Improved Therapeutic Benefits by Combining Physical Cooling With Pharmacological Hypothermia After Severe Stroke in Rats

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Background and Purpose—Therapeutic hypothermia is a promising strategy for treatment of acute stroke. Clinical translation of therapeutic hypothermia, however, has been hindered because of the lack of efficiency and adverse effects. We sought to enhance the clinical potential of therapeutic hypothermia by combining physical cooling (PC) with pharmacologically induced hypothermia after ischemic stroke.

Methods—Wistar rats were subjected to 90-minute middle cerebral artery occlusion by insertion of an intraluminal filament. Mild-to-moderate hypothermia was induced 120 minutes after the onset of stroke by PC alone, a neurotensin receptor 1 (NTR1) agonist HPI-201 (formally ABS-201) alone or the combination of both. The outcomes of stroke were evaluated at 3 and 21 days after stroke.

Results—PC or HPI-201 each showed hypothermic effect and neuroprotection in stroke rats. The combination of PC and HPI-201 exhibited synergistic effects in cooling process, reduced infarct formation, cell death, and blood-brain barrier damages and improved functional recovery after stroke. Importantly, coapplied HPI-201 completely inhibited PC-associated shivering and tachycardia.

Conclusions—The centrally acting hypothermic drug HPI-201 greatly enhanced the efficiency and efficacy of conventional PC; this combined cooling therapy may facilitate clinical translation of hypothermic treatment for stroke. (Stroke. 2016;47:1907-1913. DOI: 10.1161/STROKEAHA.116.013061.)

Key Words: cell death • hypothermia • middle cerebral artery • neurotensin receptor 1 • shivering • stroke

Stroke remains a major brain disorder with few effective treatments.\(^1\) New approaches are urgently needed to develop additional and better treatments for patients with acute stroke. Therapeutic hypothermia has shown promising neuroprotective effects against strokes in preclinical and clinical studies.\(^2-6\) Various clinical trials have been completed with mostly encouraging results.\(^7\)\(^-\)\(^8\) Currently, physical cooling (PC) methods are used in hypothermia therapy. It is generally recognized that forced cooling of physical means is inefficient and cumbersome.\(^9\)\(^-\)\(^12\) The common surface cooling using external blankets is slow for humans (3.5–6.5 hours) and requires general anesthesia to counteract vasoconstriction and strong shivering reactions. In addition, precise control of core temperature is difficult.\(^9\) More recent methods using intravenous heat exchange or infusion may provide better and faster control of core temperature.\(^10\)\(^,\)\(^11\) Other (mostly proprietary) techniques currently being explored include head cooling caps or helmet devices and intra-arterial cooling methods.\(^12\) These interventions again require specialized cooling technologies/devices that are not widely or readily available to patients with acute stroke.

Cooling-associated compensatory reactions such as shivering have to be suppressed and regulated for efficient hypothermia-based stroke treatment.\(^13\) General anesthesia is normally required, but it can increase the risk of lung infection and has negative consequences on recovery.\(^14\) The preparation of general anesthesia is time consuming, so it delays the acute neuroprotective treatments. In addition to general anesthesia, sedating narcotics and muscle paralytics have been tried to counteract shivering.\(^15\) However, these drugs often produce..."
serious respiratory depression. These drawbacks can significantly disrupt the remedy of hypothermia treatment and reduce the efficacy for patients with acute stroke.

In the quest for better hypothermia therapies, alternative methods such as pharmacologically induced hypothermia have been evaluated. Pharmacological methods that quickly reduce brain/body temperature with well-controlled cooling and rewarming rates should significantly improve the efficacy and feasibility of hypothermia treatments. Antipyretic drugs such as aspirin and Tylenol have been tested for pharmacologically induced hypothermia; however, these drugs are inefficient at inducing hypothermia. Even in combination with forced-air surface cooling, antipyretic drugs elicit only a small (≈1°C) decrease in core body temperature. Adenosine nucleotides also induce hypothermia in rodents, and the neuroprotective effect of ATP against ischemic stroke has been tested. Although ATP induced dose-dependent hypothermic effects, the infarct volume grew even larger in ATP-treated rats, accompanied with other detrimental side effects such as seizure, hemorrhagic transformation, and higher mortality. The negative effects were attributed to increased blood glucose, severe acidosis, and hypocalcaemia induced by ATP. More recently, the concept of regulated hypothermia involving reduction of the set point in the thermoregulatory center of the brain was proposed as a more efficient and safer approach than systemic cooling. Severe acidosis, and hypocalcaemia induced by ATP. More recently, the concept of regulated hypothermia involving reduction of the set point in the thermoregulatory center of the brain was proposed as a more efficient and safer approach than systemic cooling. Severe acidosis, and hypocalcaemia induced by ATP. More recently, the concept of regulated hypothermia involving reduction of the set point in the thermoregulatory center of the brain was proposed as a more efficient and safer approach than systemic cooling. Severe acidosis, and hypocalcaemia induced by ATP.

