SUPPLEMENTARY METHOD 1

Animal model

Male, specific pathogen-free, Sprague Dawley rats were obtained from Bio-Genomics (Seoul, Korea). The rats were 7–8 months old and weighed 250–350 g. The animals were housed in a conventional state under adequately controlled temperature (23°C) and humidity (60%) with a 12-hour light/12-hour dark cycle for 1 week and provided with free access to food and water. The procedures for handling and caring for animals adhered to the guidelines that are in compliance with current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996), and they were approved by the Institutional Animal Care and Use Committee of Kangwon National University. All of the experiments were designed to minimize the number of animals used and the suffering caused by the procedures used in the present study.

Rats were anesthetized with an intraperitoneal injection of 30 mg/kg Zoletil® (Virbac, Carros, France). Additionally, 4 mg/kg gentamicin was subcutaneously injected before skin incision to prevent infection, and 3 mg/kg ketoprofen was administered after the procedures to relieve pain. Perioperatively, routine check-ups of body weight, diet, behavior and neurological status was performed daily for 4 days after procedures and every 2 days after procedures. When an abrupt loss of body weight of more than 20% of the preoperative weight or abnormal behavior was observed, the rat was euthanized.

After shaving and sterilization of the cervical skin area with betadine solution, the right common, external and internal carotid arteries were exposed. The right external carotid artery was tied with a thread to permanently occlude it, and the proximal and distal parts of the right common carotid artery were temporarily clamped with clips. After puncturing the temporarily occluded vessel segment with a 24-gauge intravenous cannula, an intra-arterial infusion catheter was placed in the right common carotid artery. The head of the catheter was plugged with a heparin cap and fixed at the surrounding soft tissues. Then, the catheter was irrigated with heparinized saline to remove the air and blood clots via the heparin cap, and the temporary clips were finally removed. When intra-arterial drug injection via the intra-arterial catheter was completed, the catheter was removed, and the puncture site was sutured with 10-0 nylon to control bleeding (Fig. 1).

Blood-brain barrier (BBB) opening

To model the condition of a chronically ischemic brain with a partially disrupted BBB, temporary BBB opening was attempted via intra-arterial infusion of mannitol. The rat model was designed to determine the optimal time interval between mannitol administration and drug infusion, in which temporary BBB opening was maximized and the drug was able to pass through the BBB into the brain parenchyma.

A 20% mannitol solution (CJ Health Care, Daeso, Korea) was administered at a rate of 0.25 mL/kg/sec for 30 seconds with an infusion pump via the intra-arterial catheter. Subsequently, at predetermined time intervals of 0, 5, 10, 15 and 20 minutes after mannitol administration, a 2% solution of Evans Blue (EB; Sigma, St. Louis, MO, USA) in normal saline (4 mL/kg of body weight) was injected via the intra-arterial catheter. Twenty-four hours after EB infusion, the rats were transcardially perfused with 0.1 M phosphate-buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4)[3]. Then, the whole brain was harvested, and the degree of EB extravasation was evaluated in the right hemisphere. A total of 25 rats (five rats per predetermined interval) were used. The degrees of EB extravasation were scored as follows : grade 0, no EB extravasation; grade 1, weak extravasation; grade 2, moderate extravasation; and grade 3, strong extravasation (Fig. 2).

Drug administration

A 1-mL stock solution of 5 mg/mL paclitaxel (Sigma) was made by mixing 5 mg paclitaxel powder with 1 mL 25 mg/mL dimethyl sulfoxide (Sigma). Then, 2-mL substocks of 0.05 μg/μL paclitaxel were finally made by mixing 2 μL of the paclitaxel stock solution with 2 mL of normal saline. Similarly, a 200-μL stock solution of 5 mg/mL rapamycin (Sigma) was made by dissolving 1 mg rapamycin powder with 200 μL of 25 mg/mL DMSO. Then, 2-mL substocks of 0.05 μg/μL rapamycin were finally prepared by mixing 20 μL of the rapamycin stock solution with 2 mL of normal saline.

