Transarterial regional hypothermia provides robust neuroprotection in a rat model of permanent middle cerebral artery occlusion with transient collateral hypoperfusion

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Title.

Transarterial regional hypothermia provides robust neuroprotection in a rat model of permanent middle cerebral artery occlusion with transient collateral hypoperfusion

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ABSTRACT (248 words)

The robust neuroprotective effects of transarterial regional hypothermia have been demonstrated in the typical transient middle cerebral artery occlusion (tMCAO) model, but have not yet been tested in other ischemic stroke models, even though clinical ischemic conditions are diverse. In order to clarify these effects in a different ischemic stroke model, we employed a rat model of permanent MCAO (pMCAO) with transient collateral hypoperfusion (tCHP), which was achieved by direct MCA ligation through craniotomy and 1-hour bilateral common carotid artery occlusion at the beginning of pMCAO. The infusion of 20 ml/kg of 4°C cold saline (CS) or 37°C warm saline (WS) into the ipsilateral internal carotid artery (ICA) was performed for 15 minutes in intra- or post-tCHP. Neurological scores, infarct/edema volumes, and neuronal apoptosis and reactive gliosis were compared between the CS and WS groups and a non-infusion control group after 48 hours of reperfusion. Although brain temperatures were only reduced by 2-3°C for 15 minutes, the CS group had significantly better neurological scores, smaller infarct/edema volumes, and less penumbral neuronal apoptosis and reactive gliosis than the control and WS groups. The post-tCHP CS group exhibited prominent neuroprotective effects, even though infarct volumes and neuronal apoptosis were reduced less than those in the intra-tCHP CS group. In conclusion, we demonstrated the neuroprotective effects of transarterial regional hypothermia in an ischemic model of pMCAO with tCHP. Even though MCAO is persistent, cold infusion via the ICA is neuroprotective for the penumbra, suggesting the wider therapeutic application of this therapy.

Keywords: apoptosis, gliosis, hypothermia, partial reperfusion injury, permanent middle cerebral artery occlusion, transarterial regional hypothermia
1. Introduction

Although numerous experimental studies have demonstrated that hypothermia therapy exerts robust neuroprotective effects on ischemic stroke (Dumitrascu et al., 2016; Maier et al., 1998; van der Worp et al., 2007; Yenari and Han, 2012), clinical trials (Hemmen et al., 2010; Lyden et al., 2014) have failed to show any therapeutic benefit due to the adverse systemic influences accompanying this therapy (Esposito et al., 2014). Therefore, transarterial regional hypothermia is strongly expected to become a novel attractive treatment for acute ischemic stroke because of its rapid cooling action and fewer systemic side effects (Dumitrascu et al., 2016; Esposito et al., 2014; Kurisu et al., 2015). Consistent with previous findings (Chen et al., 2013; Ding et al., 2003; Ding et al., 2004a; Ding et al., 2004b; Luan et al., 2004; Zhao et al., 2009), we also clarified that transarterial regional hypothermia exerts strong neuroprotective effects on ischemia-reperfusion (I/R) injury (Kurisu et al., 2015). However, the effects of this therapy have only been examined in a typical ischemic stroke model, the transient middle cerebral artery occlusion (tMCAO) model (Chen et al., 2013; Ding et al., 2003; Ding et al., 2004a; Ding et al., 2004b; Kurisu et al., 2015; Luan et al., 2004; Zhao et al., 2009), which is a complete reperfusion model (Fluri et al., 2015; Takahashi et al., 2012; Zhao and Steinberg, 2011). The effects of this therapy have not yet been investigated in other ischemic stroke models, even though ischemic stroke in humans is extremely diverse in its pattern of occlusion and reperfusion in actual clinical settings. Therefore, we attempted to examine the therapeutic effects of transarterial regional hypothermia in a different ischemic stroke model (Fluri et al., 2015; Takahashi et al., 2012; Zhao and Steinberg, 2011).
In the present study, we investigated the neuroprotective effects of transarterial regional hypothermia on permanent MCAO (pMCAO) with transient collateral hypoperfusion (tCHP). In order to evaluate the effectiveness of transarterial regional hypothermia in terms of time interval and perfusion conditions, the treatment was performed in intra- or post-tCHP. We also assessed neuronal apoptosis and reactive gliosis by immunofluorescence staining to observe pathophysiological reactions in the ischemic core and penumbra.