We have generated novel NTR1 agonists, such as HPI-201 and HPI-363 (formally known as ABS-201 and ABS-363) that induce mild-to-moderate hypothermia in a dose-dependent manner. These compounds centrally act on the hypothalamus and effectively induce regulated hypothermia only minutes after administration without triggering shivering. These NTR1 compounds showed marked neuroprotective effects against brain damage and resulted in improved functional recovery after ischemic stroke, hemorrhage stroke, and traumatic brain injury.

In the present investigation, we explored the possibility that the PC methods in clinical practices could benefit by being combined with a centrally acting hypothermic drug such as HPI-201 to suppress the antagonistic shivering reaction associated with PC. This could enable quicker initiation and better control of the hypothermic effect, resulting in enhanced therapeutic benefits without sedation of the patient.

**Methods**

**Chemicals and Ischemic Stroke Model of Rats**

HPI-201 was synthesized using procedures described previously. The drug solution was prepared in saline for intraperitoneal injection. Wistar rats (8 weeks, 270–290 g) were housed in standard cages in a 12-hour light/12-hour dark cycle and given food and water ad libitum. Rats were randomly divided into 4 groups (n=7–9/group): (1) stroke-control group, (2) stroke plus HPI-201 group, (3) stroke plus PC group, and (4) stroke plus PC and HPI-201 group. We calculated the sample sizes to be at least 6 animals/group to reject the null hypothesis (H0) at P<0.05 with a power of 0.80. Animals were anesthetized with 2% isoflurane in 70% N2 and 30% O2, and maintained at 1.5% isoflurane anesthesia using a facemask. Occlusion of the right side middle cerebral artery (MCA) was achieved by advancing a 4-0 surgical nylon monofilament (3.0 cm length) with an expanded (heated) tip from external carotid artery into the lumen of the internal carotid artery to block the origin of the MCA. Reperefusion was allowed by withdrawal of the suture after 90-minute MCA occlusion. During 90-minute MCA occlusion, body temperature was monitored and maintained at 37±0.5°C in a humidity and temperature controlled incubator (Thermocare, Incline Village, NV). The animal protocols were approved by the Emory University Institutional Animal Care and Use Committee (IACUC), in compliance with National Institutes of Health (NIH) guidelines.

**Hypothermia Induction**

PC was achieved by placing animals in a rat holder covered by ice for the first 10 to 15 minutes and then in 24°C chamber during the maintenance period. PC or HPI-201 was administrated 120 minutes after the onset of ischemia. On the basis of our previous experiments, the first bolus injection of HPI-201 (10 mg/kg, IP) was followed by additional injections at half of the initial dose (5 mg/kg) to maintain hypothermia for 6 or 10 hours. The intervals between the injections were ≥1.5 hours, adjusted according to body temperature changes. Rectal temperature was monitored using a rectal probe (Harvard Apparatus, Holliston, MA). The correlation of body and brain temperature had been demonstrated in our previous reports.

**Brain Infarction and Cell Death Measurements**

On sacrificing animals, the brains were removed immediately, and brain tissue was cut into 6 serial 2-mm thick coronal sections. The sliced tissues were immersed in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO) in PBS at 37°C for 10 minutes. Infarct volume ratio was calculated using the indirect method. A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit (Promega Corporation, Madison, WI) was used to examine cell death by detecting fragmented DNA in 10-µm thick coronal fresh frozen sections as described previously. All data analysis for immunofluorescent measurements and behavior tests were performed in a double-blind manner by an investigator who was given no information about the experimental groups.

