tan[1], Zheng-Zhao Liu[1,5], Ben Wu[1], Xiong-Ke Hu[1], Tuan-Hui Chen[1], Chun-Yuan Chen[1#] and Hui Xie[1-5,11#]

1. Movement System Injury and Repair Research Center, Xiangya Hospital, Central South University, Changsha, Hunan 410008, China.
2. Hunan Key Laboratory of Organ Injury, Aging and Regenerative Medicine, Changsha, Hunan 410008, China.
3. Department of Sports Medicine, Xiangya Hospital, Central South University, Changsha, Hunan 410008, China.
4. Department of Orthopedics, Xiangya Hospital, Central South University, Changsha, Hunan 410008, China.
5. National Clinical Research Center for Geriatric Disorders, Xiangya Hospital, Central South University, Changsha, Hunan 410008, China.
6. Department of Spine Surgery, Xiangya Hospital, Central South University, Changsha, Hunan, 410008, China.
7. Department of Forensic Science, School of Basic Medical Sciences, Central South University, Changsha, Hunan 410013, China.
8. Department of Orthopaedic Surgery, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA
9. Department of Clinical Pharmacology, Xiangya Hospital, Central South University, Changsha, Hunan 410008, China.
10. The Second Xiangya Hospital, Central South University, Changsha, Hunan 410008, China.
11. China Orthopedic Regenerative Medicine Group (CORMed), Changsha, Hunan 410008, China.

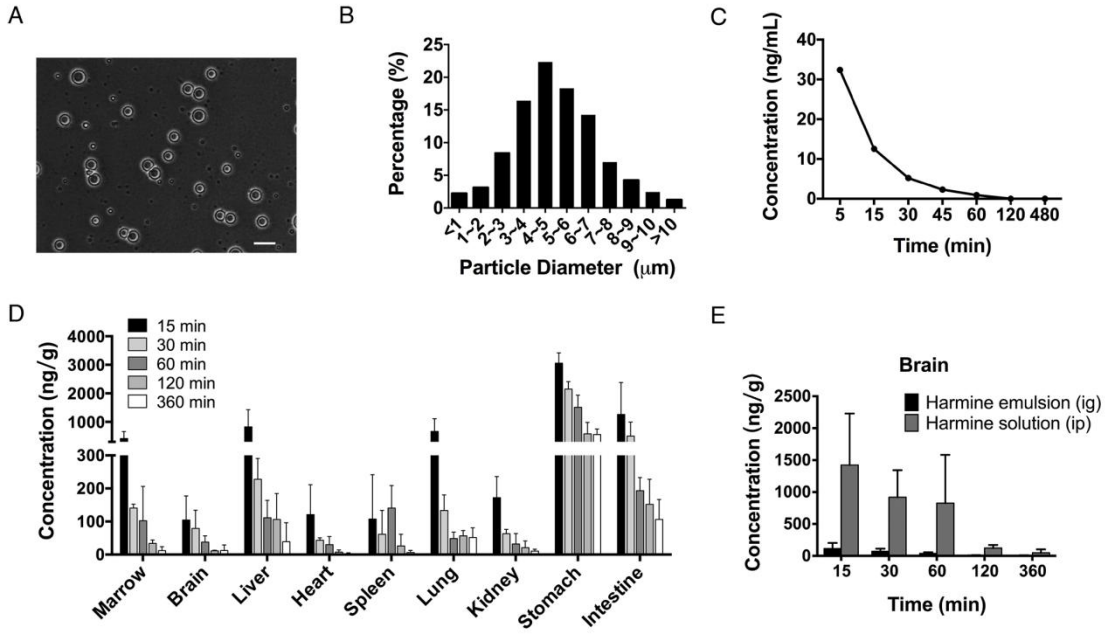

Figure S1. Size distribution, pharmacokinetics and tissue distribution of harmine emulsion. (**A**) Representative images of harmine emulsion under optical microscopy. Scale bar: 10 μm. (**B**) Particle size distribution of harmine emulsion in (**A**). The mean diameter of emulsified harmine particles was 5.02 ± 1.79 μm. (**C**) Blood concentration-time curve of harmine in mice following a single oral dose of harmine emulsion. The half-life of elimination phase calculated from the blood concentration-time curve was $t_{1/2}$ = 11.04 min. n = 3. (**D**) Tissue distribution of harmine in mice following a single oral dose of harmine emulsion at indicated times. (**E**) Brain concentrations of harmine in mice intragastrically (ig) administrated with harmine emulsion or intraperitoneally (ip) injected with harmine solution at indicated times.

