Importance of inflammatory mechanism in therapeutic effect of hypothermia on stroke

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The Graduate School, Yonsei University

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Directed by Professor Chul Hoon Kim

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This certifies that the Doctoral Dissertation of Jung Ho Lee is approved.

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가장 먼저, 아무것도 모르던 제가 이렇게 박사학위를 받을 때까지, 실험과 논문 작성 과정, 그리고 그 밖의 모든 과학자로서 필요한 지식과 경험을 쌓게 해주신 지도교수님 김철훈 교수님께 깊이 감사드립니다. 처음 교실에 들어왔을 때부터, 따스한 말씀을 아끼지 않으셨던 안영수 교수님, 항상 삶, 특히 과학자로서의 삶에 대한 여러 소중한 조언을 주신 김동구 교수님, 처음엔 많이 엉성했던 제 논문을 꼼꼼히 봐주셔서 부족하나마 지금의 형식을 갖추게끔 해주신 이배환 교수님, 연구에 좋은 조언을 많이 주셨고, 항상 뵐 때마다 많이 걱정해주시던 김세훈 교수님께 감사드립니다.

가까이 뵈었던 시간은 적지만, 잊지 말아야 할 말씀들을 많이 해주셨던 김경환 교수님, 교실생활에서는 따뜻하게, 연구에 대해서는 날카롭게, 많은 조언과 힘을 주신 이민구 교수님, 언제나 환한 웃음으로 인사를 받아주시는 박경수 교수님, 열심히 연구하시는 모습이 인상적인 김주영 교수님, 김형범 교수님, 처음 교실에 들어왔을 때는 선배로서, 지금은 교수님으로, 많은 격려를 주시는 지헌영 교수님, 제 연구결과를 조금씩 발표할 때마다, 따뜻한 조언을 주셨던 문석준 교수님께 감사드립니다.

제 박사학위논문이 나올 때까지 모든 실험과정을 계속 함께 해주시고, 가끔 힘들어 했을 때 많은 힘이 되어주신 응급의학과 유제성 교수님께 특별히 감사드립니다.

긴 랩생활에서 힘들고 기쁜 시간을 함께했던 제호, 가끔 의대 주변을 거닐며 함께 커피를 마셨던 한웅수 선생님, 많은 실험을 가르쳐주었던 석진이, 의대시절부터 어쩌다보니 여기에서까지 함께 공부하게 된 호진군과 홍인이 형님, 힘이 되는 말씀을 해주셨던 문여정 선생님, 열심히 연구해야 함을 보여준 임영신 선생님, 그리고 랩에서 함께 했던 권오빈, 최재용, 손선영, 조아련, 신소라, 윤은장 선생님과 지금도 랩을 굳건히 지키고 있는 윤종진, 임지수, 노현종에게도 감사드립니다.

교실 생활의 세세한 부분까지에 많은 도움을 주신 임종수 선생님, 여러 어려운 일들을 해결해주셨던 민선자 선생님, 동물실험에 큰 도움을 주셨던 김건태 선생님께 교실 생활하는 내내 감사했습니다.

마지막으로 힘든 목회생활을 마치시고, 이제 고향으로 돌아가 조용히 전원생활을 즐기시는 부모님과 오랜 시간을 함께 해주지 못함을 참아준 아내에게 큰 감사의 마음을 전합니다.

2016. 12. 이정호

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ABSTRACT

Importance of inflammatory mechanism in therapeutic effect of hypothermia on stroke

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In ischemic stroke, the inflammatory response plays an important role in infarct evolution and in the overall severity. Therapeutic hypothermia is a promising neuroprotectant, but the mechanisms underlying this neuroprotection are unknown. High mobility group B1 (HMGB1) is a critical mediator in many human diseases. Previous studies showed that HMGB1 induces the inflammatory response in *in vitro* and *in vivo* stroke model. I hypothesized that hypothermia reduces HMGB1 release and inhibits its effect in activation of the inflammatory response to ischemic injury.

Rats underwent middle cerebral artery occlusion with or without hypothermia. In middle cerebral artery occlusion model rats, hypothermia greatly reduced the infarct size. Immunofluorescence and enzyme linked immunosorbent assay showed that hypothermia inhibited extracellular HMGB1 release from the ischemic brain into systemic circulation. By real time PCR, I showed that hypothermia reduced the expressions of infarct-modifying inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tissue necrosis factor (TNF)-α, in peri-ischemic and ischemic regions. When glycyrrhizin, a direct HMGB1 binding inhibitor was injected, similar effects were also observed in our animal stroke model with hypothermia. In conclusion, I have demonstrated that hypothermia inhibits the release of HMGB1 and reduces the inflammatory responses of the penumbra; I suggested that the inhibition of HMGB1 release via hyporthermia in the brain provides an important mechanism underlying neuroprotective effects on ischemic and peri-ischemic regions in stroke.

Key words: stroke, hypothermia, inflammation, cytokine

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Ⅰ. INTRODUCTION

Ischemic stroke causes brain damage and significant chronic disability. The ischemic penumbra is the region that surrounds the ischemic core. If a therapeutic intervention fails, the ischemic penumbra can also be recruited into the ischemic core. Thus, the penumbra is an important target for stroke therapeutics. Ischemic stroke induces an innate immune response. High mobility group box protein 1 (HMGB1) is one of the most important mediators of this innate immune response. Previous studies showed that HMGB1 induces the inflammatory response and

brain damage in *in vitro* and *in vivo* model. Therapeutic hypothermia is one of the most effective neuropretectant. Although, there is no consensus regarding which hypothermia-supressed inflammatory mediator generates the neuroprotection. I designed this study to investigate the mechanism by which hypothermia reduces the inflammatory response in acute ischemic stroke. The current study suggests that therapeutic hypothermia inhibits the ischemic brain damage propagation by inhibiting HMGB1 release.

1. Stroke

Stroke, also known as cerebrovascular disease, is caused by the interruption or severe reduction of the blood flow to a brain region. Cerebrovascular disease includes two types of disease: ischemic stroke and hemorrhagic stroke. Ischemic stroke accounts for nearly 90 % of stroke cases. When an intracranial vessel is occluded, the brain regions supplied by that vessel become deprived of oxygen and glucose. If blood flow is not restored within 10 min, cell death can occur in the poorly supplied brain region. Patients may experience various neurological symptoms, including paralysis, impaired speech, and loss of vision. Neurons, the most important cell type in the brain do not recover after damage from ischemic injury. Therefore, when therapeutic strategies fail to block the ischemic damage, stroke patients show poor neurological outcomes.[1] Societal and economic burden for the treatment and the rehabilitation of stroke patients is great. Acute ischemic stroke is a leading cause of mortality and serious long-term disability worldwide.[2] Clear elucidation of the damaging mechanisms of stroke and developing therapeutics for effective treatment are important for human health.

2. Ischemic penumbra

A. The definition and meaning of 'ischemic penumbra' in ischemic stroke

The ischemic penumbra is the target region for stroke therapeutics; Astrup et al. first defined this region in 1981.[3] According to the original definition, the ischemic penumbra is the brain region with reduced cerebral blood flow surrounding the infarct core. The penumbra shows an impaired neuronal functionality, but a preserved structural integrity. If the therapeutic approach for blocking the ischemic damage fails, the ischemic penumbra may be recruited into the developing infarct core. This is an important characteristic of the penumbra in stroke therapeutics. Thus, saving the penumbral region is the prime purpose of stroke therapeutics.

B. Biologic markers for ischemic penumbra

Although many researchers have conducted multiple studies regarding the penumbra, there is no definite histologic marker for the penumbral region.[4] Researchers are now developing histological markers that clearly show the penumbral region under experimental conditions. Perfusion-diffusion mismatch is the most widely used method for detection of the penumbra through magnetic resonance imaging (MRI) in stroke patients and experimental ischemic stroke models. Perfusion-diffusion mismatch is defined as the mismatch between the volume of abnormal tissue in perfusion-weighted MRI images and that in diffusion-weighted MRI images. Perfusion-diffusion mismatch region shows decreased cerebral blood flow but no detectable brain damage. Perfusion-

diffusion mismatch is typically presumed to represent the penumbra, but there are several limitations.[5,6] Abnormality in perfusion-weighted imaging can overestimate the infarct region, and the initial diffusion lesion does not necessarily show or encompass the irreversible infarct region. Hence, in order to evaluate the effects of potential therapeutics in animal stroke models, researchers want to have histologic markers for the penumbra.

3. Recombinant tissue plasminogen activator

Recombinant tissue plasminogen activator (tPA) is the only FDA-approved therapeutic for stroke patients. tPA reestablishes blood flow and saves damaged brain regions by breaking down a vessel-occluding thrombi. The National Institute of Neurological Disorders and Stroke (NINDS) tPA Stroke Study reported that intravenous tPA treatment showed a clear benefit versus placebo in stroke patients.[7] However, tPA treatment usage is limited by this important disadvantage: it is beneficial only when treatment is delivered within 3~4.5 hr after the onset of symptom. This “time is brain” concept reflects that the aggravation of damage by ischemia and reperfusion is time-sensitive and phase specific. Consequently, less than 10 % of patients with acute ischemic stroke are candidates for thrombolysis.[8] tPA treatment can also induce intracerebral hemorrhage in stroke patients.[9] Development of new adjunctive therapies targeting neuroprotection beyond thrombolysis is needed to attenuate the brain ischemic injury.