**Quantification of Immunostaining Positive Cells**

Cell count was performed as described previously. Systematic random sampling was used to ensure accurate and nonredundant cell counting. Six brain sections per animal were collected at 90-µm distance between sections for nonoverlapping multistage random sampling. Six fields were chosen in each section in the penumbra region and viewed at 20× for cell counting. ImageJ (NIH, Bethesda, MD) was used to analyze each picture.

**BBB Leakage Measurement**

Two percent Evans blue (EB) dye (Sigma) solution was administered intravenously at a dosage of 1 ml per rat 6 hours before euthanization to visualize the blood-brain barrier (BBB) leakage. Coronal sections were used to examine the EB extravasation by photographing under the tetramethylrhodamine excitation wavelength (red) at 1.25× for fluorescent microscopy (BX51, Olympus, Japan). After photographing, brain tissues were incubated overnight in formamide (5 ml/g dry weight), and the amount of EB released into the formamide was quantified by florescence spectrophotometry.

**Functional Assessments**

The adhesive removal test measured sensorimotor function as previously described. A small adhesive dot was placed on each forepaw, respectively, and the amount of time in seconds needed to contact and remove the tape from each forepaw was recorded. Before surgery, rats that showed no response or prolonged time (≥20 s) to remove
the dot during the training period were excluded from future tests. The experimental recording in stroke rats stopped at 6 minutes. The cylinder test measured the use of limbs in body support.29 At 21 days after stroke, the rats were placed in a glass cylinder (9.5-cm diameter and 11-cm height), and the number of times each forelimb or both forelimbs used to support the body on the wall of the cylinder was counted for 5 minutes (see details in the online-only Data Supplement). The corner test evaluated the integrity of the whisker barrel neuronal activity.23 Two cardboard plates (30×20×0.3 cm) were attached at a 30° angle in a home cage. The number of right and left turns was counted. In cylinder and corner tests, prestroke rats that showed biased behaviors/turns were excluded from future tests.

Shivering and Heart Rate Measurements

The HomeCageScan System (Clever Sys Inc, Reston, VA) was used to monitor shivering activities as previously described.21 Animals were video recorded from 30 minutes after hypothermia induction. Subtle twitch–like behavior was detected by the monitoring camera and analyzed by the HomeCage Software 3.0 (see details in the online-only Data Supplement). A SurgiVet pulse oximeter (Smiths Medical PM, Inc, Norwell, MA) was placed on the rear paw to monitor heart rate at 1 hour after starting cooling and 2 hours before finishing cooling.

Statistical Analysis

GraphPad Prism 6 (GraphPad Software, San Diego, CA) was used for statistical analysis and graphic presentation. One-way ANOVA followed by Bonferroni correction was used for multiple-group comparisons. Two-way ANOVA followed by Bonferroni correction was used for repeated measurements. Significant differences between the groups were identified by a P value of <0.05. All data are presented as mean±SEM.

Results

Hypothermia Induction by PC and Hypothermic Compound

Adult rats (male, 250–280 g) were subjected to 90-minute MCA occlusion induced by insertion of a silicone-coated monofilament via the external carotid artery.27 Previously, we demonstrated that HPI-201 reduced body and brain temperature of mice in a dose-dependent manner.23 To select an optimized dose of HPI-201 in rats, we tested 4 dosages of intraperitoneally delivered HPI-201 (1, 10, 25, or 50 mg/kg) at 2 hours after the onset of MCA occlusion in rats (Figure 1A). On the basis of the efficacy and time courses of the HPI-201–induced hypothermic effect, we selected a dosage of 10 mg/kg to induce a mild hypothermia in stroke rats.

