Supporting information

Simultaneous Antioxidant and Neuroprotective Effects of Two-dimensional (2D) MXene-loaded Isoquercetin for Ischemic Stroke Treatment

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Materials and Methods

1.1 Synthesis of BSA-ISO

BSA powder (120 mg) was dispersed in SDS (60 mg) and DTT (4.4 mg) solution (3 ml) to eliminate hydrophobic forces and intramolecular disulfide bonds, which would result in the release of free thiols. The above BSA containing free thiols was heated to 90 °C for 2 h to obtain the reduced BSA (rBSA). The rBSA was dissolved in MES buffer (0.1 M, pH 4.8) and

fully magnetically stirred (800 rpm, 37 °C) with ISO for 4 h to prepare BSA-ISO. Finally, BSA-ISO was characterized after ultrafiltration

1.2 Synthesis of Ti_2C@BSA-ISO

MXene-COOH (1 mg/mL) was activated by equal amounts of EDC/NHS (EDC:NHS=1:1) and added to the above BSA-ISO stock solution for 12 h to obtain Ti_2C@BSA-ISO. Ti_2C@BSA-ISO was ultrafiltered and stored at 4 ℃.

1.3 Stability test

Ti_2C@BSA-ISO nanoparticles were dispersed in PBS solution and subjected to DLS analysis after standing for 12, 24, 36, and 48 hours to assess their size and stability.

1.4 ISO release behavior analysis

The release behavior of ISO from Ti_2C@BSA-ISO was tested in PBS buffer (pH 7.4) using dialysis bags. The concentration of ISO in the released medium was measured by UV-Vis at 350 nm.

1.5 Cell culture:

Embryos at the embryonic day 16-18 (E16-E18) were used for isolating primary hippocampus neurons. Briefly, the maternal rats were subjected to isoflurane anesthesia, followed by the extraction of embryos. The extracted embryos were then euthanized by excessive anesthesia using isoflurane. Next, after dissection of the cortex in Hank's balanced salt solution and digestion in trypsin (0.125 % w/v), the cells were resuspended in the neurobasal medium (2 % B27 serum-free supplement, 0.5 mM glutamine, 0.5 % v/v penicillin/streptomycin) after centrifugation at 1000 × rpm at 4 ℃ for 5 min. Afterward, the cells at a density of 5×10^4 cells/cm^2 were cultured in poly-l-lysine pre-coated plates at 37℃ in a 5% CO_2 humidified incubator with Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (FBS, Gibco, Gaithersburg, US) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin (Solarbio, Beijing, China)).

1.6 Oxygen-Glucose Deprivation/reoxygenation

The neurobasal medium was removed and substituted with DMEM medium, which was glucose-free or serum-free. The plates were then incubated at 37℃ in an anaerobic chamber containing a mixture of 95% N_2 and 5% CO_2 for 3 h. For reperfusion, cultures were subsequently incubated for 24 h with neurobasal medium in a 95% air and 5% CO_2 oxygen-containing incubator.

1.7 Middle cerebral artery occlusion

The rats were divided into three groups: control, sham, and MCAO (n=6, n=6, and n=36 respectively). The control group received no treatment. The rats in the MCAO groups were subjected to pentobarbital intraperitoneal injection to be anesthetized. Next, the external carotid artery was inserted with the Beijing Cinontech Co. Ltd-provided nylon thread (2636A4/2838A4) to jam the blood flow. After that, the middle cerebral artery was occluded, and the nylon thread was withdrawn after 1.5 h of ischemia to allow reperfusion. The Doppler flowmeter was utilized for measuring the regional cerebral blood flow. MCAO establishment was confirmed when the local cerebral blood flow was declined to 20 pre-ischemia. The rats in the sham group underwent the same treatment as those in the MCAO group but without the step of the occlusion of middle cerebral artery.

All animal procedures were performed in accordance with the guidelines for Care and Use of Laboratory Animals of Hainan Medical University and approved by the Animal Ethics Committee of Hainan Medical University. The assigned approval number is HYLL-2023-383.

1.8 TTC (2,3,5-Triphenyte-trazolium Chloride) Staining

For TTC staining, 2-mm thick coronal sections were prepared from the frozen brains and subsequently immersed for 30 min in 1% TTC solution (Amresco, USA) in normal saline at 37℃. The software ImageJ (NIH Image, Bethesda, MD) was used to analyze the infarct area of each brain slice. The total infarct area was obtained by summing up the area of each slice

from this brain, and the infarct ratio was calculated by dividing the total infarct area by the total brain area.

1.9 Neurological Deficit Tests:

The neurological deficits of rats were determined based on a score. The score was zero for the total normal functions of the rats (the middle artery was not effectively jammed); the score was 1 if the contralateral forepaw was altered, 2 if, during the walking, the rats were circling to the ipsilateral side, 3 when the rats were leaving towards the opposite side, 4 was when the rats were unconscious and unable to walk (severely injured or near death). Thus, rats with scores between one and three were used for subsequent experiments. The rats were evaluated at indicated time points after the MCAO operation.

1.10 Nissl staining:

For Nissl staining, 20 μm thick brain sections from the cortex and the hippocampal CA1 area were taken on poly-L-lysine coated slides, dried, and subsequently put in graded ethanol concentrations for hydration. Next, the sections were stained for 10 min with toluidine blue. After clearing in xylene, the sections were mounted in DPX and then counterstained until transparent background of the sections before being examined under light microscopy (Eclipse Ci, Nikon, Japan) and photographed.