2. Results

2.1. Brain Temperature and Physiological Parameters

Brain temperature was continuously monitored for 1 hour during surgery in the intra-tCHP cold saline (CS), intra-tCHP warm saline (WS), and control groups (Fig. 1). It was rapidly and significantly lowered by the CS infusion in both the cortex (from 34.1±1.2°C to 32.5±0.9°C, p<0.01) and striatum (from 36.4±0.8°C to 34.3±1.0°C, p<0.01) during the CS infusion period. Since it was rapidly elevated after finishing the infusion, the brain temperature-lowering effect was only maintained for 15 minutes. Rectal temperature did not change during the observational period.

Physiological parameters were monitored twice: at the pre-ischemic baseline and the time of sacrifice (48 hours after surgery). No significant differences were observed in physiological parameters between any of the five groups tested (Table 1).

2.2. Neurological function

Neurological function was examined in rats in each group using an 18-point scale score
(Garcia et al., 1995) 48 hours after surgery (Fig. 2A). Neurological scores were significantly better in the intra- and post-tCHP CS groups than in the control and intra- and post-tCHP WS groups (p<0.01).

2.3. **Infarct and Edema formation**

Representative images of brain sections stained with 2,3,5-triphenyltetrazolium chloride (TTC) are shown in Figure 2B. Infarct volumes were significantly smaller in the intra-tCHP CS group (8.1±4.7%, p<0.01) and post-tCHP CS group (15.7±5.9%, p<0.01) than in the control and intra- and post-tCHP WS groups (~35%) (Fig. 2C). Edema volumes were also significantly smaller in the intra- and post-tCHP CS groups (p<0.01) than in the control and intra- and post-tCHP WS groups (Fig. 2C). Comparisons between the intra- and post-tCHP CS groups revealed that infarct volumes were significantly smaller in the intra-tCHP CS group than in the post-tCHP CS group (p<0.05) (Fig. 2C).

2.4. **Appearance of neuronal apoptosis in the penumbra**

Double immunostaining for cleaved caspase 3 (CC3) and NeuN was performed in order to observe cell apoptosis and viable neuronal cells in regions of interest (ROIs). Representative images of double NeuN and CC3 staining in the penumbra are shown in Figure 4A. The frequent appearance of CC3-positive cells and a reduction in the number of NeuN-positive cells in the penumbra were observed in the control and intra- and post-tCHP WS groups, but less so in the intra- and post-tCHP CS groups (Fig. 3A). A quantitative analysis of CC3-positive cells revealed that apoptotic cell numbers were significantly less (p<0.01) in the intra- and post-tCHP CS groups than in the control and
intra- and post-tCHP WS groups (Fig. 3B). The number of viable neuronal cells was significantly higher (p<0.01) in the intra- and post-tCHP CS groups than in the control and the intra- and post-tCHP WS groups (Fig. 3B). A comparison between the intra- and post-tCHP CS groups revealed that the suppressive effects of neuronal apoptosis were significantly stronger in the intra-tCHP CS group than in the post-tCHP CS group (p<0.01). These suppressive effects by the CS infusion were not observed in the ischemic core (data not shown).

