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Nanoparticles Improved Resveratrol Brain Delivery and its Therapeutic Efficacy against Intracerebral Hemorrhage†

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S1. Experimental section

1.1 Res-NPs Preparation

The Res-NPs were prepared using the antisolvent precipitation method. To optimize the Res-NPs formulation, Res-NPs were prepared under multiple conditions that involved varying type and concentration of stabilizer, the concentration of Res, the concentration of organic solvent, the antisolvent-to-solvent volume ratio and the stirring speed.

1.2 In vitro Dissolution Assessment of Res-NPs

The in vitro dissolution assessment of Res-NPs was evaluated by a dialysis method. Briefly, 1 ml of Res or Res-NPs was transferred into a dialysis bag (2000 Damolecular weight cutoff) with 50 mL of hydrochloric acid (HCl) pH 1.2 or PBS buffer pH 7.4 conditions. After shaking at a rate of 100 r/min in 37 °C, 1 mL of medium was collected and replaced with 1 mL of fresh medium at different time points (0, 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 h). The Res content was determined using an Agilent 1100 series HPLC system (Santa Clara, CA, USA) with a ZORBAX Eclipse Plus C18 (4.6 × 250 mm, 5 μm) analytical column. After 20 μL of sample was injected into the column, the analytes were eluted by methanol and water (25:75, v/v) at a flow-rate of 1 mL/min for 20 min. UV absorption was conducted at 250 nm.

1.3 Effect of NPs on ICH

A total of 24 C57BL/6 mice were divided into three groups: the sham surgery group (Sham), the ICH model group (ICH) and the NPs treatment group (NPs) (n=8 per group). The ICH mouse model was generated following the protocol described. After anesthetization by intraperitoneal injection of 2% pentobarbital sodium (40mg/kg), the mice were placed in the prone position in a stereotaxic frame (RWD, China). A burr hole (1 mm) was made using a dental drill (2.0 mm lateral right of the bregma, and 3.5 mm deep below the skull surface). Type IV collagenase (0.1U) dissolved in 0.4 μl saline was injected into the hole at a rate of 0.2 μl/min to induce intracranial hemorrhage in the ICH group and the NPs group. After that, the drill was kept in place for 10 min to prevent reflux. Then the drill was withdrawn at a rate of 1 mm/min. The mice in the Sham

group received an equal amount of normal saline instead of Type IV collagenase.

1.4 Effect of Res-NPs on mouse primary neurons treated with erastin

The primary neurons were prepared from the cerebral cortex of newborn (P0) C57BL/6 mice as described previously with minor modifications. Briefly, the brain tissues were collected and transferred to sterile Petri dishes containing dissection solution (HBSS on ice). The cortical tissues were isolated after careful separation of meninges and blood vessels under a stereomicroscope. Then the cortical tissues were washed by dissection solution 3 times and cut into pieces. After that, the cortical tissues were incubated with 0.125% Trypsin solution (Gibco, USA) containing 20 U/ml DNase I (Sigma-Aldrich, USA) for 20 min in 37 °C. The digestion was terminated by 10% fetal bovine serum (FBS). The cortical tissues were gently triturated with a flame-polished Pasteur pipette to procure a homogeneous cell suspension. After filtering using a 70 μM cell strainer, the cell suspension was centrifuged at 800 r/min for 5 min. A total of 8×10^4 cells/well were seeded into a 48-well plate coated with polylysine. After 2 h, the medium was changed to fresh complete Neurobasal medium consisting of Neurobasal-A medium (GIBCO), supplemented with 2% B27 (Thermo Scientific), 1% penicillin-streptomycin (GIBCO) and 1X glutaMAX (Thermo Scientific). The primary cortical neurons were placed in a 37 °C incubator and culture media were changed freshly every 3 days. On days 5 after culture, the primary culture yielded mixed cells with neuronal, glia and non-neuronal were employed in this study.

The effect of Res-NPs on primary neurons treated with erasin (20 μM) was evaluated by the live and dead cell staining assay test kit. The primary neurons were treated with the predetermined concentrations of Res-NPs (0, 5, 10, 20 μM) for 24 h. Then the supernatant was discarded and the cells were incubated with staining working solution (2 μM calcein AM, 8 μm PI) for 30 min in the dark. Finally, images were acquired with a fluorescence microscope (model DMi8, Leica, Germany).