Two hours after the onset of ischemia, ice-induced PC, the NTR1 agonist HPI-201, and the combination of PC plus HPI-201 (PC/HPI-201) were administered, respectively. As expected after a severe ischemic stroke, hyperthermia (38–39°C) or poststroke pyrexia occurred in stroke rats (Figure 1B). Administration of PC or HPI-201 (10 mg/kg, IP)
Prevented pyrexia and decreased the body temperature to 30°C within 60 minutes or 35°C within 30 minutes, respectively (Figure 1B). In the stroke rats that received the combined PC/HPI-201 treatment, the body temperature was reduced to 32°C in ≈30 minutes, which constituted a 30-minute acceleration of the cooling process by PC alone (Figure 1B). To maintain the cooling effect, animals in the PC group were kept in a cooling chamber, whereas rats subjected to pharmacologically induced hypothermia received additional injections of HPI-201 at half the initial dosage (5 mg/kg) with intervals of 1.5 to 2 hours (Figure 1B). The body temperature was maintained at 32°C to 33°C >6 hours, which, in previous studies, resulted in significant neuroprotection against ischemic damage.23

We observed that PC caused larger variations in temperature control, accompanied by visible shivering of the animals (Figures 1B and 2A). On termination of PC treatment and after the last injection of HPI-201, the rewarming rate was faster in PC-treated rats (≈0.07°C/min) than that in HPI-201–treated rats (≈0.04°C/min; Figure 1). The combination of PC and HPI-201 helped to slow the rewarming pace (Figure 1B).

HPI-201 Prevented PC-Induced Shivering and Tachycardia Responses

Shivering is a defensive reaction to forced cooling that interferes with the cooling process and severely reduces the efficacy of PC methods.30 To compare shivering responses, a HomeCage Monitoring system was used to detect shivering behavior. PC triggered extensive shivering in stroke rats, whereas HPI-201–treated rats showed no shivering behavior (Figure 2A). Combination of PC and HPI-201 fully inhibited the PC-induced shiver reaction (Figure 2A).

The heart rate was measured using a pulse oximetry at 1 hour after the cooling onset and 2 hours before stopping cooling. Heart rate increased from the control of 300 to ≈600 bpm in PC-treated rats. However, HPI-201 caused only a minor increase in heart beat rate at early time points, whereas the PC/HPI-201 combination kept the heart rate at control levels (Figure 2B and 2C).

Figure 2. HPI-201 prevented physical cooling (PC)–induced shivering and tachycardia responses. A, Using the HomeCage monitoring system, the time that animals spent shivering was recorded in stroke control, PC, HPI-201, and PC/HPI-201 combined groups for 30 minutes. Excessive shivering responses were seen in PC-treated animals 30 to 60 minutes after the onset of cooling, whereas shivering was not detected in rats that received the combination of PC plus HPI-201. *P<0.05 vs stroke controls, #P<0.05 vs PC alone; n=7 to 8 per group. B and C, Heart rates were measured to examine the tachycardia response at 1 and 4 hours(s) after the onset of cooling using pulse oximetry. The PC group exhibited significant increases in heart rates, whereas HPI-201 did not show any increase. The increased heart rate by PC was prevented by coapplied HPI-201. *P<0.05 vs stroke controls, #P<0.05 vs PC alone; n=7 to 8 per group.

Figure 3. Neuroprotective effects of hypothermic treatments after severe stroke in rats. A, 2,3,5-Triphenyltetrazolium chloride (TTC) staining of brain sections from physical cooling (PC), HPI-201, and PC/HPI-201 groups, respectively, 72 hours after 90-minute middle cerebral artery occlusion. B, Quantified data revealed significant reductions in the infarct volume of hypothermia treatment groups. The combined PC and HPI-201 treatment further reduced the infarction. *P<0.05 vs stroke controls, #P<0.05 vs PC or HPI-201 alone; n=6 to 7 per group. C, Immunofluorescent images of TUNEL-positive cells (green) and total cells (Hoechst 33342, blue). Scale bars, 50 µm. D, All hypothermic treatments significantly reduced the number of dead cells. The PC and HPI-201 combined treatment provided additional protection against cell death. *P<0.05 vs stroke controls, #P<0.05 vs PC or HPI-201 alone; n=7 to 8 per group.
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Neuroprotective Effects of Different Hypothermia Treatments

At 3 days after stroke, a large infarct formed in saline-treated rats (Figure 3A). Using TTC staining, this was quantified as 23% brain damage in the ipsilateral hemisphere. PC and HPI-201 treatments each significantly reduced the infarct volume (Figure 3A and 3B). The combination of PC plus HPI-201 showed even greater protective effect of reducing the infarction (Figure 3A and 3B). To see if longer and more pronounced hypothermia could provide more protection, we also tested a group of animals with 10 hours hypothermia; there was no significant difference in the neuroprotective effects between 6 and 10 hours treatments of HPI-201 (Figure 3B).