2.5. Appearance of reactive gliosis in the penumbra

Immunostaining for glial fibrillary acidic protein (GFAP) was performed in order to evaluate the activation of astrocytes. Representative images of GFAP staining in the penumbra are shown in Figure 4A. The up-regulated expression of GFAP, representing glial activation, in the penumbra was observed in the control and intra- and post-tCHP WS groups, but not in the intra- or post-tCHP CS group (Fig. 4A). A quantitative analysis of the area of GFAP-positive staining revealed that it was significantly smaller (p<0.01) in the intra- and post-tCHP CS groups than in the control and intra- and post-tCHP WS groups (Fig. 4B). Immunostaining for ionized calcium binding adapter molecule 1 (Iba1) was also performed in order to evaluate microglial activation. Representative images of Iba1 staining in the penumbra are shown in Figure 5A. The up-regulated expression of Iba1, mainly amoeboid microglia, in the penumbra was observed in the control and intra- and post-tCHP WS groups, but not in the intra- or post-tCHP CS group (Fig. 5A). A quantitative analysis of the number of Iba1-positive cells revealed that it was significantly lower (p<0.01) in the intra- and post-tCHP CS groups than in the control and intra- and post-tCHP WS groups. These suppressive
effects on reactive gliosis by the CS infusion were not observed in the ischemic core (data not shown).

3. Discussion

The present study demonstrated that transarterial regional hypothermia exerts robust neuroprotective effects in the pMCAO with tCHP model, as well as the typical tMCAO model, as reported previously (Chen et al., 2013; Ding et al., 2003; Ding et al., 2004a; Ding et al., 2004b; Kurisu et al., 2015; Luan et al., 2004; Zhao et al., 2009). This therapy significantly improved neurological deficits and decreased infarct/edema volumes. The results of the immunohistological analysis showed that neuronal apoptosis and reactive gliosis were significantly suppressed in the penumbra, but not in the ischemic core. These therapeutic effects may be expected even if therapy is initiated after the tCHP period.

The model used in the present study is known to produce focal cerebral infarction with high reproducibility (Fluri et al., 2015; Saito et al., 2013; Sugiyama et al., 2011), and is achieved through a combination of persistent antegrade hypoperfusion by direct MCAO and transient retrograde collateral hypoperfusion by bilateral common carotid artery occlusion (CCAO). Since leptomeningeal collateral flow in the rodent brain is well developed, direct MCAO only is not sufficient to ensure the induction of infarction. Therefore, tCHP by bilateral CCAO is necessary for producing complete and reproducible infarction. Cerebral blood flow may be partly re-perfused after the period of tCHP. Based on temporal and spatial changes in perfusion, the model is called a partial or incomplete reperfusion model, in contrast to the tMCAO model, which is
called a complete reperfusion model (Fluri et al., 2015; Takahashi et al., 2012; Zhao and Steinberg, 2011). When considering the perfusion state, the model of pMCAO with tCHP may mimic the conditions of incomplete revascularization after intravenous t-PA therapy or endovascular thrombectomy, which is frequently encountered in clinical settings.

Transarterial regional hypothermia shows rapid and selective brain cooling in the model of pMCAO with tCHP as well as in the tMCAO model (Ding et al., 2003; Ding et al., 2004a; Ding et al., 2004b; Kurisu et al., 2015; Luan et al., 2004; Zhao et al., 2009). A comparison of the depth and duration of the cooling effect revealed that it was weaker in the model of pMCAO with tCHP than in the tMCAO model, but still mediated significant temperature lowering (2 to 3°C for 15 minutes). The CS infusion from the ICA in the model of pMCAO with tCHP may have exerted a cooling effect through collateral blood flow, even though the main trunk of the MCA remained occluded. Since the model of pMCAO with tCHP is likely to represent incomplete revascularization, as described above, the same cooling effect may be expected with incomplete revascularization in clinical settings.