1.5 Collagenase IV activity colorimetric assay

We assessed the effect of Res-NPs on collagenase IV enzymatic activity according to the kit instructions of a collagenase activity colorimetric assay kit (Sigma, St Louis, MO). In brief, a total of 2 μL different concentration of Res-NPs or collagenase IV inhibitor 1-10P were added respectively with 10 μL of provided collagenase IV (0.35 U/mL) into a test well. Enzyme control was prepared as a parallel well added with 10 μL of collagenase IV. The volume of test drugs and enzyme control wells were adjusted to 100 μL with collagenase assay buffer. They were incubated at room temperature for 10 minutes. For each reaction, 100 μL of reaction mix containing 40 μL of collagenase substrate and 60 μL of collagenase assay buffer was prepared. A total of 100 μL of the reaction mix was added into each well. The absorbance was measured in kinetic mode at 345 nm in a microplate reader at 37 ℃ for 5–15 minutes. Percent inhibition was calculated using the following equation: $\%\text{inhibition}=(\text{Activity}_{(\text{Enzyme})}-\text{Activity}_{(\text{Drug})}) / \text{Activity}_{(\text{Enzyme})} \times 100\%$.

1.6 Assessments of brain Res concentrations after i.v injection

In this study, C57BL/6 mice were randomly divided into two groups (Res and Res-NPs, n=3, i.v., dose of 5 mg/kg Res) to test the drug accumulation in the brain after i.v injection. At different time

points (2 h, 4 h, and 6 h, respectively), the mice were anesthetized, and the brain excised, homogenized, centrifuged and the concentration of Res in these samples was measured by HPLC.

S2. Results and Discussion

2.1 **Res-NPs preparation.** As shown in Fig. S1(A), compared with SDS, PVP K90 and F68, HPMC E50 was the best choice as the stabilizer for Res-NPs preparation and was used in the following study. Then the effect of different concentrations of HPMC E50 on particle short-term stability was detected, and 1.5 mg/ml of HPMC E50 was determined to be the optimal concentration for Res-NPs preparation (Fig. S1B). Furthermore, we detected the effects of different concentrations of Res on particle short-term stability, and 20 mg/ml was the best choice (Fig. S1C). Furthermore, we examined the effects of different concentrations of organic solvent on particle short-term stability, and 30 mg/ml PEG-PLGA was the optimal concentration to prepare Res-NPs (Fig. S1D). As shown in Figure S1E, 1:50 antisolvent-to-solvent volume ratio showed the best short-term stability of Res-NPs. Finally, as shown in Figure S1F, 1000 rpm/min was the optimal stirring speed to prepare Res-NPs.