1.11 TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining:

TUNEL staining was used to determine neuronal apoptosis in the brain. First, paraffin brain tissue sections were dewaxed with xylene and gradient alcohol, and TdT and FITC-dUTP were prepared into TUNEL staining solution in a ratio of 1:9. Then, the TUNEL staining solution was applied to brain sections and incubated for 60 minutes at room temperature. NeuN was used to label neurons through immunofluorescence staining.

1.12 Immunofluorescence staining:

The brains of each group were fixed in 4% paraformaldehyde, dehydrated with sucrose, and cut into 10-μm-thick slices. These slices were then double-stained with anti-NLRP3 and anti-

caspase-1 antibodies, washed, and incubated with secondary antibodies. Finally, the nuclei were stained with DAPI and the slides were examined using a confocal laser scanning microscope.

1.13 Western blot analysis:

Western blot procedures used a standard protocol with specific antibodies against NLRP3, ASC, caspase-1, GSDMD, and IL-1β. The blots were visualized using a ChemiDoc™ Touch Imaging System (Bio-Rad, CA, USA). Band intensity was measured with NIH Image J software, and β-actin served as the internal control.

1.14 Statistical analysis

GraphPad Prism Software Inc. version 9 was used for statistical analysis. One-way ANOVA with Tukey's multiple comparison test detected differences among three groups based on one variable (P-value cutoff = 0.05).

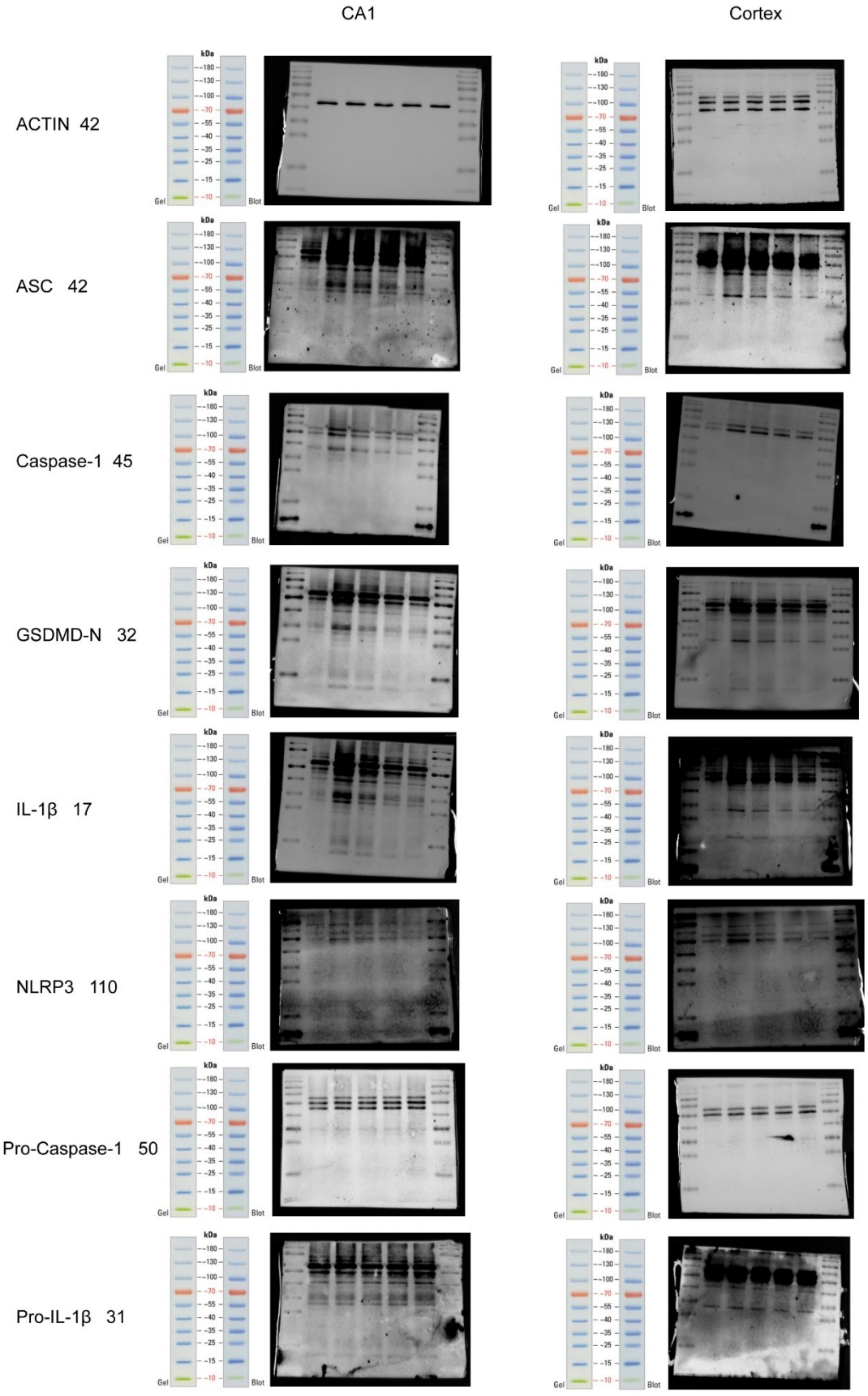

Fig. S1 The raw data of western blot.