General hypothermia needs to have a sufficient depth and duration of cooling in order to attain significant neuroprotection (Dumitrascu et al., 2016; Maier et al., 1998; van der Worp et al., 2007; Yenari and Han, 2012). Maier et al (Maier et al., 1998) demonstrated that more than 4°C of cooling and more than 30 minutes of therapy are required to successfully attain significant neuroprotection in ischemic stroke models. General hypothermia is an artificial hibernation-like state that produces neuroprotective effects by lowering the metabolic demand in all cellular activities in the brain. The process of lowering the metabolic demand may require a certain amount of time to show
neuroprotective effects. In contrast to general hypothermia, transarterial regional hypothermia provided significant neuroprotection with less depth and duration of the cooling effect (2 to 3°C for less than 30 minutes). We speculate that transarterial regional hypothermia may utilize a different mechanism to that of general hypothermia in order to mediate neuroprotection. Since CS is directly infused into the vascular system, transarterial regional hypothermia may have a more direct and intensive cooling effect on the microvascular endothelium and its surroundings (Kurisu et al., 2015). This cooling effect may be important for the therapeutic mechanism, resulting in the difference observed. In our previous study (Kurisu et al., 2015), the cooling effect achieved by a CS infusion initially inhibited the acute microvascular aquaporin-4 surge and then attenuated microvascular narrowing, blood-brain barrier disruption, and the activation of other inflammatory reactions in the acute phase of ischemia/reperfusion injury. The preservation of microvascular integrity is likely to maintain the microcirculation and exert neuroprotective effects. In the present study, neuroprotective effects were detected as the suppression of neuronal apoptosis and reactive gliosis in the area of the penumbra, which appears to be a target of transarterial regional hypothermia as with other neuroprotective therapies.

Timing of the treatment is relevant in the clinical application. While we used the most ideal intra-tCHP treatment as an initial proof of concept study of transarterial regional hypothermia, we recognize that post-tCHP treatment may be more clinically relevant. Since we show that post-tCHP treatment is effective as well as intra-tCHP treatment in our model, further studies should address whether post-tCHP treatment is feasible and effective.

The present study demonstrated that transarterial regional hypothermia achieves its
cooling effect through collateral blood flow and mediates robust neuroprotection in the partial or incomplete reperfusion model. These results suggest that this therapy is effective for patients who remain in the partial reperfusion state after intravenous t-PA therapy or endovascular thrombectomy. Although optimal and advanced methods for intravenous thrombolysis and/or endovascular treatments have been adopted, approximately one-third to two-thirds of patients treated with acute recanalization therapy develop thrombolysis in cerebral infarction (TICI) grade 2, regarded as partial perfusion (Higashida et al., 2003). A previous study demonstrated that transarterial regional hypothermia exerted significant neuroprotective effects in the tMCAO model (Kurisu et al., 2015); therefore, the additional favorable results in the present study support this therapy having wider therapeutic applications in ischemic stroke patients. Furthermore, our results revealed that neuroprotective effects are expected even if this therapy is initiated after the severe hypoperfusion state (post-tCHP). Therefore, this therapy has potential as an adjunctive strategy to acute recanalization therapy because most patients have access to transarterial regional hypothermia after recanalization therapy in actual clinical settings.

There are some limitations to this study for the clinical application of transarterial regional hypothermia. The most significant limitation is that the precise therapeutic time window of this therapy remains unknown. In clinical settings, most patients receive and complete acute recanalization therapy several hours after the onset of ischemia (Higashida et al., 2003). A markedly larger delay is anticipated to receive transarterial regional hypothermia in actual clinical settings. Thus, further studies that focus on how a delay in the initiation of therapy affects its effects are warranted. In order to prolong the therapeutic time window, another strategy may be required, such as the effective
supply of oxygen with cooled artificial oxygen carriers (Shimbo et al., 2014) or cooled neuroprotective agents (Chen et al., 2013). Furthermore, we need to establish acceptable and appropriate therapeutic conditions in terms of, for example, timing, infusion volumes, and infusion routes for successful clinical applications. Even though the present and previous studies (Ding et al., 2004a; Kurisu et al., 2015) showed that this approach would lead to fewer systemic effects, it is still unclear whether this same approach (e.g., volume, infusion rate) could be safely applied in the clinical setting. Therefore, studies using other animal species similar to humans are necessary in order to examine these factors (Kurisu et al., 2015). Another limitation of the present study is lack of data on the microvascular reactions, such as, expression of AQP4 and the ultrastructure of blood-brain barrier disruption in this stroke model. Although we speculate that the same microvascular reactions demonstrated in our previous study (Kurisu et al, 2016) occur and are partly inhibited by transarterial regional hypothermia in this stroke model as well, further experiments are needed to clarify this issue. Furthermore, long term (longer than 48 hours) therapeutic effects of transarterial regional hypothermia have not been addressed in the present study. The durability of any therapeutic effects remains to be determined, and should be addressed in the future studies.