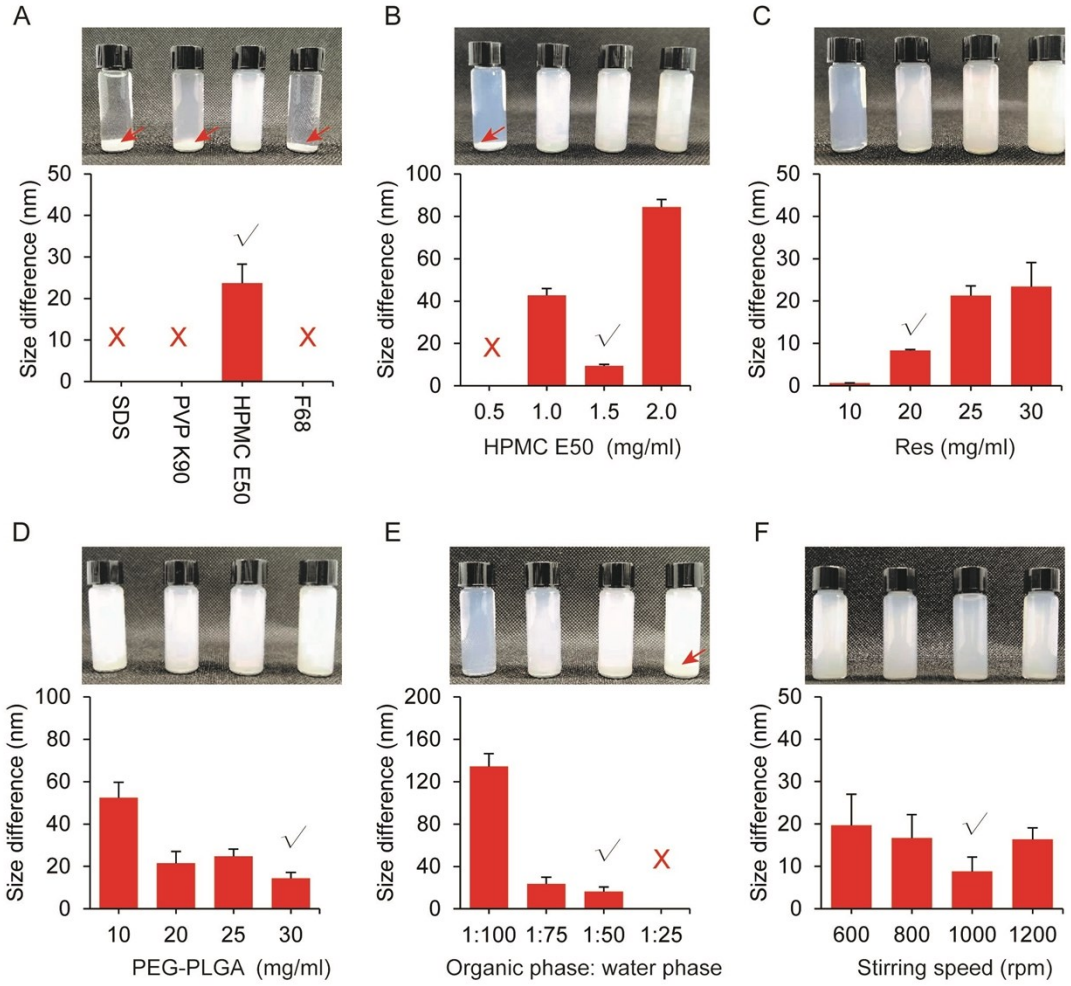

Fig. S1 - Effect of various parameters on the short-term stability of Res-NPs (n=3). (A) (1) SDS, (2) PVP K90, (3) HPMC E50, and (4) F68. (B) HPMC E50 concentrations. (1) 0.5 mg/ml, (2) 1.0 mg/ml, (3) 1.5 mg/ml, and (4) 2 mg/ml. (C) Res concentrations. (1) 10 mg/ml, (2) 20 mg/ml, (3) 25 mg/ml, and (4) 30 mg/ml. (D) Organic phase: water phase. (1) 1:100, (2) 1:75, (3) 1:50, and (4)

1:25. (E) PEG-PLGA concentrations. (1) 10 mg/ml, (2) 20 mg/ml, (3) 25 mg/ml, and (4) 30 mg/ml. (F) Stirring speed. (1) 600 rpm/min, (2) 800 rpm/min, (3) 1000 rpm/min, and (4) 1200 rpm/min. Red arrow indicates the agglomerates.

2.2. Assessment of the *in vitro* dissolution of Res-NPs

The results of Res-NPs *in vitro* dissolution assessment demonstrated that Res-NPs presented biphasic release behavior in HCl pH 1.2 and PBS buffer pH 7.4 conditions. After the initial burst for 4 h, the release rate of Res slowed down and became an almost zero-order release (Fig S2). The initial burst release of Res might be due to the diffusion of Res that was absorbed on the surface of NPs, while the subsequent phase of sustained release might be due to slow release through diffusion and dissolution.