TUNEL staining was performed to assess cell death in the penumbra region. TUNEL-positive cells were significantly reduced by PC or HPI-201 alone, whereas PC/HPI-201 treatment showed additional reduction in cell death (Figure 3C and 3D).

Protections of Hypothermia Treatments on BBB

BBB integrity was assessed using the EB leakage assay. PC, HPI-201, and PC/HPI-201 combination all significantly reduced the EB leakage at 3 days after stroke. Obvious synergistic effects were observed in the PC/HPI-201 group that showed a significantly stronger effect on BBB protection versus either PC or HPI-201 administered individually (Figure 4).

Functional Recovery After PC, HPI-201, and PC/HPI-201 Treatments in Stroke Rats

To compare the functional outcomes after stroke in PC, HPI-201, and combined treatment groups, behavior tests were performed 3 to 21 days after stroke. In adhesive dot removal test, stroke control animals showed prolonged times to contact and remove the sticky dot from the affected forelimb because of impaired neuronal function in the sensorimotor cortex (Figure 5A and 5B). The rats that received PC or HPI-201 treatment showed significantly faster responses in removing the sticky dot, whereas the combined PC/HPI-201 treatment further improved the functional recovery (Figure 5A and 5B).

In cylinder and corner tests, stroke damage resulted in a noticeable bias in forelimb uses (Figure 5B and 5C). PC and HPI-201 significantly improved the use of impaired limb from 3 to 21 days after stroke, whereas PC/HPI-201 cotreatment showed the strongest improvement in the test (Figure 5C). In the corner test, the stroke group exhibited obvious bias in the turn direction (Figure 5D). All hypothermic treatments showed significant corrections in the turn behavior. The combined PC/HPI-201 treatment again showed the strongest effect in correcting the turn behavior ≤21 days after stroke (Figure 5D).

Figure 4. Protective effects of hypothermic treatments against blood-brain barrier (BBB) damage after stroke The BBB integrity was evaluated as the amount of Evans blue (EB) leakage at 3 days after stroke. A, Representative images of EB fluorescence in the brain from stroke and stroke plus physical cooling (PC)/HPI-201 rats. B and C, Quantified analysis of EB leakage in different groups. All hypothermic treatments significantly reduced the EB leakage, whereas the best protection was seen with the combination treatment of PC plus HPI-201. *P<0.05 vs stroke controls, #P<0.05 vs PC or HPI-201 alone, n=7 to 8 per group.

Figure 5. Functional recovery after several ischemic stroke in rats. Sensorimotor and locomotor functions were tested 3 days after stroke. A and B, The adhesive removal test revealed a marked dysfunction of the ipsilateral hemisphere, showing as prolonged response and removal times in stroke animals. All hypothermic groups improved the functional deficits; however, the combination treatment of physical cooling (PC) plus HPI-201 provided additional recovery. *P<0.05 vs stroke controls, #P<0.05 vs PC or HPI-201 alone; n=7 to 9 per group. C, Cylinder test showed impaired forelimb usages ≤21 days after stroke. Hypothermia improved the activity, but the combination treatment showed the strongest effect. *P<0.05 vs stroke controls, #P<0.05 vs PC or HPI-201 alone; n=7 to 9 per group. D, Biased turning behavior was seen in stroke animals. Rats receiving PC or HPI201 hypothermic treatment showed improved turning score, whereas the PC and HPI-201 combined treatment showed additional improvement in the turning test. *P<0.05 vs stroke controls, #P<0.05 vs PC or HPI-201 alone; n=7 to 9 per group.
Discussion

In this study, we compared the efficacy and efficiency of PC and the hypothermic NTR1 agonist HPI-201 in a severe ischemic stroke rat model. Both PC using ice and pharmacological cooling using HPI-201 were able to reduce the body temperature of stroke rats. This effect is clinically meaningful because pyrexia occurs after severe strokes, and the extent of hyperthermia directly correlates with the outcomes and mortality of patients with stroke.31,32 More importantly, we demonstrate that HPI-201 in combination with PC leads to multiple benefits, including faster cooling, no shivering induction, a slower rewarming phase, enhanced neuroprotective effects, and improved functional recovery. These data support that the strategy of combining pharmacological hypothermia with PC should increase the clinical feasibility, efficacy, and safety in the treatment of patients with stroke.