In conclusion, we herein demonstrated that transarterial regional hypothermia exerted robust neuroprotective effects in the pMCAO with tCHP model. This model mimics the conditions of partial reperfusion accompanying persistent arterial occlusion, which is commonly encountered in actual clinical settings, particularly after acute recanalization therapy. The results of the present study strengthen the therapeutic potential of transarterial regional hypothermia for clinical application to various ischemic stroke
patients and situations.

4. Experimental procedures

4.1. Stroke Model Rats

This study was approved by the Animal Studies Ethics Committee at the Hokkaido University Graduate School of Medicine. All procedures used in this study were performed in accordance with the institutional guidelines for animal experiments. All efforts were made to minimize animal suffering and the number of animals sacrificed. A total of 75 rats were studied in the experiments and 65 rats (86.7%) were included in the data analysis. The reasons for exclusion were premature deaths due to intraoperative surgical complications (n=8) and postoperative anesthetic complications (n=2).

Male Sprague Dawley rats (210-280g, CLEA Japan, Inc. Tokyo, Japan) were used in this study. The ischemic stroke model employed consisted of two surgical procedures: pMCAO by direct MCA ligation through craniotomy and tCHP by 1-hour bilateral CCAO (Saito et al., 2013; Sugiyama et al., 2011).

Rats were anesthetized with 4.0% isoflurane in a mixture of 70% N₂O and 30% O₂. The bilateral CCAs were exposed. The right cervical region was then dissected until the external carotid artery (ECA) and internal carotid artery (ICA) were both secured. In order to perform transarterial regional hypothermia therapy, the secured ECA was ligated and cut off for the cannulation of a microcatheter (Kurisu et al., 2015; Shimbo et al., 2014). A 1.5-cm vertical skin incision was then made between the right eye and ear. The temporal muscle was scraped from the temporal bone, and 5×5-mm temporal craniotomy was performed using a small dental drill. After dura opening, the right MCA
was ligated using 10-0 nylon thread and cut off to accomplish pMCAO. Bilateral CCAO was performed for 1 hour using microsurgical clips. This procedure provides further hypoperfusion in the MCA territory by reducing collateral flow. The high reproducibility of focal cerebral infarction has been verified in this model (Fluri et al., 2015; Saito et al., 2013; Sugiyama et al., 2011).

4.2. Transarterial Regional Hypothermia and Experimental Protocol

Transarterial regional hypothermia was performed by the infusion of 20 ml/kg of 4°C CS through the microcatheter placed in the right ICA via the ECA for 15 minutes (Kurisu et al., 2015). In order to confirm whether hemodilution by saline infusion exerts neuroprotective effects, 37°C WS infusion was performed in the same manner. The experimental protocol and animal groups are shown in Figure 6A. Rats were randomly selected prior to the surgery, and rats undergoing MCAO were divided into the following five groups in a randomized fashion. (Fig. 6A); intra-tCHP CS, intra-tCHP WS, post-tCHP CS, post-tCHP WS, and control groups. The saline infusion in the intra- or post-tCHP group was started after the onset or end of tCHP, respectively. During the treatment, brain and rectal temperatures were monitored. Needle thermistor probes (BAT-12 Microprobe Thermometer; Physitemp Instruments, Inc., NJ USA) were placed into the cortex (depth of 1 mm) and striatum (depth of 4 mm) through burr holes 3 mm lateral and posterior to the bregma. Body temperature was measured through the rectum. Throughout anesthesia, rats were placed on a heating pad in order to maintain the rectal temperature at 37.0±0.5°C. Physiological parameters, including mean arterial blood pressure, body temperature, blood pH, pCO₂, pO₂, Hb, and Hct were measured before surgery and 48 hours after ischemia. All rats were sacrificed 48 hours after surgery for
subsequent experiments. Neurological deficits, infarct and edema volumes (n=8 / each group) were assessed within the same rats. Immunohistological analysis was performed within the different rats (n=5 / each group). Analysis of the result obtained from each rats were performed by a different investigator (M.G) blinded to the experimental manipulations.