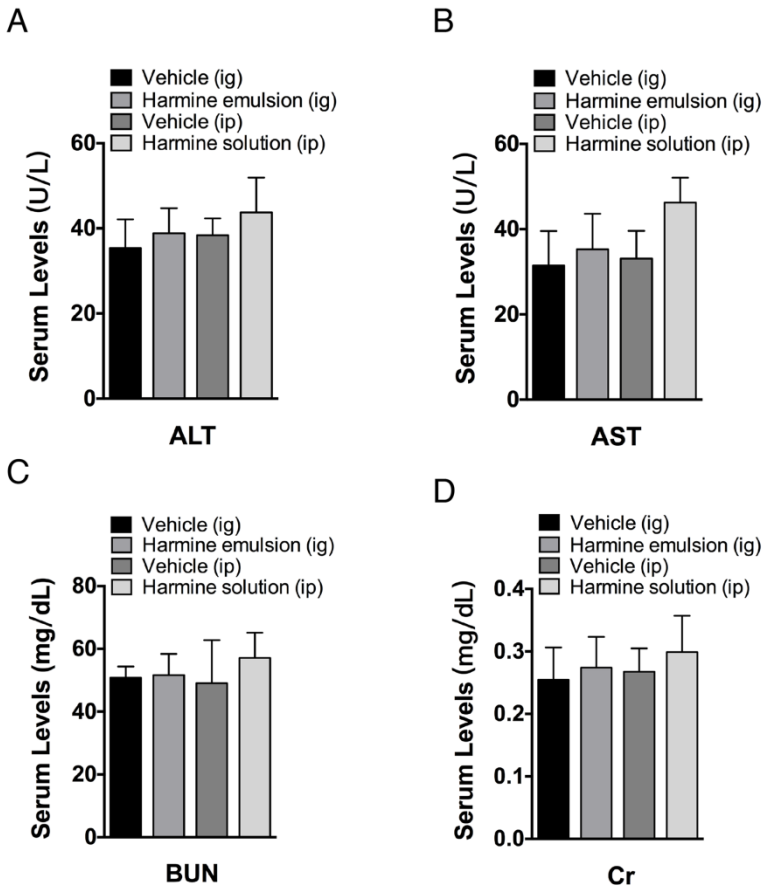

Figure S2. Oral administration of harmine emulsion induces non-significant effects on liver and kidney. (**A-B**) The serum levels of liver function indicators including ALT and AST in mice intragastrically (ig) administrated with harmine emulsion or intraperitoneally (ip) injected with harmine solution. Mice in control group were treated with their respective vehicles. (**C-D**) The serum levels of renal function indicators including blood urea nitrogen (BUN) and creatinine (Cr) in mice in different treatment groups. *****$P < 0.05$ compared with the vehicle-treated mice**.**

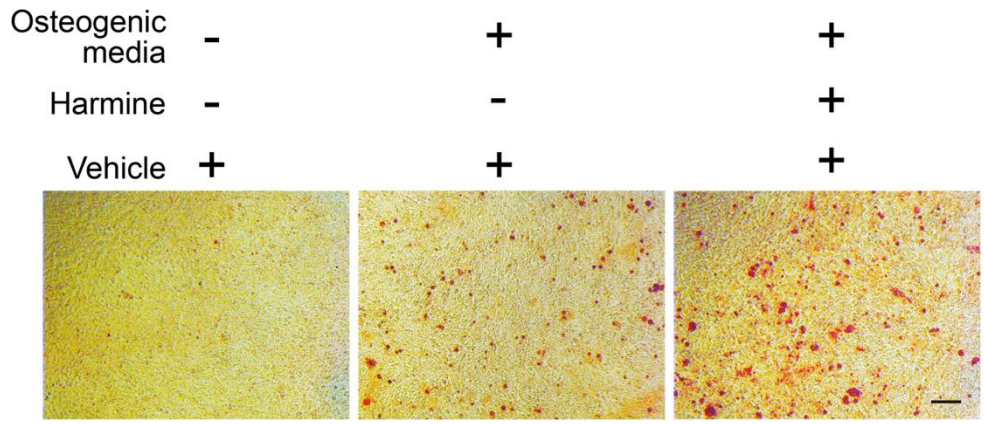

Figure S3. Harmine promotes osteogenic differentiation of mesenchymal stem cells (MSCs). MSCs were cultured in osteogenic medium supplemented with 3 μg/mL harmine or an equal volume of vehicle (DMSO). Alizarin red S (ARS) staining was performed after 14 days of induction. Scale bar: 100 μm.

Methods

Pharmacokinetics and tissue distribution of harmine

Animals and treatments

12-week-old female C57BL/6 mice (weighing 25-30 g) were used for these experiments. To test the pharmacokinetics of intragastrically administrated harmine emulsion, twenty-four mice were oral gavaged with 10 mg/kg of harmine emulsion (0.625 mg/mL) and then averagely and randomly assigned into 8 groups. The mice were respectively sacrificed at 5 min, 15 min, 30 min, 1 h, 2 h, 6 h, 12 h and 24 h after the administration of harmine emulsion. Blood samples were collected by removing eyeballs after euthanasia. Bone marrow samples from femora of mice were collected in heparin. The brain, liver, heart, spleen, lung, kidney, stomach and intestine were collected from the mice treated with harmine for 15 min, 30 min, 1 h, 2 h and 6 h, in order to test the tissue distribution of intragastrically administrated harmine emulsion. To detect the accumulation of harmine in brain upon its intraperitoneal administration in water soluble form, fifteen mice were injected with 10 mg/kg of harmine solution (0.625 mg/mL) and randomly divided into five groups with three mice per group. Brain samples were collected after the mice treated with harmine for 15 min, 30 min, 1 h, 2 h and 6 h, respectively.