4. Inflammation

A. Beneficial and detrimental effects of inflammation

Inflammation is important for the survival of organisms. Inflammatory mechanisms protect organisms from injury and infection, which are major threats in evolution. When pathogens from the external environment enter an organism, the organism senses those pathogens and induces controlled responses to remove the pathogens. Under these circumstances, well-controlled inflammatory responses are beneficial for the host defense and tissue repair. However, when inflammation becomes chronic or excess, inflammatory mechanism is detrimental. Chronic or excess inflammatory mechanisms are important mediators in the pathophysiology of many human diseases.[10]

B. Mechanisms of ischemic damage

After the onset of blood flow reduction, the ischemic damage occurs by many important mechanisms.[11] In the acute phase, blood flow decreases and a ionic disturbance occurs. Intracellular calcium levels elevate; then the glutamate release from brain cells increases and the elevation of extracellular glutamate level induces the cytotoxicity. Cytotoxic edema and necrosis occur within a few hours of ischemia. In the subacute phase (hours and days from the onset of ischemic injury), neuronal apoptosis occurs and reactive oxygen species are produced; monocytes, macrophage, and neutrophils induce inflammatory responses. After weeks or months, necrotic debris is removed and new vessels are formed. Lost circuits are re-connected and neurovascular remodeling and functional recovery occur.

C. Inflammatory mechanisms in stroke

Inflammatory response has a prominent role in stroke pathophysiology. Many types of inflammatory cells and mediators are shown to have negative roles in ischemic brain injury. Among these, pro-inflammatory cytokines are important mediators in ischemic damage. Interleukin (IL)-1β, IL-6, tissue necrosis factor (TNF)-α are representative examples of stroke-related cytokines.[12,13] Inhibiting the action of these cytokines yields protective effects on the infarct size and several subsequent damaging mechanisms of stroke. Inhibition of these cytokines by neutralizing antibodies or newly developed pharmacological inhibitor is a potential candidate therapy for stroke patients.

Cerebral ischemic injury is highly associated with extreme inflammatory responses and the neuronal cell loss. Ischemic insult is sterile injury that activates innate immunity, which induces a devastating inflammation in the absence of foreign invaders.[14,15] In these sterile injuries, high mobility group box protein-1 (HMGB1) is an important mediator.

5. High Mobility Group Box protein-1 (HMGB1)

A. Extracellular HMGB1 acts as a pro-inflammatory cytokine

HMGB1 was first discovered as a nuclear non-histone DNA binding protein. For decades, HMGB1 was known merely as a structural protein related to the stability of DNA and a transcriptional modulation. However, in 1999, HMGB1 was identified as a late mediator in endotoxin lethality.[16] HMGB1 is released by two different mechanisms. Active release of HMGB1 occurs when immunologically competent cells (i.e., monocytes and macrophages) are activated during infection

by pathogens or toxins. Active release of HMGB1 generally occurs within 24 to 48 hr after the infective threat. Passive release of HMGB1 occurs when cells are damaged by the necrotic injury; HMGB1 is passively released a few minutes after the onset of the sterile injury, which includes the ischemic injury. Contrary to its role in the nucleus, after active or passive release, HMGB1 acts as a pro-inflammatory cytokine. While HMGB1 propagates signals through many types of receptors, the two most important receptors in the pathophysiology of human disease are toll-like receptor (TLR) 4 and receptor for advanced glycation end-product (RAGE). In monocytes and macrophages, HMGB1-TLR4 signaling induces an elevated pro-inflammatory cytokine expression by the nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).[17,18] In endothelial cells and immune cells, HMGB1 effects cell migration, cell growth, and the differentiation through RAGE signaling.[19,20] The exact signaling pathway of HMGB1-RAGE signaling is elusive until now. HMGB1 has a pivotal role in inflammatory responses through these receptors.

B. HMGB1 in stroke patients and stroke animal models

In many human diseases including sepsis, arthritis, and myocardial infarction, HMGB1 induces inflammatory response and tissue damage. In one clinical study, Goldstein et al.[21] revealed that serum HMGB1 levels are significantly elevated (218.0 ± 18.4 ng/ml) in patients with acute ischemic stroke within 24 hr after the onset of symptoms compared to those of healthy controls (16.8 ± 10.9 ng/ml) ($p < 0.001$), and this elevation is correlated with the length of time after the onset of symptom. Higher plasma HMGB1 levels are a valuable prognostic marker for mortality and neurologic outcomes in ischemic stroke.[22] Other previous studies

reported elevated serum HMGB1 levels after ischemic injury by MCAO.[23,24] These studies show that HMGB1 may be important mediator in stroke.

C. The role of HMGB1 in stroke pathophysiology

The role of HMGB1 in ischemic brain damage has been established partly by *in vivo* and *in vitro* studies. Kim et al.[25] demonstrated that extracellular HMGB1 released from injured neurons in post-ischemic brain activates microglia, astrocytes, and microvascular endothelial cells. Actively secreted HMGB1 from these cells expands the brain inflammatory response and significantly aggravates brain damage.[25-27] HMGB1 also has an effect on the systemic inflammatory response after brain ischemic injury.[28] HMGB1 induces complex behavior sickness syndrome in a mouse model, and inhibits adaptive immune responses by the induction of bone marrow egression and the proliferation of bone marrow-derived suppressor cells.[28] HMGB1 functionally exhausts mature monocytes and induces lymphopenia. HMGB1 is an important mediator in immune suppression after extensive ischemia and its mechanisms are detrimental in stroke prognosis. Although many negative roles of HMGB1 in stroke are well known, there are discrepancies regarding the effect of HMGB1 inhibition via antibodies on infarct size of stroke animal models. In one study, the injection of HMGB1 monoclonal antibodies before MCAO reduced the size of the infarct region.[29] Conversely, in another study using MCAO mouse model, there was no significant change on infarct size after the injection of neutralizing HMGB1 antibody.[28]

6. Therapeutic hypothermia

A. Therapeutic hypothermia is a promising neuroprotectant

Lowering body temperature to preserve tissue in various medical conditions is not a new concept. Indeed, hypothermia has been used as a therapeutic agent for millennia.[30] Although therapeutic hypothermia has been shown to be a robust neuroprotectant for brain injury,[30] its use for acute ischemic stroke has been limited due to incomplete clinical data. However, after two clinical studies demonstrated the benefit of therapeutic hypothermia in patients with cardiac arrest, hypothermia has been clinically applied during post-resuscitation care as an efficient therapy for improving neurologically intact tissue.[31,32] The current guidelines recommend that therapeutic hypothermia (cooling the core temperature to 32°C or 34°C) for 12 to 24 hr should be considered for comatose adult patients after cardiac arrest.[33]

B. The mechanisms of neuroprotection in therapeutic hypothermia in stroke

Many studies have suggested several mechanisms of the neuroprotective effect of therapeutic hypothermia in various time phases of stroke.[11] In the acute phase, therapeutic hypothermia increases cerebral blood flow,[34] reduces glutamate release,[35] improves glucose metabolism within brain cells,[36] and alters immediate early gene expressions and cellular stress responses.[37] In the subacute phase of stroke, hypothermia protects brain cells from apoptotic cell death by the reduction of pro-apoptotic protein expressions (e.g., BAX) and increasing anti-apoptotic protein expression (e.g., BCL-2).[38] Hypothermia also inhibits caspase-dependent

pathways and extrinsic apoptotic pathways.[39] Additionally, the inflammatory responses are inhibited by therapeutic hypothermia during the subacute phase. Inflammatory cell infiltration is reduced in the infarct region,[40] transcriptional activation of immune-related genes is inhibited by hypothermia,[41] and radical oxygen production is reduced.[42] The disruption of the blood-brain barrier can induce inflammatory competent cell infiltration (neutrophil, T cell) into infarct region. Hypothermia inhibits blood-brain barrier disruption by reducing extracellular protease activity.[43-45] In the chronic phase, hypothermia induces the remodeling process of the infarct region and enhances precursor cell differentiation. Hypothermia also induces angiogenesis, neurite outgrowth, and neuronal connectivity.[46,47] As described above, therapeutic hypothermia has protective effects by many putative mechanisms. A better understanding of these hypothermic mechanisms can open new avenue for stroke research, and it can also improve identifying proper indications for introducing hypothermia to stroke patients. Once the mechanisms of hypothermic neuroprotective effects are well elucidated, new adjunctive hypothermic therapeutics may be easily developed around that mechanism. However, yet, there is no consensus regarding which hypothermia-suppressed inflammatory mediator generates the most protection.

7. Aims of the study

The aim of this study was to investigate the neuroprotective effects of therapeutic hypothermia on post-ischemic inflammation in a clinically relevant MCAO rat model. As HMGB1 exerts its effect as the trigger and booster of inflammatory responses in the ischemic injury, therapeutic hypothermia may contribute to the inhibition of HMGB1 release from brain cells. However, to my

knowledge, no study has determined whether HMGB1 release from ischemic cortex cells is inhibited by hypothermia, or found the consequence of reduced extracellular HMGB1 after brain ischemia. I hypothesized that the protective mechanism of therapeutic hypothermia on the post-ischemic brain injury after MCAO is inhibition of extracellular HMGB1 release, which mitigates its pro-inflammatory role in the acute stage of ischemia.

Ⅱ. MATERIALS AND METHODS

1. Animal preparation

Healthy male Wistar rats weighing between 295-315 g were used for all experiments. A single-source breeder (Orientbio Inc., Seongnam, Korea) consistently supplied healthy animals with uniform age and weight. All animal experiments were approved by Institutional Animal Care and Use Committee (IACUC) of Yonsei University Health System. Prior to experimentation, rats were offered food and water ad libitum.