The doses of the drugs (paclitaxel and rapamycin) were translated from humans into animal equivalent doses using a formula based on body surface area : animal equivalent dose (mg/kg) = human dose (mg/kg) × human K_m / animal K_m[4]. The 60 kg-weighted human and 250 g-weighted rat K_ms were 37 and 6, respectively. For example, 250 g-weighted rat equivalent doses of 600, 1200 and 2400 μg doses in a 60 kg-weighted

human are 15.4, 30.8, and 61.7, respectively. As a dose of 600 μg is the highest dose contained on the surface of commercial DEBs, comparative doses were determined as previous doses[5]. Each rat equivalent dose was calculated using the formula based on the body weight of each rat and injected.

Paclitaxel and rapamycin were injected together with EB via the intra-arterial catheter in the optimal time interval after mannitol administration. Calculated animal doses of drugs equivalent to human doses of 600, 1200, and 2400 μg were infused as an intra-arterial bolus, and then, brain tissues were obtained 24 hours and 14 days after drug infusion. A total of 70 rats were needed; the rats were divided into six groups per drug (five rats per group), including two sham groups (five rats per group) that were examined 24 hours and 14 days after drug-free solution infusion following mannitol administration.

Neurological and histological examination

Neurological status was evaluated using Bederson et al.[1]'s grading system as follows : grade 0, no observable deficit; grade 1, forelimb flexion; grade 2, decreased resistance to lateral push without circling; and grade 3, same behavior as grade 2 with circling.

For the histological examination, rats were anesthetized with sodium pentobarbital (30 mg/kg, i.p.) and transcardially perfused with 0.1 M PBS (pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and postfixed in the same fixative for 6 hours. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter, frozen tissues were serially sectioned on a cryostat (Leica, Wetzlar, Germany) into 30-μm coronal sections, and they were then collected into six-well plates containing PBS.

To confirm neuronal death, cresyl violet staining (Sigma) was performed. The sections were mounted on gelatin-coated microscope slides. Cresyl violet acetate (Sigma) was dissolved at 1.0% (w/v) in distilled water, and glacial acetic acid was added to this solution. Before and after staining for 2 minutes at room temperature, the sections were washed twice in distilled water. After dehydration, the sections were mounted with Canada balsam (Kanto, Tokyo, Japan).

Fluoro-Jade C (FJ C; Histochem, Jefferson, AR, USA) histofluorescence staining was conducted to localize neuronal degeneration[2]. In brief, the sections were first immersed in a solution containing 1% sodium hydroxide in 80% alcohol, followed by 70% alcohol. The sections were then transferred to a 0.06% potassium permanganate solution and then transferred to a 0.0004% FJ C staining solution. After washing, the sections were placed on a slide warmer (approximately 50°C) and then examined using an epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) with blue (450–490 nm) excitation light and a barrier filter. With this method, neurons undergoing degeneration brightly fluoresce compared to the background[6].

To obtain accurate immunohistochemistry data, the sections were processed at 24 hours and 14 days after drug infusion by immunohistochemistry under the same conditions. The sections were sequentially treated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 30 minutes and 10% normal goat serum in 0.01 M PBS for 30 minutes. The sections were next incubated with diluted rabbit anti-glial fibrillary acidic protein (GFAP) (diluted 1 : 1000; Biogenesis, San Ramon, CA, USA) for astrocytes and rabbit anti-ionized calcium-binding adapter molecule 1 (Iba-1) (diluted 1 : 500; Wako, Osaka, Japan) for microglia overnight at 4°C and subsequently exposed to biotinylated goat anti-rabbit IgG and streptavidin peroxidase complex (diluted 1 : 200; Vector, Burlingame, CA, USA). The sections were visualized by staining with 3,3'-diaminobenzidine in 0.1 M Tris-HCl buffer (pH 7.2) and mounted on gelatin-coated slides. After dehydration, the sections were mounted with Canada balsam (Kanto).

The degrees of neuronal damage were scored based on the cresyl violet and FJ C histofluorescence staining data as follows : grade 0, no neuronal damage; grade 1, mild damage; grade 2, moderate damage; and grade 3, severe damage. The degrees of inflammation were scored based on the GFAP and Iba-1 immunohistochemistry data similar to the degrees of neural damage.

Supplementary References

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