HPI-201 did not trigger the defensive response of shivering, which made the induction and maintenance of cooling more effective and controllable. We further examined the effects of combined treatments of PC plus HPI-201 on the induction of hypothermia, brain protection, and functional outcomes. Impressively, adding HPI-201 during PC effectively prevented the adverse shivering and tachycardia responses. Of clinical importance, the elimination of shivering could allow PC to be carried out without general anesthesia. We also demonstrated significant additional effects of the combined treatment in neuroprotection and functional recovery. All these advantages provide promising benefits in clinical applications of hypothermia therapy.

Previous data from human and animal studies have shown that cooling can cause both tachycardia and bradycardia.33–35 Generally, deep hypothermia (<30°C) leads to further depression of the metabolic rate and bradycardia.33–35 In this study, the PC-induced cooling (30–35°C) caused tachycardia response, but not bradycardia, likely because of the relatively moderate level of cooling. Although it is unclear what kind of influences tachycardia may have in hypothermia, cooling-induced tachycardia can be deleterious because of the high risk of cardiac arrhythmias.36 HPI-201 showed a significant effect of preventing tachycardia. Although further study are needed to reveal possible direct and indirect mechanisms related to the HPI-201 regulation of heart function, it seems that the prevention of tachycardia by HPI-201 is an additional benefit for clinical application of the combined hypothermia therapy.

The NTR1 agonist HPI-201 acts on the receptors located in the hypothalamus and causes a downward shift of the temperature set point in the thermoregulatory center.25,26,36 On the basis of the understanding of the cooling mechanisms, we hypothesized that coapplication of an NTR1 agonist to target the central thermoregulatory system could have synergistic effects of temperature reduction and associated neuroprotective benefits. Previously, we demonstrated hypothermic and neuroprotective effects of HPI-201 in a focal ischemic stroke mouse model with restricted damage to the sensorimotor cortex.25 In this study, a severe ischemic stroke rat model was selected to vigorously test the efficacy of the pharmacologically induced hypothermia treatment. The severe stroke not only damages the ipsilateral cortex but also forms infarction in the subcortical structures, such as hypothalamus, hippocampus, and basal ganglia. It initially was a concern whether NRT1 agonists could show cooling effects when the thermoregulatory center in one side of the brain was destroyed by severe ischemia. The experimental result suggests that HPI-201 administration still causes hypothermia, although the cooling efficacy is relatively weaker compared with its effects in normal and small stroke animals. This dilemma, however, should be relatively less significant when the drug is used as a supplemental treatment with PC.

Conclusions

Hypothermia therapy using physical means has been widely used in patients, and currently it is under evaluation in clinical trials for the treatment of several central nervous system and cardiovascular disorders, including stroke and traumatic brain injury. This study demonstrates for the first time the beneficial effect of a combination therapy using PC and a hypothermia-inducing compound for the treatment of stroke. Combination of HPI-201 and PC improves the cooling effect while preventing cellular and brain damage, and improving functional recovery with reduced side effects after stroke in rats. The benefits of HPI-201 shown with PC could be a promising approach for more effective and safer hypothermia therapies for patients with brain injury.

Sources of Funding

This investigation was supported by American Heart Association (AHA) Grants 12GRNT12060222 (Dr Yu), 14POST20130024 (Dr Won), 15POST25680013 (Dr Lee), 15POST25710112 (Dr Wei), National Institutes of Health (NIH) grants NS090529 (Dr Dix/Dr Yu), NS062097 (Dr Wei), NS075338 (Dr Wei), NS085568 (Dr Wei/Dr Yu), and a Veteran Affair (VA) National Merit Grant RX000666 (Dr Yu).