2. Experimental model of MCAO

The rats were subjected to permanent MCAO, as described previously.[48] Previously established rodent model of focal brain ischemia with MCAO by intraluminal suture was used.[49] Anesthesia was induced with 5 % isoflurane in mixture of 0.7 L/min nitrous oxide and 0.3 L/min oxygen. During the surgical procedure for MCAO, anesthesia was maintained using 1~2 % isoflurane with the aforementioned gas mixture. Rectal temperature was monitored and maintained with surface cooling ice packs, a temperature monitoring system, and a feedback-controlled heating pad (Harvard apparatus, Holliston, MA, USA). Under an operating microscope, the left common carotid artery (CCA) was carefully exposed by dissection through a midline neck incision. The ligation and coagulation of the external carotid artery (ECA) was performed after isolation of the ECA and its branches. The internal carotid artery (ICA) was carefully isolated from the adjacent vagus nerve. After ligation of the pterygopalatine artery, the CCA also was ligated with 6-0 black silk. Next, the proximal portion of the ICA was loosely tied with 6-0

black silk, and a microvascular clip was clipped across the distal portion of the ICA. After making an incision with micro-scissors in the proximal ICA, the intraluminal 4-0 MCAO suture (Doccol Corporation, Sharon, MA, USA) was inserted in a hole, and loosely tied 6-0 black silk was tightened on the proximal portion of the ICA. After removing the microvascular clip, the intraluminal 4-0 MCAO suture was advanced approximately 22 mm beyond the CCA bifurcation from the proximal ICA lumen to the distal direction. After sham and MCAO surgeries, tracheostomy was performed with an intravenous catheter (IV Catheter 16G, Sewoon Medical Co., Seoul, Korea) through a midline neck incision, and mechanical ventilation (tidal volume, 3.0 ml; respiratory rate, 80/min) was conducted using a rodent ventilator (SAP-830/AP, CWE, Inc., Ardmore, PA, USA). The incision was closed by simple suture technique with 4-0 nylon.

3. Experiment protocol and temperature management

First, the animals were randomly divided into four groups: sham+normothermia (n = 11), sham+hypothermia (n = 12), MCAO+normothermia (n = 15), and MCAO+hypothermia (n = 12). In hypothermia groups, surface cooling started after 15 min of ischemia (maintaining at 33.0 ± 0.5°C) by placing ice packs on the torso of rats. Vecuronium (0.9 mg/kg) was injected intramuscularly after mechanical ventilation to inhibit shivering. After sham and MCAO operations, the target core temperature was carefully monitored and maintained for 4 hr using feedback-controlled heating pads and surface cooling with ice packs. In the normothermia and hypothermia groups, targeted core temperatures were maintained at 37.5 ± 0.5°C and 33.0 ± 0.5°C, respectively. During the experiments, all animals were treated in the same manner, except for the hypothermia and MCAO. Figure 1A shows the

experimental protocol.

Second, the pharmacological direct inhibitor against HMGB1 (glycyrrhizin) was used to demonstrate the functional significance of HMGB1 release in ischemic injury. Animals were randomly divided into four groups: sham+vehicle (n = 7), sham+glycyrrhizin (n = 7), MCAO+vehicle (n = 9), and MCAO+glycyrrhizin (n = 9). A mixture of glycyrrhizin (100 mg/kg) and 5 ml of saline was injected intraperitoneally 30 min before MCA occlusion. All groups were treated similarly throughout the experiment with the exception of intraperitoneal injection for the glycyrrhizin groups and MCA occlusion for the MCAO groups.

Third, HMGB1 neutralizing antibody was injected by intracerebroventricular route. Five μg of antibody in 5 μl of phosphate buffered saline was injected 30 min before MCA occlusion by infusion pump. Two groups were treated similarly throughout the experiment with the exception of antibody injection.

4. Infarct volume measurement

Rats were decapitated under anesthesia 4 hr after MCA occlusion or sham operation. Whole brains were dissected and sliced into 2 mm thick coronal sections using a matrix device (ASI instrument, Warren, MI, USA). Coronal sections were stained with 1 % TTC (2, 3, 5-triphenyl-tetrazolium chloride, Sigma-Aldrich, St. Louis, MO, USA) solution (in saline) at 37°C for 10 min. After staining, sections were immersed in 4 % paraformaldehyde solution (in phosphate-buffered saline) and fixed for 18 hr. After fixation, the caudal face of each section was scanned with a flatbed scanner (EPSON, Nagano, Japan). The scanned image was analyzed with ImageJ 1.48v software (National Institutes of Health, Bethesda, MD, USA). The

white regions in the ipsilateral hemisphere were measured as the infarcted area using an auto-threshold ImageJ method.[50] Infarct volumes (mm^3) were calculated by multiplying the summed section of the white areas by the section thickness.

5. Enzyme-linked immunosorbent assay (ELISA)

A 23 gauge needle was inserted into the right atrium at 4 hr post MCA occlusion or sham operation. Approximately 1 ml of blood was withdrawn and collected with a serum separator tube (BD Vacutainer® SST™ Advance, BD, Plymouth, UK). To separate serum, collected blood was centrifuged for 20 min at 2000 rpm and serum was stored at -80°C. HMGB1 concentration was determined using the HMGB1 ELISA kit (IBL international GMBH, Hamburg, Germany).

6. Immunofluorescence

For immunofluorescence, 2 mm thick rat brain slices (bregma 0.7 mm to -1.3 mm) were immersed in a 4 % paraformaldehyde solution, and then subjected to cryoprotection by 30 % sucrose in phosphate-buffered saline. The slice was frozen with optical cutting temperature (OCT) compound (CellPath, Mid Wales, UK), then cryosections were cut at a thickness of 20 μm in a cryostat machine (Leica Biosystems, Buffalo Grove, IL, USA). Sections from 0.2 mm to -0.3 mm relative to bregma were chosen for staining. Sections were first permeabilized with PBS containing 0.3 % Triton X-100, 3 % goat serum, 0.02 % sodium azide, 10 mg/ml bovine serum albumin (BSA) for 1 hr and incubated overnight at 4°C with anti-HMGB1 polyclonal antibody (1:100, ab18256; Abcam, Cambridge, UK) in PBS containing 3 % goat serum, 0.02 % sodium azide, 10 mg/ml BSA. Next, sections were washed with PBS for 5 min, three times, and incubated for 1 hr at room

temperature with Alexa 566 conjugated goat anti-rabbit antibody (1:500, Invitrogen). Then sections were washed with PBS three times and mounted using Vectashield with DAPI solution (Vectorlab, Burlingame, CA, USA). Slides were observed under a confocal microscope (LSM 700, Carl Zeiss GmbH, Jena, Germany). TTC staining results showed the lower half of the primary somatosensory cortex and the secondary somatosensory cortex in the left hemisphere of each rat were damaged by MCA occlusion; these regions are observed as the ischemic regions. The corresponding regions in the right hemisphere were defined as the contralateral regions.

7. Real-time polymerase chain reaction (PCR)

Three regions of rat brain tissue from each brain slice (bregma -1.3 mm to -3.3 mm) were used for the real-time PCR experiments. All TTC stating results showed infarcted ipsilateral primary sensory cortex barrel field regions. This region was defined as the ischemic region. TTC results showed the ipsilateral primary and secondary motor cortices and cingulate cortex were not infarcted, but rather adjacent to the infarcted region. This region was chosen as the peri-ischemic region. Contralateral primary sensory cortex barrel field region tissue was used as the control region. Tissue RNA was extracted using a Hybrid-R kit (Geneall biotechnology, Seoul, Korea). cDNA was prepared from 1 μg of RNA with a 1st strand cDNA Synthesis Kit (Takara bio, Shiga, Japan). Specific PCR primers for IL-1α, IL-β, IL-6, TNF-α were designed from corresponding mRNA sequences using Primer Express 3.0 software (Applied Biosystems, Foster city, CA, USA). PCR amplification was performed on an ABI 7300 system (Applied Biosystems, Foster city, CA, USA) and involved the use of SYBR Premix Ex Taq (Takara, Shiga,

Japan).

8. Statistical analysis

All data are presented as mean ± s.e.m. Differences between groups were analyzed with one-way analyses of variance (ANOVA) followed by Bonferroni *post hoc* test for multiple comparisons between groups. Values of $p < 0.05$ were considered significant.

Ⅲ. RESULTS

1. Therapeutic hypothermia was successfully induced and maintained in the MCAO model

All experiments were conducted according to the schedule schematically illustrated in Figure 1A. Focal brain ischemia was induced by MCAO through intraluminal suture. After MCAO, therapeutic hypothermia was applied to the MCAO rat model and core temperature of 33.0 ± 0.5°C was maintained for the next 4 hr (Figure 1B). In normothermia groups, normothermia was maintained by a temperature monitoring system and feedback-controlled heating pad. After 4 hr, rats were sacrificed and samples for further molecular studies were prepared.

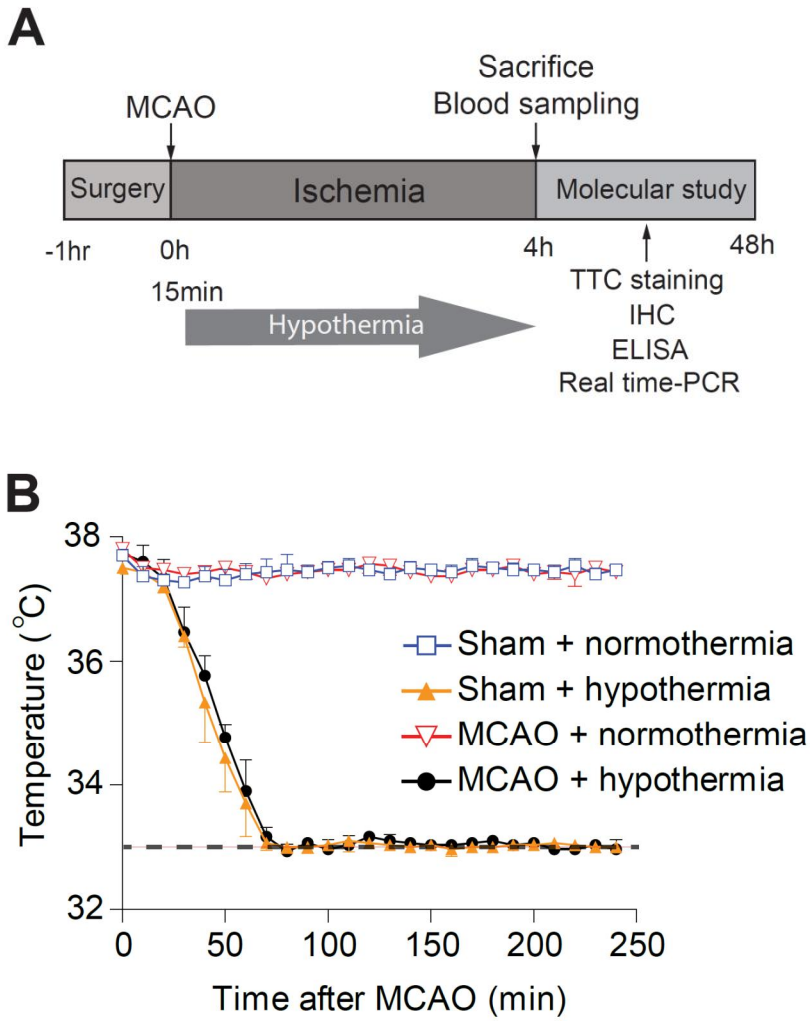
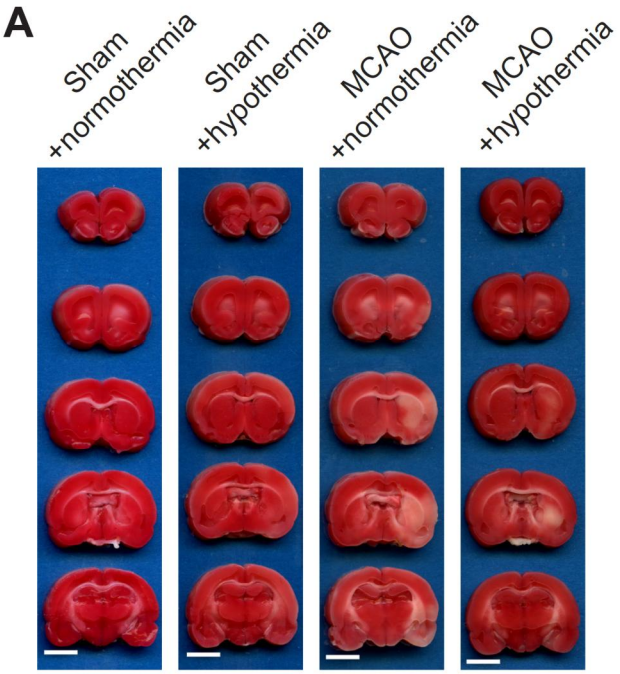