4.3. Neurological Assessment

A neurological assessment was performed 48 hours after surgery using the 18-point scale score (n=8/group). The 18-point scale score (Garcia et al., 1995) system comprised 6 different neurological assessments: (1) spontaneous activity, (2) symmetrical limb movement, (3) forepaw out stretching, (4) climbing, (5) body proprioception, and (6) response to vibrissae touch. The scores for each test (maximum 3 points) were summed (Garcia et al., 1995).

4.4. Measurement of Infarct and Edema Volumes

Rat brains (n=8/ each group) were harvested 48 hours after surgery, and infarct and edema volumes were then measured as described previously (Shimbo et al., 2014). In brief, 5 2-mm-thick serial coronal sections were prepared from rat brains and stained with 2% TTC (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) for 20 minutes. Brain sections were photographed and quantitatively analyzed with ImageJ software (NIH, Bethesda, MD, USA). Infarct volumes were calculated as a percentage volume of the normal left hemisphere according to the following formula: (left hemisphere volume – right non-infarct volume)/left hemisphere volume (%). Edema volumes were calculated as a percentage volume of the normal left hemisphere according to the
following formula: (right hemisphere volume – left hemisphere volume)/left hemisphere volume (%).

4.5. Fluorescence Immunohistochemistry

The harvested brain was immersed in 4% paraformaldehyde for 2 days and embedded in paraffin. Four-micrometer-thick coronal sections at the level of the striatum (A 1.0-mm to P 1.0-mm of the bregma) were prepared for subsequent analyses. Deparaffinized sections were processed through antigen retrieval for 2 minutes in a pressure pot. Fluorescence immunohistochemistry was performed (n=5/each group).

In order to assess treatment effects on neuronal apoptosis, sections were double labeled for CC3, the marker for apoptotic cells, plus NeuN, the marker for neuronal cells. Sections were reacted with primary antibodies against CC3 (rabbit monoclonal, 1:200, Cell Signaling Technology, Danvers, MA, USA) and NeuN (mouse monoclonal, 1:100, Millipore, Billerica, MA, USA) at room temperature for 1 hour. Sections were then reacted with Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:200, Life Technologies, Carlsbad, CA, USA) and Alexa Fluor 488-conjugated goat anti-mouse IgG (1:200, Life Technologies) at room temperature for 1 hour.

GFAP staining and Iba1 staining were performed to assess reactive gliosis, including the activation of astrocytes and microglia. Sections were reacted with a primary antibody against either GFAP (mouse monoclonal, 1:500, BD Bioscience, San Jose, CA, USA) or Iba1 (rabbit polyclonal, 1:1000, Wako, Tokyo, Japan) at room temperature for 1 hour. Each section was then reacted with Alexa Fluor 594-conjugated goat anti-mouse IgG (1:200, Life Technologies) for GFAP staining and Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200, Life Technologies) for Iba1 staining at room temperature for 1
Fluorescent signals were observed through appropriate filters using a fluorescence microscope (BX61; Olympus, Tokyo, Japan) and digitally photographed using a cooled charged-couple device camera (model VB-6000; Keyence Corporation, Osaka, Japan). In the quantitative analysis, 2 and 4 ROIs were randomly set in the areas of the ischemic core and penumbra, respectively. The area of the penumbra was defined as the area where the ischemic region spared when a suitable treatment, i.e., transarterial reginal hypothermia in this study, was performed (Shichinohe et al., 2015; Takahashi et al., 2012) (Fig. 6B).