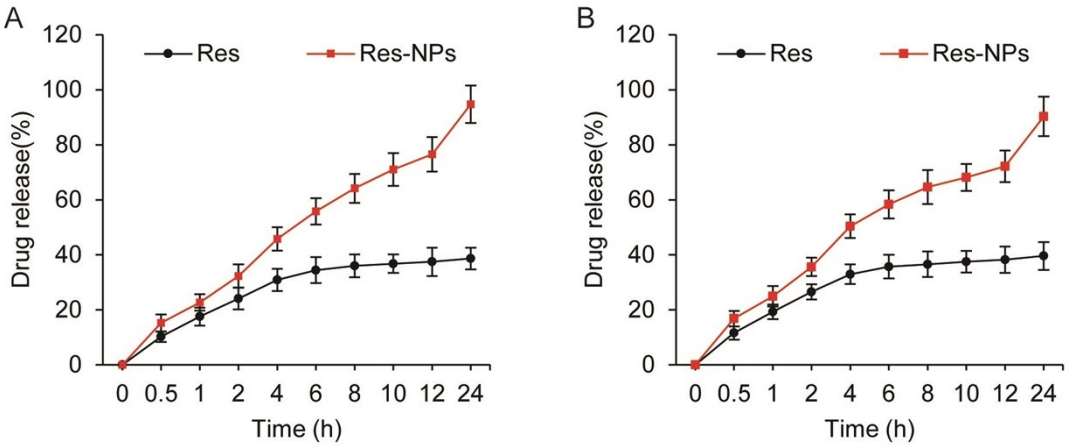

Fig. S2 -In vitro dissolution profiles of Res-NPs. (A) In vitro dissolution profiles of Res-NPs in HCl pH 1.2. (B) In vitro dissolution profiles of Res-NPs in PBS buffer pH 7.4.

2.3. NPs showed no protective effect on ICH

The brain tissue sections showed that compared with the Sham group, the hematoma volume in ICH group and Mix group (NPs) were markedly increased. NPs showed no protective effect on ICH-induced brain injury (Fig S3).

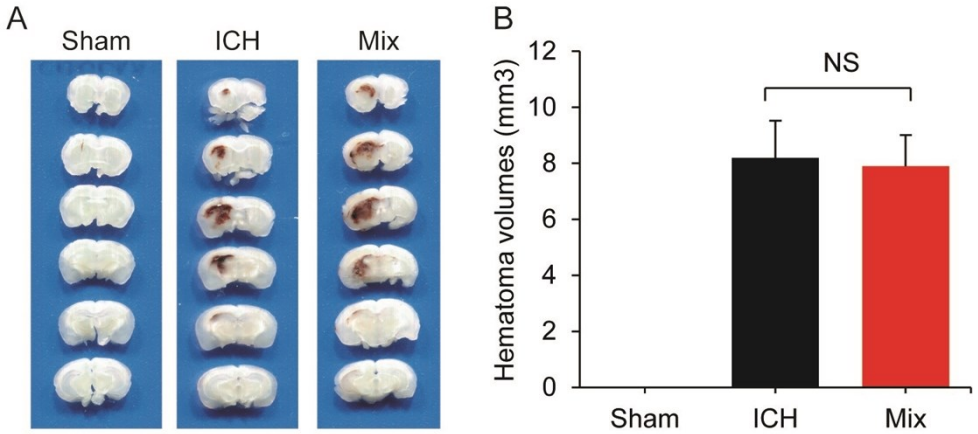

Fig. S3 -Effect of NPs on ICH. (A) Images of hematoma of brain sections in each group. Scale bar: 0.5 cm. (B) The hematoma volumes of ICH mice in each group (means ± SD, n = 8).

2.4. Res-NPs showed protective effect on primary neurons treated with erastin

The results of live/dead cell staining assay showed that compared with the control, erastin obviously inhibited the cell viability of primary neurons. While Res-NPs administration remarkably enhanced the cell viability of primary neurons. These results strongly suggested that Res-NPs effectively protected primary neurons against erastin-induced cytotoxicity.