Figure 1. Induction and maintenance of hypothermia in MCAO model rats. (A) Illustration that schematically shows schedule of experiments. (B) Monitored traces of rectal temperature of rats in experiments. (MCAO: middle cerebral artery osslucion)

2. Therapeutic hypothermia descreases infarct volume of post-ischemic brain

To evaluate the neuroprotective effects of mild hypothermia on post-ischemic brain, infarct volumes of MCAO+hypothermia group rats were compared with those of MCAO+normothermia group rats by TTC staining.

The infarct volumes in the ischemic hemispheres of the MCAO-treated rats were much larger than those of control (sham-operated) rats and hypothermia-only treated rats. Mean infract volumes were evaluated at 4 hr of ischemia; the administration of mild hypothermia (33°C) significantly decreased the mean infarct volumes (73.60 ± 37.67 mm^3, $n = 4$) of MCAO rats compared with those of the MCAO+normothermia group (256.40 ± 29.01 mm^3, $n = 4$, $p < 0.001$) (Figure 2A and B). This MCAO model results indicate that mild hypothermia treatment has protective effects in the acute stage (4 hr after onset of MCAO) of ischemic injury.



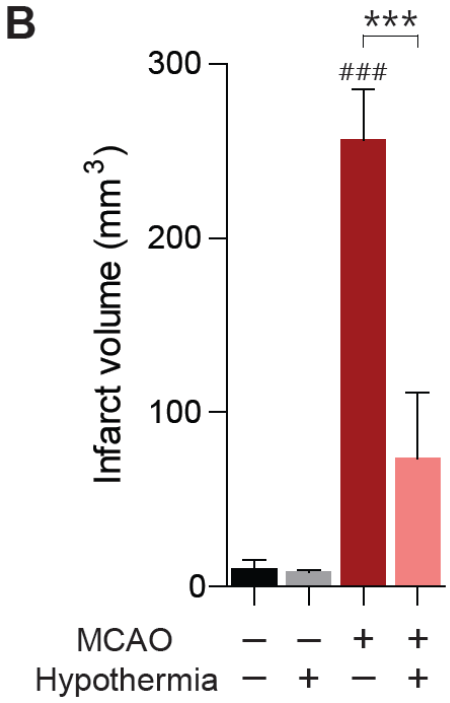


Figure 2. Reduction of infarct volume of post-ischemic brain by hypothermia. (A) Representative pictures of TTC staining results. (B) Quantification plot for infarct volume measured in TTC-stained brain slices of rat treated with MCAO and/or hypothermia. Number of rats in each group is as follows. Sham+normothermia (n = 3), sham+hypothermia (n = 4), MCAO+normothermia (n = 5), MCAO+hypothermia (n = 4). ### $p < 0.001$ versus non-MCAO, non-hypothermia group, *** $p < 0.001$, MCAO without hypothermia group versus MCAO with hypothermia group, one-way ANOVA followed by Bonferroni *post hoc* test.

3. Hypothermia inhibits the extracellular release of HMGB1 from the ischemic cortex

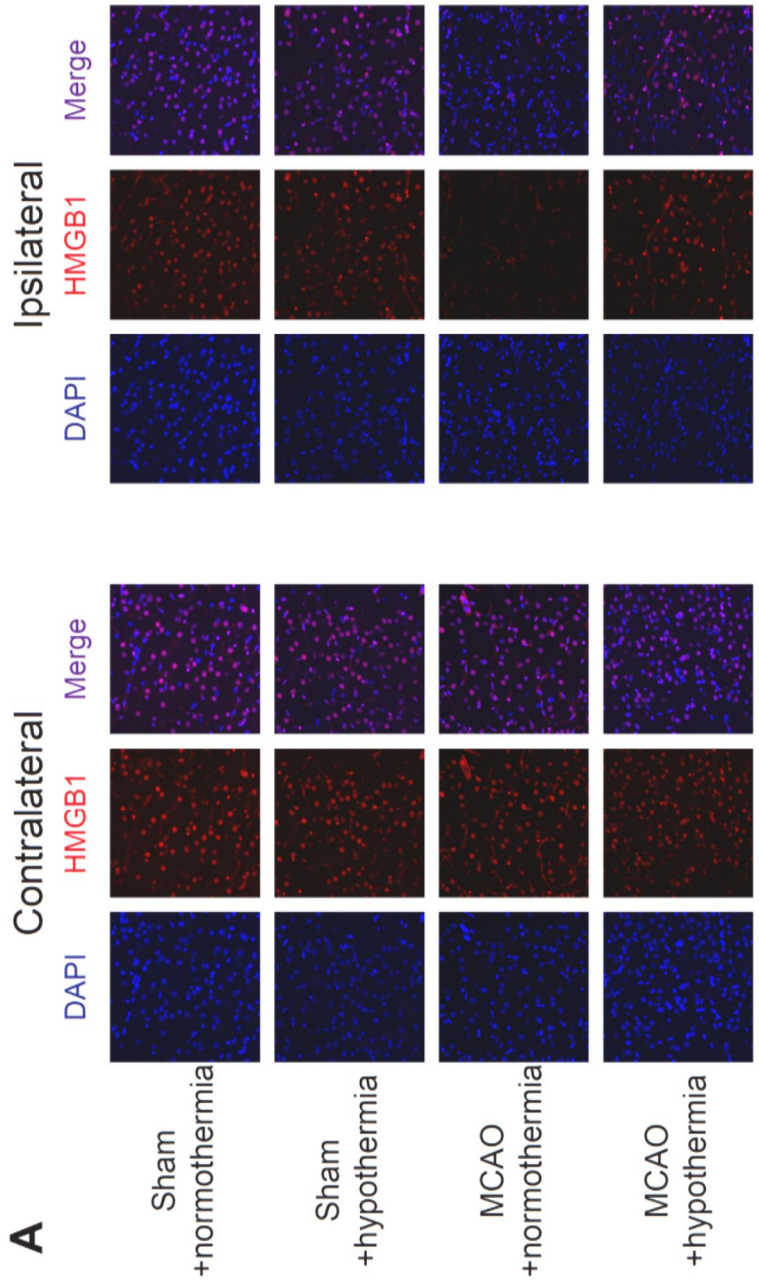
To address whether hypothermia inhibits the extracellular release of HMGB1 after ischemic injury, immunofluorescence was used to compare the amounts of nuclear HMGB1 in the brains of MCAO- and hypothermia- treated rats with those of MCAO-only treated rats.

When ischemic injury is achieved using MCAO, HMGB1, which is usually located in the nucleus, is released from most cortical cells.[27] A prior study showed that HMGB1 immunoreactivity was markedly reduced in the ischemic cortex.[27] In immunofluorescence, therapeutic hypothermia significantly attenuated the extracellular release of HMGB1 on the post-ischemic rat cortex, compared with ischemic regions that showed a reduced number of HMGB1-positive cortical cells (Figure 3A and B).

The percentage of HMGB1 positive cells/DAPI positive cells was 30.31 ± 1.60 % in

the cortex of the ischemic hemisphere versus 65.04 ± 3.53 % in the corresponding cortical region of the hypothermia-treated ischemic hemisphere ($p < 0.001$).

These results indicate that hypothermia has strong inhibitory effects on the release of HMGB1 from the ischemic cortex in ischemic injury.