Disclosures

Dr Dix is a cofounder and chief scientific officer of JT Pharmaceuticals. The other authors report no conflicts.

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Stroke. 2016;47:1907-1913; originally published online June 14, 2016; doi: 10.1161/STROKEAHA.116.013061

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SUPPLEMENTAL MATERIAL

TTC staining of infarct volume measurement

To measure the infarction, the rats were sacrificed at 3 days after the induction of MCAo. The brains were removed immediately and brain tissue was cut into six serial 2-mm coronal sections. Sliced brain tissues were immersed in a 2% solution 2,3,5-triphenyltetrazolium chloride (Sigma) in PBS at 37°C for 10 minutes, and then stored in 10% buffered formalin for 24 hrs. Digital images of the caudal aspect of each slice were obtained by a flatbed scanner. Infarct, ipsilateral hemisphere, and contralateral hemisphere areas were measured using ImageJ software (NIH, Bethesda, MD). Infarct volume ratio was calculated using the indirect method.19

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

A TUNEL assay kit (Promega Corporation, Madison, WI, USA) was used to examine cell death by detecting fragmented DNA in 10-µm-thick coronal fresh frozen sections as described previously.11 After fixation and permeabilization, brain sections were incubated in the equilibration buffer for 10 min. Recombinant terminal deoxynucleotidyl transferase (rTdT) and nucleotide mixture were then added on the slide at 37°C for 60 min in the dark. Reactions were terminated by 2X SSC solution for 15 min. Nuclei were counterstained with Hoechst 33342 (1:20,000; Molecular Probes, Eugene, OR, USA) for 5 min.

Quantification of immunostaining positive cells

Cell count was performed as described previously.11 Systematic random sampling was employed to ensure accurate and non-redundant cell counting. Six brain sections per animal were collected at >90 µm distance between sections for non-overlapping multistage random sampling. Six fields were chosen in each section in the pericontusion region and viewed at 20x for cell counting. ImageJ was used to analyze each picture. All analysis was performed in a blinded fashion.

BBB leakage measurement

BBB permeability was investigated by monitoring extravasation of Evans blue (EB) dye as previously described.11 The red auto-fluorescence of EB dye-albumin conjugate is evident in tissue sections examined by fluorescence microscope. Sterilized 2% EB dye (Sigma) solution was administered intravenously (i.v.) at a dosage of 1 ml per rat 6 hrs before sacrifice to visualize the BBB leakage. Coronal sections were used to examine the EB extravasation by photographing under the TRITC (red) excitation wavelength at 1.25x for fluorescent microscopy (BX51, Olympus, Japan). Photoshop Professional was used to make an image mosaic (Adobe® Photoshop® CS5, San Jose, CA, USA). In addition, for quantification, whole brain tissues were incubated overnight in formamide (5 ml/g dry weight) and the amount of Evans Blue released into the formamide was quantified by florescence spectrophotometry (excitation 620 nm; emission 680 nm).

Cylinder test
A unilateral injury to the motor cortex results in an asymmetry in the forelimb used for support during rearing, which can be measured using the cylinder test. The rats were placed in a glass cylinder (9.5 cm diameter and 11 cm height) and the number of times each forelimb or both forelimbs were used to support the body on the wall of the cylinder was counted for 5 min at 3 days after stroke. Two mirrors were placed behind the cylinder to view all directions. The number of impaired and non-impaired forelimb contacts was calculated as a percentage of total contacts.

**Shivering behavior**

The HomeCageScan System (Clever Sys Inc., Reston, VA, USA) was performed to measure the shivering activities during hypothermia as previously described. The system had 4 cameras that monitored 4 cages, with each cage (191mm x 292mm x 127mm) containing one rat. The behavior patterns were recorded for 1 hr at 30 min after starting hypothermia. Subtle twitch-like behavior was detected by the monitoring camera and analyzed by the Home Cage Software 3.0 for shivering activity. To distinguish the shivering behaviors from twitch activities, the detection factors in the data acquisition system were modified as the following: (1) animal size threshold (pixel): 160; (2) contrast threshold: 24; (3) muscle twitch max length: 30; (4) twitch/sleep merge limit: 10. Shivering was measured during a three hours period soon after the hypothermic treatment in normal animals.