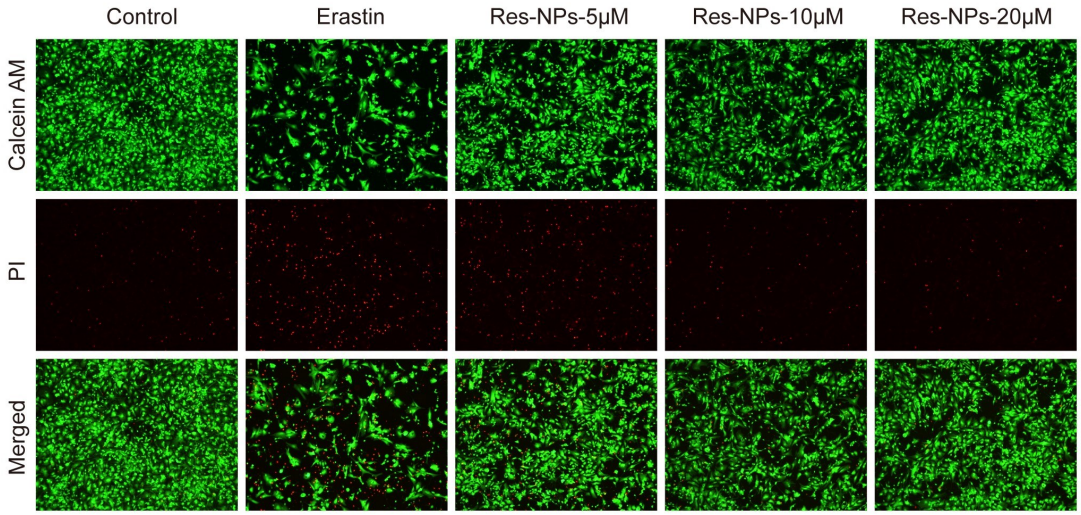

Fig. S4 -Effect of Res-NPs on primary neurons treated with erastin.

2.5 Res-NPs showed no inhibitory effect on the activity of collagenase IV

These results suggested that compared with the collagenase IV inhibitor 1-10P, Res-NPs showed no inhibitory effect on the activity of collagenase IV. Importantly, these findings further confirmed that Res-NPs can protect the brain after an initial hematoma.

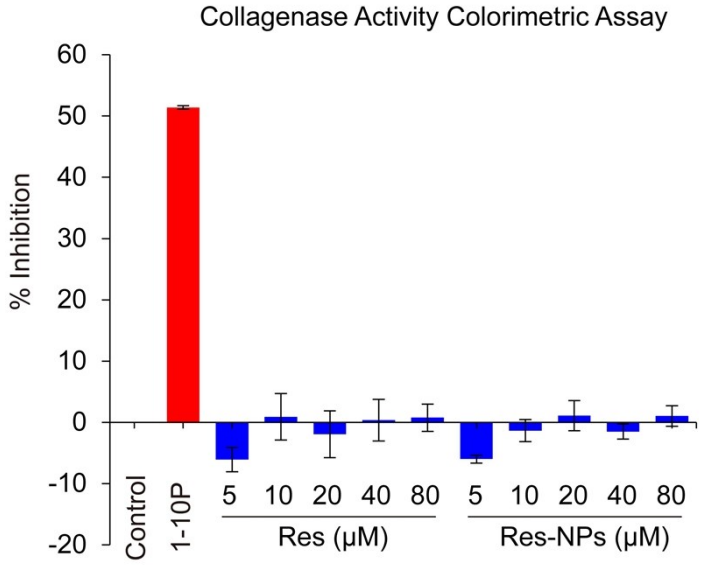

Fig. S5 -Effect of Res-NPs on the activity of collagenase IV.

2.6 Assessments of brain Res concentrations after i.v injection

Compared with Res treatment, a higher concentration of Res was observed in the brains of mice treated with Res-NPs after 2 h, 4 h, and 6 h. These results supported that NPs could deliver a

higher concentration of Res to the brain.

Table S1 Quantification of the concentration of Res accumulated in the brain after treatment with Res and Res-NPs respectively. Data are expressed as means ± SD (n=3).

Time (h)	Res group (μg/g)	Res-NPs group (μg/g)
2	0.13 ± 0.01	0.31 ± 0.04*
4	0.10 ± 0.02	0.44 ± 0.05*
6	0.09 ± 0.01	0.47 ± 0.05**

*$p < 0.05$ and **$p < 0.01$ vs. the Res group.