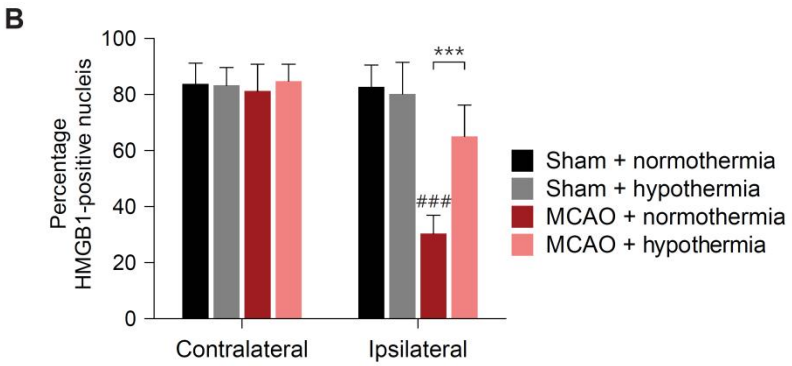


Figure 3. Hypothermia inhibits the reduction of the number of cells with nuclear HMGB1 immunoreactivity in post-ischemic brain. (A) Immunofluorescence staining result of MCAO- and/or hypothermia-treated rat brain. (B) Quantification plot of immunofluorescence results. Number of rats in each group is as follows. Sham+normothermia (n = 4), sham+hypothermia (n = 4), MCAO+normothermia (n = 6), MCAO+hypothermia (n = 4). ### $p < 0.001$ versus non-MCAO, non-hypothermia group, *** $p < 0.001$, MCAO without hypothermia group versus MCAO with hypothermia group, one-way ANOVA followed by Bonferroni *post hoc* test.

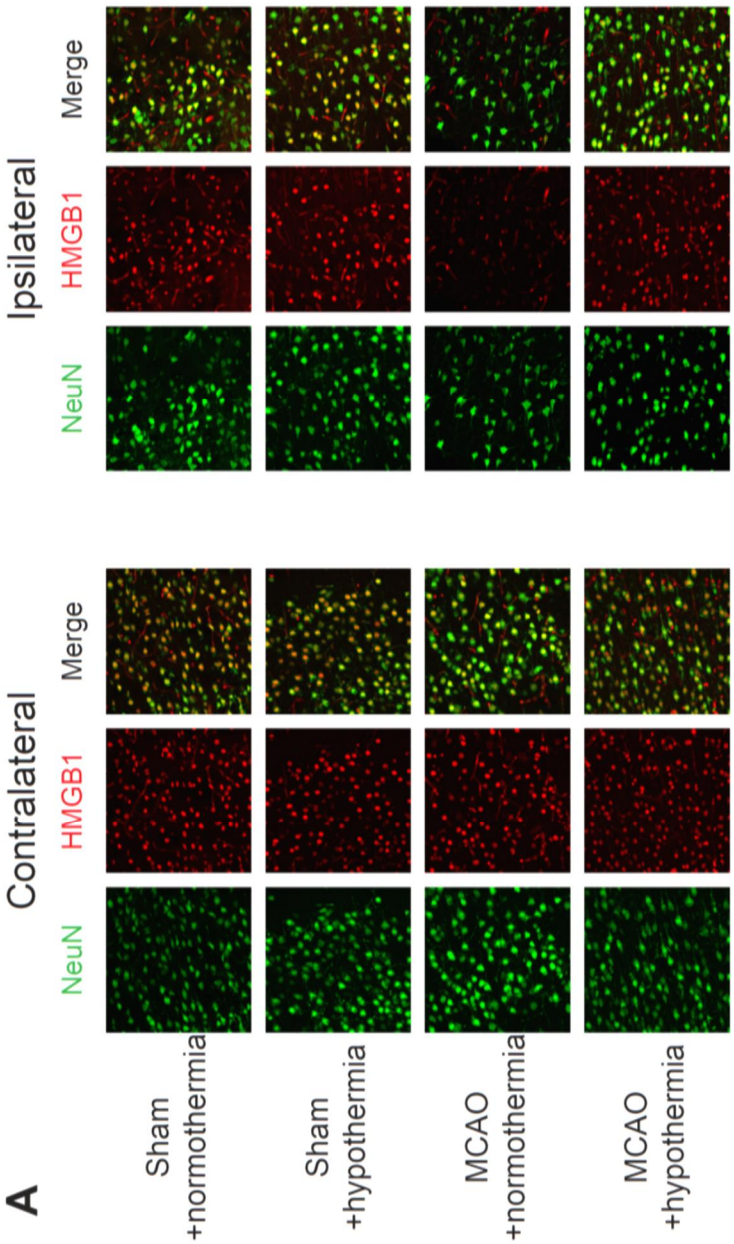
4. HMGB1 is released preferentially from neurons and hypothermia inhibits this release

Rat brain sections were immunostained with HMGB1 and NeuN, a widely used neuronal marker, to identify which cell type is important in HMGB1 release and whether hypothermia has an effect on the release of HMGB1 by this cell type.

In immunofluorescence analysis, HMGB1 was depleted in the ischemic cortex,

consistent with my previous immunofluorescence results in Figure 3 (Figure 4A and B). The percentage of HMGB1-positive cells that colocalized with the neuronal marker NeuN was reduced to 39.0 ± 3.1 % in rat brains after MCAO ischemic injury (Figure 4B). Treatment of hypothermia attenuates the reduction of the percentage of HMGB1-positive cells that is colocalized with the NeuN in MCAO rat brains (Figure 4B).

These results indicate that HMGB1 is preferentially released from neurons and that hypothermia inhibits this release.



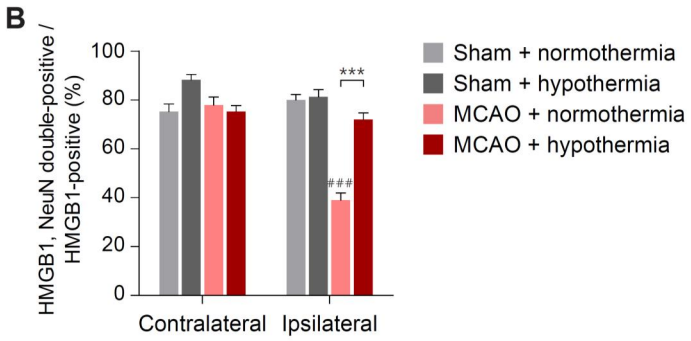


Figure 4. . Hypothermia inhibits the reduction of the percentage of HMGB1-positive cells with NeuN immunoreactivity in post-ischemic brain. (A) Representative immunofluorescence pictures. (B) Quantification plot of immunofluorescence results. Number of rats in each group is as follows. Sham+normothermia (n = 4), sham+hypothermia (n = 3), MCAO+normothermia (n = 5), MCAO+hypothermia (n = 3). ### $p < 0.001$ versus non-MCAO, non-hypothermia group, *** $p < 0.001$, MCAO without hypothermia group versus MCAO with hypothermia group, one-way ANOVA followed by Bonferroni *post hoc* test.

5. Therapeutic hypothermia reduces MCAO-induced elevation of serum HMGB1

To evaluate the effect of hypothermia on the release of HMGB1 into circulation, I measured serum HMGB1 level with an ELISA assay. The ELISA results showed that HMGB1 was significantly elevated in the serum of rats after receiving ischemic injury by MCAO. However, therapeutic hypothermia significantly inhibited the elevation of serum HMGB1 levels after ischemic insult, returning the levels to a

normal physiological range (Figure 5).

These results indicate that therapeutic hypothermia has an inhibitory effect on the extracellular release of HMGB1 from post-ischemic brain cells into the circulatory system.

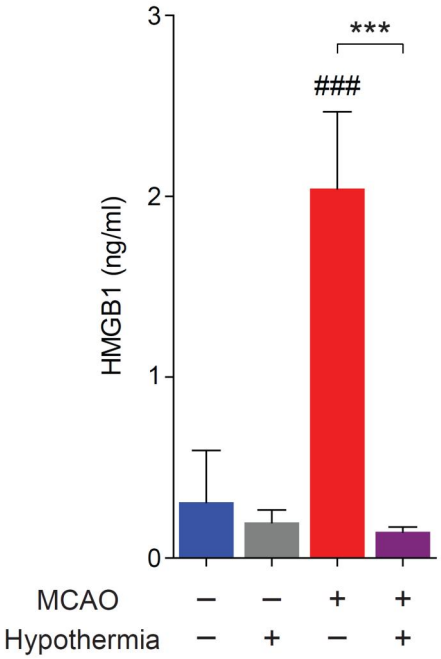


Figure 5. Hypothermia inhibits the elevation of serum HMGB1 level in MCAO-treated rat model. Number of rats in each group is as follows. Sham+normothermia (n = 4), sham+hypothermia (n = 4), MCAO+normothermia (n = 4), MCAO+hypothermia (n = 4). ### $p < 0.001$ versus non-MCAO, non-hypothermia group, *** $p < 0.001$, MCAO without hypothermia group versus MCAO with hypothermia group, one-way ANOVA followed by Bonferroni *post hoc* test.

6. Glycyrrhizin, a pharmacological HMGB1 inhibitor, ameliorates MCAO-induced brain ischemic injury

To further demonstrate that HMGB1 plays a direct and critical role in brain ischemic injury, I examined the effects of glycyrrhizin as a pharmacological HMGB1 inhibitor, which directly inhibits HMGB1 actions in post-ischemic injury.

I first asked whether inhibition of HMGB1 action by glycyrrhizin has an effect on the size of ischemic damage. Infarct volumes of vehicle-treated MCAO rats and those of MCAO+glycyrrhizin group rats were measured. The inhibition of HMGB1 activity using glycyrrhizin markedly attenuated the infarct volumes of the post-ischemic cortex in MCAO-treated rats (257.20 ± 21.93 mm^3 in vehicle-treated MCAO group versus 77.35 ± 27.19 mm^3 in the glycyrrhizin-treated MCAO group) (Figure 6A and B). This result indicates that extracellular HMGB1 plays a direct and critical role in aggravation of ischemic injury.