Sample Preparation

Blood and bone marrow samples were centrifuged at 1,000 ×g for 15 min and the serum and marrow supernatant were collected and stored at −80 °C until analysis. The organ samples and bone marrow were homogenized in double distilled water (ratio of sample weight and water volume was 1:5). The homogenate was centrifuged at 1,200 ×g for 30 min and the supernatant was collected. Each organ supernatant (100 μL) was mixed with 10 μL of internal standard solution (diphenhydramine; 1 μg/mL in 50 % methanol) and then 500 μL of acetonitrile was added for protein precipitation. After centrifugation at 15,000 ×g for 10 min at 4 ℃, the supernatant was collected and desolvated under nitrogen. Afterwards, the residue was redissolved in 120 μL of 50% methanol and vortexed for 1 min. After one more centrifugation at 15,000 ×g for 10 min at 4 ℃, 5 μL of supernatant was subjected to the high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis.

The calibration standards samples were prepared by adding appropriate working

solutions of harmine into blank serum or homogenate to yield concentrations of 1, 2, 5, 10, 20, 50, 100, 200, 500, and 1000 ng/mL. Calibration curves of the serum or homogenate were constructed by plotting the peak area ratios of harmine and internal standard. Linearity was demonstrated by testing the calibration solutions ranging from 0 to 1000 ng/mL. A correlation coefficient (R^2) exceeding 0.99 was desirable for calibration curves.

HPLC-MS/MS analysis

The HPLC chromatographic equipment used was an Acquity Ultra performance LC with a sample manager and binary solvent manager. A Phenomenex Kenetex C18 (50 × 2.1 mm; 5 μm) was used. The mobile phase was composed of (A) acetonitrile and (B) 0.1% formic acid, as gradient, for 7 min, at a flow-rate of 0.2 mL/min as follows: 0–0.1 min, 8% A; 0.1–2 min, 8–60% A; 2–2.5 min, 60–90% A; 2.5–4.5 min, 90% A; 4.5–5 min, 90-8% A; 5–7 min, 8% A. The injection volume was 5 μL. The AB SCIEX Triple Quad^{TM} 4000 LC/MS/MS system (Framingham, MA, USA) equipped with an electrospray ionization (ESI) interface was used for quantification. In positive ion mode, the mass spectrometer was performed using MRM to monitor the mass transition pairs: m / z 213.2 → 169.3 for harmine, m / z 256.2 → 167.2 for diphendydramine (IS). The optimized parameters were as follows: curtain gas (CUR), ion source gas1 (GS1) and ion source gas 2 (GS2) were 20, 40 and 45 psi, respectively; source temperature was maintained at 200 ℃; ion spray voltage was at 5500 V; the collision energy (CE) for harmine and IS was 43 and 20 eV, respectively. MS data acquisition was performed using Analyst TF 1.6.1 software (AB SCIEX, Framingham, MA, USA). 2.3.3. After determining the serum concentrations of harmine, the blood concentration-time curve was established and the half-life of elimination phase ($t_{1/2}$) was calculated. The concentrations of harmine in different tissues were illustrated in histogram form.

Toxicity analysis of harmine

Sixteen female C57BL/6 mice (12-week-old; weighing 25-30 g) were averagely and randomly assigned into four groups. Mice were intragastrically administrated 10 mg/kg of harmine emulsion (0.625 mg/mL; emulsion group) or intraperitoneally injected with 10 mg/kg of harmine solution (0.625 mg/mL; solution group). Mice in control group were treated with an equal volume of their respective vehicles. The

treatments were conducted daily for 2 months. After administration, the mice were observed thoroughly for the onset of any neural toxicities. The mice were monitored continuously for 10 days to observe any abnormal behaviors. 2 month later, blood samples of mice were collected by removing eyeballs after euthanasia and serum samples were separated by centrifugation at 1,000 ×g for 15 min). The levels of serum ALT and AST were tested using ELISA kits from Elabscience (Wuhan, China). The levels of serum creatinine and blood urea nitrogen (BUN) were measured using commercial kits from Jiancheng (Nanjing, China) and Solarbio (Beijing, China), respectively.

Osteogenesis induction and alizarin red S (ARS) staining

MSCs were prepared from male mouse strain C57BL/6-Tg(UBC-GFP)30Scha/J (Jackson Laboratory). For osteogenesis induction, MSCs (1.0×10^4/well) were plated in 48-well plates and cultured in high glucose DMEM containing 10% FBS. 24 h later, the medium was replaced with osteogenic medium (Cyagen Biosciences Inc, Santa Clara, USA) supplemented with 3 μg/mL harmine or an equal volume of diluents (DMSO). MSCs cultured in DMEM + 10% FBS were served as the negative control. Half of the medium was changed every 3 days. After 2 weeks, the cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min, and then stained with 2% ARS solution (Solarbio, Beijing, China) according to the manufacturer's instructions. The stained cells were examined using an inverted microscope (Leica DMI6000B, Solms, Germany).