4.6. **Quantitative analysis of neuronal apoptosis**

A quantitative analysis of neuronal apoptosis was performed using ImageJ software (NIH, Bethesda, MD, USA). In randomly selected ROIs (200× magnified field), the number of CC3-positive cells was quantified automatically with binary images processed by ImageJ software. In the same fields, the number of NeuN-positive cells was also quantified using the same method.

4.7. **Quantitative analysis of reactive gliosis**

A quantitative analysis of astrocytic and microglial activation was also performed using ImageJ software. In the analysis of astrocytic activation with an increase in cell size, the area occupied by GFAP-positive cells per field was automatically measured with binary images processed by ImageJ software in randomly selected ROIs (400× magnified field). In the analysis of microglial activation with an increase in cell number, the number of Iba1-positive cells was counted with binary images processed by ImageJ.
software in randomly selected ROIs (400× magnified field).

4.8. Statistical Analysis

All experiments were conducted in a random manner, and assessments of neurological function, infarct/edema volumes, and histological analyses were performed by an investigator (M.G.) who was blinded to the experimental manipulations. All data were presented as the mean±SD, and the data were confirmed to be normally distributed. Multiple comparisons were performed using a one-way ANOVA followed by Bonferroni’s post hoc test. \( p<0.05 \) was considered significant.

Conflict of interest

The authors declare no competing financial interests.

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REFERENCES


Yenari, M.A., Han, H.S., 2012. Neuroprotective mechanisms of hypothermia in brain


**Figure Legends**

**Fig. 1** Temperatures measured in the ipsilateral cortex, striatum, and rectum during and after transarterial regional hypothermia. *P<0.05, **P<0.01.

**Fig. 2** Effects of transarterial regional hypothermia on neurological and pathophysiological findings.  (A) Neurological findings evaluated by an 18-point scale scoring system 48 hours after ischemia. (B) Representative 2% 2,3,5-triphenyltetrazolium chloride (TTC) staining image in each group. (C) Infarct and edema volumes 48 hours after ischemia in each group. *P<0.05, **P<0.01.

**Fig. 3** Effects of transarterial regional hypothermia on neuronal apoptosis. (A) Representative images of double NeuN and cleaved caspase 3 (CC3) staining in the penumbra. (B) A quantitative analysis of the numbers of CC3-positive cells and NeuN-positive cells. Scale bar = 50 μm. *P<0.05, **P<0.01.

**Fig. 4** Effects of transarterial regional hypothermia on glial activation. (A) Representative images of GFAP staining in the penumbra. (B) A quantitative analysis of the total area of GFAP-positive cells in ROIs. Scale bar = 40 μm. *P<0.05, **P<0.01.

**Fig. 5** Effects of transarterial regional hypothermia on microglial activation. (A) Representative images of Iba1 staining in the penumbra. (B) A quantitative analysis of the number of Iba1-positive cells in ROIs. Scale bar = 40 μm. *P<0.05, **P<0.01.
Fig. 6 Scheme and figures concerning experimental methods. (A) Scheme of the experimental protocol. Rats were divided into 5 groups. (B) A representative image for examination of the ischemic core and penumbra.
Figure 3

A

control

intra-ICHP

WS

intra-ICHP

CS

B

Cloned Caspase 3 positive cells/ROI

control  WS  CS  WS  CS  post-ICHP

Neuro-positive cells/ROI

control  WS  CS  WS  CS  post-ICHP
Figure 4

A

control  |  intra-tCHP WS | intra-tCHP CS | post-tCHP WS | post-tCHP CS
GFAP   |  DAPI      | GFAP/DAPI    | GFAP   |  DAPI      | GFAP/DAPI

B

Graph showing GFAP positive area/ROI (%) for control, intra-tCHP WS, intra-tCHP CS, post-tCHP WS, and post-tCHP CS groups.