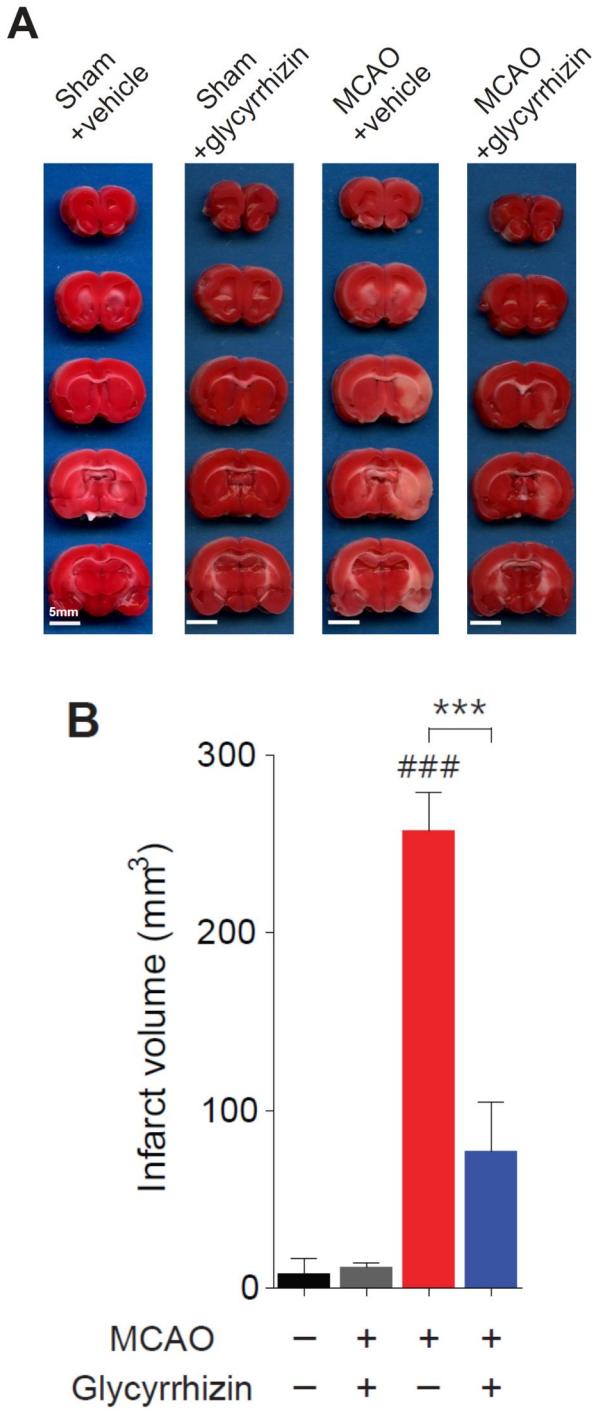
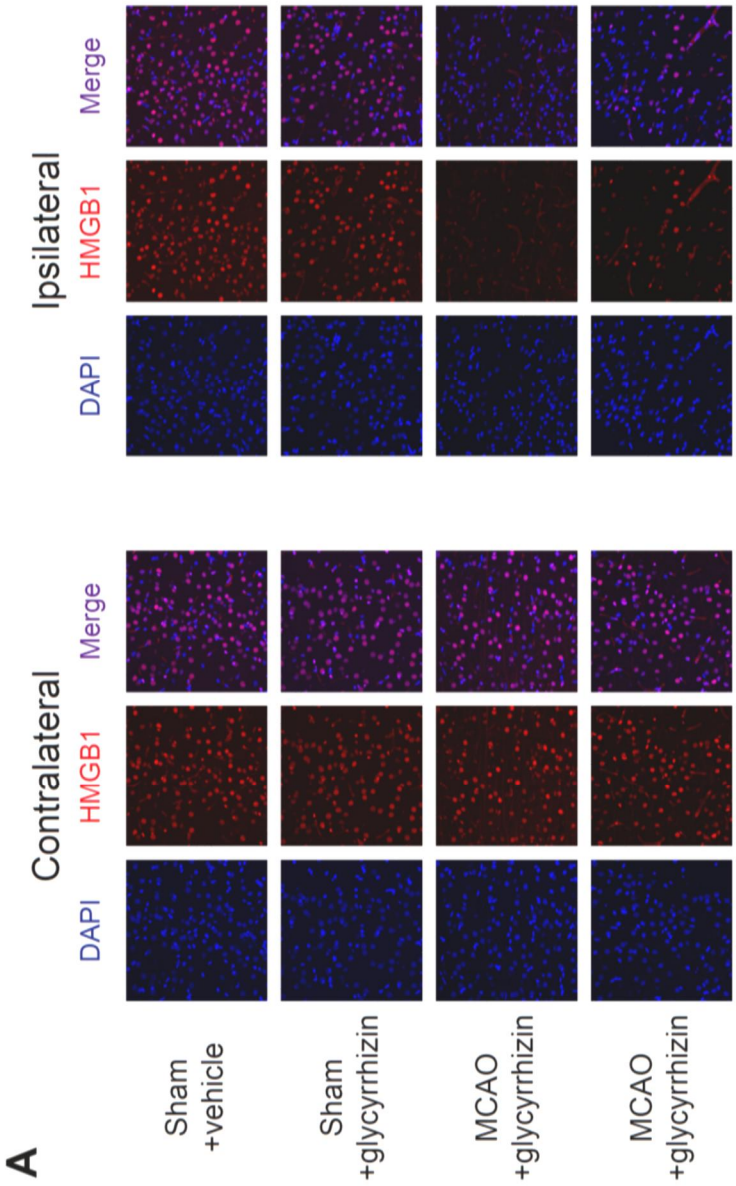


Figure 6. Glycyrrhizin reduces infarct volume in post-ischemic brain. (A) Representative pictures of TTC staining results. (B) Quantification plot of TTC results. Number of rats in each group is as follows. Sham+vehicle (n = 4), sham+ glycyrrhizin (n = 4), MCAO+vehicle (n = 5), MCAO+glycyrrhizin (n = 5). ### $p < 0.001$ versus non-MCAO, non- glycyrrhizin group, *** $p < 0.001$, MCAO without glycyrrhizin group versus MCAO with glycyrrhizin group, one-way ANOVA followed by Bonferroni *post hoc* test.

7. Glycyrrhizin inhibits HMGB1 release from the ischemic cortex

Next, I wanted to discern the effect of glycyrrhizin on extracellular release of HMGB1 from brain cells.

Using immunofluorescence, the nuclear HMGB1 immunoreactivity of post-ischemic brains of MCAO- and glycyrrhizin-treated rats was compared with those of MCAO-only treated rats. Immunofluorescence results showed a significant increase in the percentage of HMGB1-positive cells/DAPI-positive cells in the ipsilateral hemisphere of the glycyrrhizin-treated MCAO group (52.17 ± 1.59 %) compared with vehicle-treated MCAO group (30.23 ± 1.34 %) ($p < 0.001$) (Figure 7A and B).



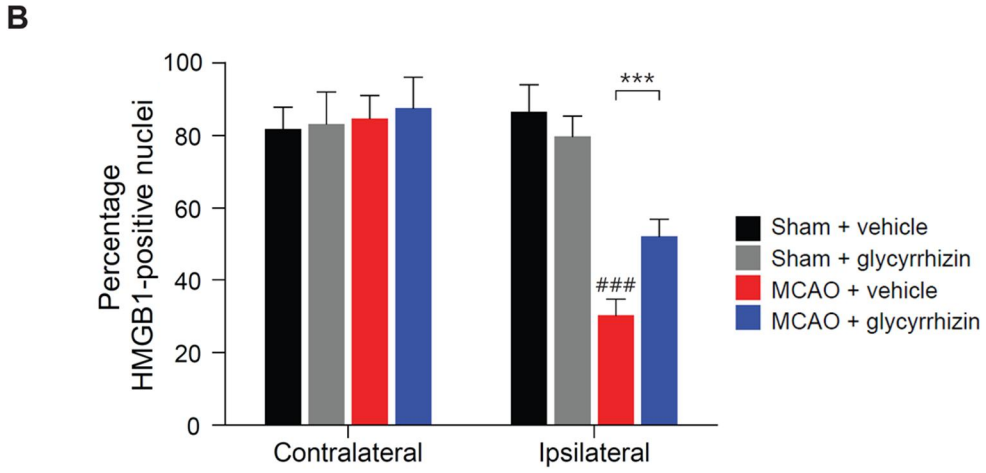


Figure 7. Glycyrrhizin inhibits reduction of nuclear HMGB1 immunoreactivity in post-ischemic brain. (A) Immunofluorescence results of HMGB1 in brain sections of MCAO and/or glycyrrhizin treated rat brain. (B) Quantification plot of immunofluorescence results. Number of rats in each group is as follows. Sham+vehicle (n = 3), sham+glycyrrhizin (n = 3), MCAO+vehicle (n = 4), MCAO+glycyrrhizin (n = 4). ### $p < 0.001$ versus non-MCAO, non- glycyrrhizin group, *** $p < 0.001$, MCAO without glycyrrhizin group versus MCAO with glycyrrhizin group, one-way ANOVA followed by Bonferroni *post hoc* test.

8. Intracerebroventricular injection of HMGB1 neutralizing antibody is protective in ischemic brain injury

Next, I wanted to discern the effect of a more specific inhibition of HMGB1 action on ischemic injury; therefore, HMGB1 neutralizing antibody was injected into the cerebral ventricle. Five micrograms of HMGB1 neutralizing antibody was injected

by infusion pump over 5 min into rats 30 min before the onset of ischemia. After 4 hr of ischemia, the TTC staining results showed HMGB1 neutralizing antibody treatment reduced the infarct size of the MCAO-treated rat cortex (Figure 8A and B). This result indicates that direct specific blocking by a neutralizing antibody is protective on ischemic injury.

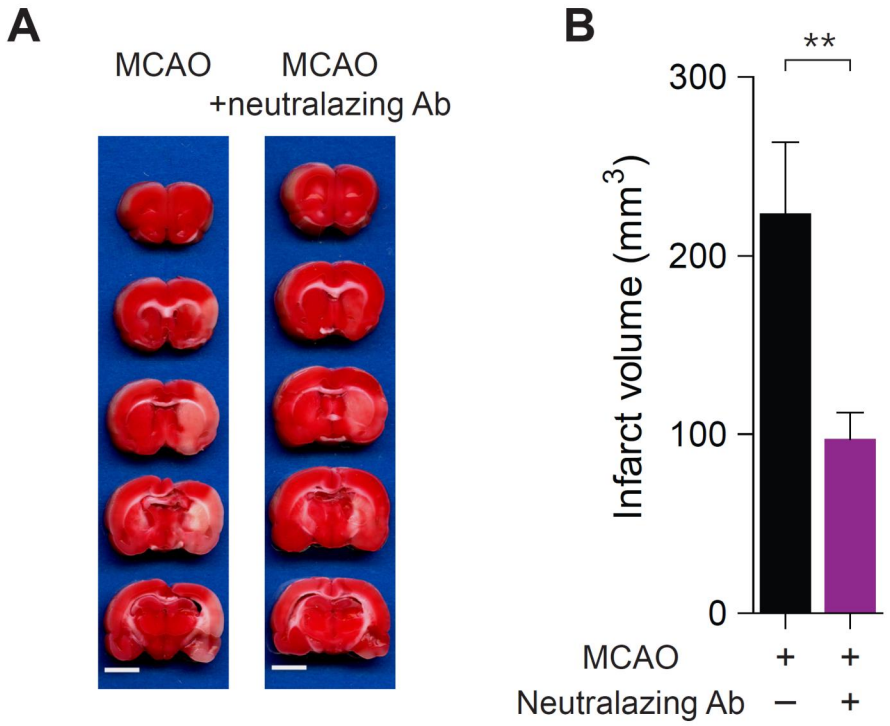
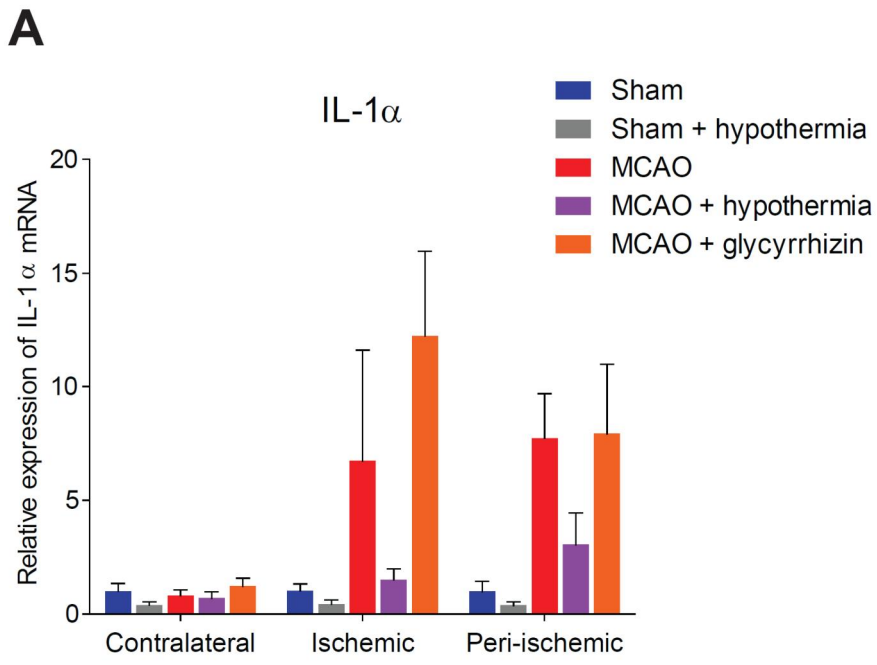
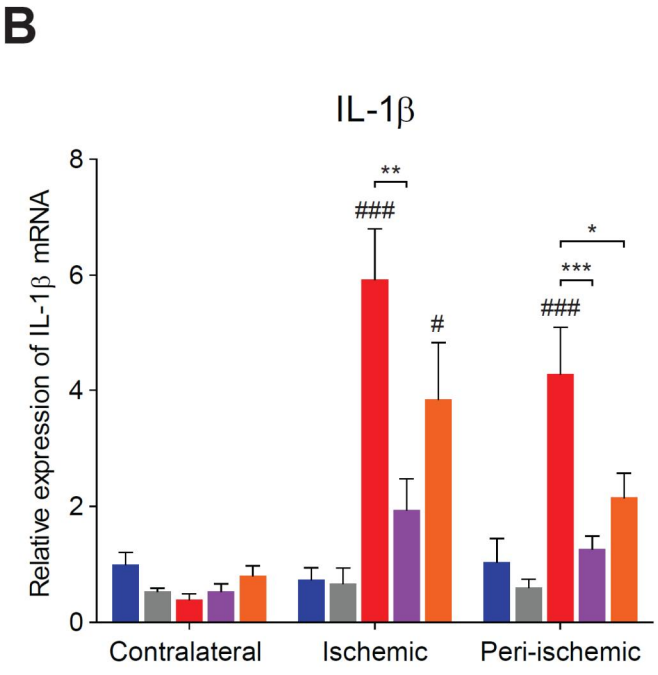


Figure 8. HMGB1 neutralizing antibody reduces infarct volume of post-ischemic brain. (A) Representative pictures of TTC staining results. (B) Quantification plot of TTC results. Number of rats in each group is as follows. MCAO (n = 4), MCAO + neutralizing antibody (n = 8). ** $p < 0.01$, MCAO without neutralizing antibody group versus MCAO with neutralizing antibody group, unpaired *t* test.

9. Both therapeutic hypothermia and glycyrrhizin inhibit the gene expression of inflammatory cytokines in peri-ischemic brain regions

I then asked whether hypothermia and pharmacological inhibition of HMGB1 action would have an effect on inflammatory responses evoked in the post-ischemic cortex. To measure effects of hypothermia and glycyrrhizin treatment on the expression of infarct-modifying cytokines, mRNA levels of four pro-inflammatory cytokine genes (IL-1α, IL-1β, IL-6, TNF-α) in the peri-ischemic and ischemic region of post-ischemic brain after 4 hr of MCAO were evaluated by real-time PCR. As shown in Figure 9, mRNA levels of IL-1β, IL-6, and TNF-α were elevated in peri-ischemic regions after ischemia, consistent with previous reports. However, theses elevations in mRNA expression by ischemic stimulation were significantly attenuated by both therapeutic hypothermia (IL-1β, IL-6, TNF-α) and glycyrrhizin treatment (IL-1β, IL-6). I found that therapeutic hypothermia attenuated the elevation of gene expression of pro-inflammatory cytokines in peri-ischemic region after cerebral ischemia, and pharmacological inhibition of HMGB1 has effects similar to that of hypothermia treatment.





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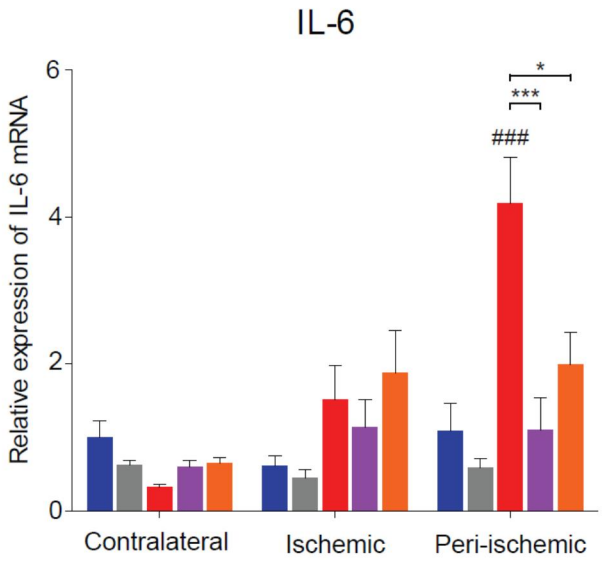

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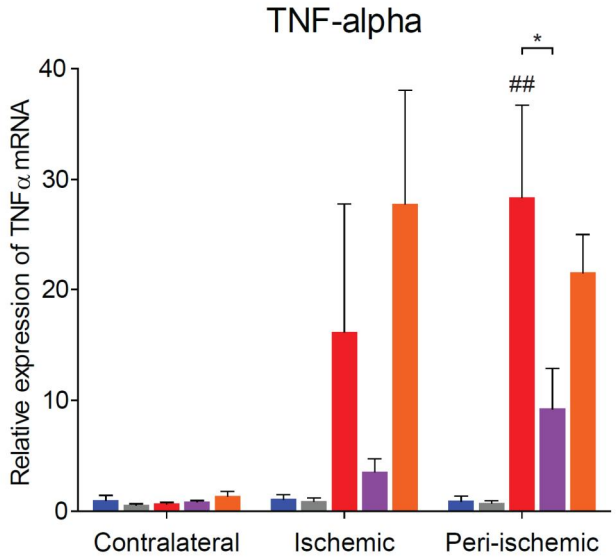

Figure 9. Real-time PCR results of post-ischemic brain tissue. (A) Real-time PCR results of IL-1α gene. (B) Real-time PCR results of IL-1β gene. (### $p < 0.001$, # $p < 0.05$ versus non-MCAO, non-hypothermia group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ one-way ANOVA followed by Bonferroni *post hoc* test). (C) Real-time PCR results of IL-6 gene. (### $p < 0.001$, versus non-MCAO, non-hypothermia group, *** $p < 0.001$, * $p < 0.05$, one-way ANOVA followed by Bonferroni *post hoc* test). (D) Real-time PCR results of TNF-α gene. (## $p < 0.01$, # $p < 0.05$ versus non-MCAO, non-hypothermia group, *$p < 0.05$, one-way ANOVA followed by Bonferroni *post hoc* test). Number of rats in each group is as follows. Sham (n = 5), sham+hypothermia (n = 5), MCAO (n = 5), MCAO+hypothermia (n = 6), MCAO+glycyrrhizin (n = 5).

Ⅳ. DISCUSSION

In this study, I investigated the effects of therapeutic hypothermia on HMGB1 release and its role in inflammatory response to cerebral ischemic injury.

First, I compared the sizes of infarct regions in MCAO model rats with or without hypothermia treatment. The TTC staining is the most widely used method for detecting infarct region in MCAO model; therefore, I utilized the TTC staining method. TTC staining results demonstrated that therapeutic hypothermia significantly attenuated the increase of infarct tissue size in the early stage (4 hr) of ischemic injury. Many previous studies have established the protective effect of hypothermia in infarct size;[51,52] my results, consistent with previous studies, show that hypothermia treatment has comparable effects in this specific MCAO experimental model.

The ELISA results show that systemic HMGB1 levels were significantly elevated at 4 hr after ischemic insult. Consequently, the serum HMGB1 levels from the group receiving hypothermia after ischemic injury were reduced to a level almost to that of the sham group. A previous study that measured serum HMGB1 levels after hypothermia treatment also shows that HMGB1 levels were reduced in a MCAO model after hypothermia treatment.[53] However, there is one difference between the previous study and this study: the previous study used a transient MCAO model and I used a permanent MCAO model. The permanent MCAO stroke model mimics patients who are not candidates for tPA treatment due to risk factor, or who were admitted to the hospital after the therapeutic window for tPA treatment (within 4 to 6 hr). As described in the introduction, HMGB1 is a prognostic marker in stroke

patients. My result suggests that therapeutic hypothermia may be a therapeutic options for stroke patients who are not candidates for tPA treatment. More clinical studies are required to elucidate the clinical efficacy of hypothermia treatment in stroke patients.

To my knowledge, my study is the first to demonstrate that hypothermia inhibits the release of HMGB1 from brain cells in the acute stage of ischemia. Immunofluorescence was chosen instead of Western blotting because immunofluorescence can show exact anatomical location of HMGB1 release. By sample preparation method of Western blot, it is difficult to clearly dissociate HMGB1 which is in cytoplasm from a locally released extracellular HMGB1. Therefore, using immunofluorescence, I also wanted to know subcellular distribution of HMGB1 in cortex cells. One report showed that the increase of HMGB1 expression after ischemic injury was inhibited after hypothermia treatment.[53] There are some similarities between the results from this previous study and this study; however, there are two important differences. In the previous study, brain HMGB1 levels were measured by ELISA assay with a whole brain sample. In this study, I used immunofluorescence to show the inhibition of HMGB1 release by hypothermia treatment. I believe that this method shows clearly the change of HMGB1 release in the MCAO rat model. Both immunofluorescence and ELISA results indicate that hypothermia may inhibit the secretion of HMGB1 from the ischemic cortex to systemic circulation.

During sterile injury, like ischemia, HMGB1 is released within 1 hr after ischemia as an early mediator of damage and induces later release of several pro-inflammatory cytokines, such as TNF-α. A previous study demonstrated that

HMGB1 is an important mediator in acute systemic immune activation and subsequent immune suppression, and that neutralizing HMGB1 action with a monoclonal antibody substantially reduced 7 d mortality in a MCAO mice model.[28] In this regard, it is worth targeting HMGB1 and the associated signal pathway as a potential therapeutic strategy and therapeutic hypothermia as a candidate for new adjunctive therapies in the acute stage of ischemic brain injury. Clinical studies for clarifying the benefits of hypothermia in stroke patients will be necessary for its clinical application.

The present study did not demonstrate whether therapeutic hypothermia preferentially blocked the passive release of HMGB1 from necrotic cells, nor the active secretion of HMGB1 from major inflammatory cell types. In developing the HMGB1 inhibition as therapy in acute stroke, a precise knowledge of release mechanisms of HMGB1 is essential. The recently discovered inflammasome is an important signaling platform that senses intracellular stress and pathogenic microorganisms. After sensing threats, the inflammasome activates the secretion of pro-inflammatory cytokines, IL-1β and IL-18.[54] Inflammasome signaling is also the pathway by which HMGB1 is actively released.[55-57] When the actions of inflammasome components are inhibited in siRNA treatment or knockout mouse models, HMGB1 release is reduced. Activity of inflammasome signaling is inhibited by hypothermia treatment in brain trauma injury models.[58] However, there are no studies investigating the change in inflammasome activity in stroke models after hypothermia treatment. Further study will be needed to evaluate the role of inflammasome signaling in the inhibition of HMGB1 release by hypothermia.

I next asked which cell type are the main sources of HMGB1 release in ischemic

injury, and whether hypothermia could inhibit HMGB1 release from that cell type. To answer this question, I co-immunostained the tissue sections with NeuN, a widely used neuronal marker, and HMGB1. The results of the immunofluorescence showed that about 80 % of cortex cells are HMGB1-positive in the nucleus. After MCAO, most HMGB1 was depleted from the cortex. Hypothermia treatment reduced the depletion of HMGB1 from ischemic neurons. This result indicates that HMGB1 is released more preferentially from neuronal cell types in the ischemic cortex 4 hr after the onset of ischemia and hypothermia inhibits this neuronal release. Previous report showed that in the acute phase (3 hr after the onset of ischemia) of stroke, neurons are more sensitive than other brain cell types to ischemic injury, which reflects preferential HMGB1 release from neurons.[27] Further study will be needed to elucidate the specific mechanistic differences between neurons and other cell types that lead to preferential release of HMGB1 in neurons.

To evaluate an importance of the inhibition of HMGB1 release in the neuroprotective effect of hypothermia on stroke, I measured the effects of a pharmacological HMGB1 inhibitor on post-ischemic injury. Glycyrrhizin inhibits HMGB1 action pharmacologically by direct binding to the A and B box domains of HMGB1.[59]

The TTC staining results showed that the inhibition of HMGB1 action by glycyrrhizin reduced the infarct size significantly compared to vehicle-treated MCAO rats. In previous studies, glycyrrhizin treatment reduced the infarct size in an MCAO model, which is consistent with my results;[24,26] however, I employed a permanent MCAO animal stroke model that was not used in previous studies. Therefore, my results are more clinically relevant to most stroke patients who are

not the candidates for thrombolysis.

The immunofluorescence results showed that HMGB1 release from the ischemic cortex was inhibited by hypothermia treatment. This result was not expected by the direct binding mechanism of glycyrrhizin when performing the experiment. However, a previous study reported that glycyrrhizin reduced the HMGB1 release in the ischemic cortex and suggested that glycyrrhizin blocks translocation of HMGB1 from the nucleus to the cytoplasm by inhibiting the phosphorylation of HMGB1.[26] This mechanism may explain the reduction in HMGB1 release by glycyrrhizin in my results.

Previous studies regarding the effects of glycyrrhizin were performed in transient MCAO models, and the effect of glycyrrhizin was assessed in subacute or chronic stages.[24,26] As mentioned in the introduction, permanent occlusion models properly represent the majority of stroke patients who are not candidates for thrombolytic therapy (tPA).[11] The present study shows that glycyrrhizin was able to ameliorate ischemic damage by inhibiting the HMGB1 actions, and by blocking the extracellular HMGB1 secretion in the acute ischemic stage of a more clinically relevant permanent MCAO model. The acute effects of glycyrrhizin on this MCAO model suggest that it may be an effective therapy for acute stage stroke patients. Further clinical studies with ischemic stroke patients will be needed for elucidating clear benefits of glycyrrhizin treatment in clinical application.

Glycyrrhizin was used as the pharmacologic inhibitor against HMGB1 action to clarify the current discrepancy in effects of neutralizing antibodies on infarct size in MCAO models. However, glycyrrhizin is a chemical inhibitor that may possibly

have effects other than direct inhibition of HMGB1 action. For example, my immunofluorescence results showed that glycyrrhizin has effects on HMGB1 release from the ischemic cortex beyond direct HMGB1 binding. To evaluate the more specific effects of HMGB1 inhibition in my MCAO model, HMGB1 neutralizing antibody was injected intracerebroventricularly in the MCAO rats.

Intracerebroventricular injection was used to induce direct access of neutralizing antibodies to the infarct brain region. Between blood vessels and the brain parenchyma is the blood-brain barrier, which can block the access of antibody or drug to the target brain region. The discrepancy in previous studies with HMGB1 neutralizing antibody may be derived from this obstacle. Hence, the neutralizing antibody was applied directly into the brain by intraventricular injection and its effects were measured by TTC staining. The TTC staining results showed that HMGB1 neutralizing antibody treatment reduced the increase of infarct size in the MCAO model. This result indicates that specific direct inhibition of HMGB1 action is protective in ischemic injury, and more clearly demonstrates that HMGB1 is an important mediator in stroke pathophysiology.

The elevation of inflammatory cytokine gene expression by HMGB1 is important in inducing brain tissue damage in stroke. Real-time PCR results demonstrate that the expressions of many inflammatory cytokines (IL-1β, IL-6, TNF-α) were elevated after ischemic injury in the peri-ischemic region, specifically.

Several previous reports have shown the elevation of inflammatory cytokines in ischemic brain;[60,61] therefore, this real-time PCR results are consistent with previous reports. My results also show that hypothermia or pharmacological HMGB1

inhibition reduces the elevations of inflammatory cytokine genes. This suppressive effect of hypothermia on expressions of inflammatory cytokines after ischemic injury was reported previously.[62-64] It is surprising that the pharmacological inhibition of HMGB1 has an effect on inflammatory cytokine gene expression comparable to hypothermia treatment which is the most promising neuroprotectant. Previous studies using *in vivo* and *in vitro* models demonstrated that glycyrrhizin reduces elevated inflammatory cytokine expressions after neuronal injury.[26,65,66] Hence, my results are consistent with previous reports; however, there are differences between the sample preparation paradigms.

Most previous studies have shown the effects of hypothermia on inflammatory cytokine levels, and mRNA or protein levels of inflammatory cytokines were measured in whole ischemic hemisphere samples. The effect of hypothermia on clinically relevant peri-ischemic regions that mimic the ischemic penumbra was not properly evaluated. I prepared tissue samples from three regions of each rat brain that were categorized as ischemic regions, peri-ischemic regions, and the contralateral regions. In particular, the peri-ischemic regions were defined according to the detected perfusion-diffusion mismatch regions in MCAO rats.[67] This findings suggest that the effect of hypothermia on inflammatory cytokine expressions in peri-ischemic regions is an important mechanism in the neuroprotection of hypothermia, and that blocking HMGB1 action has similar effects to hypothermia.

There is a discrepancy between the effects of hypothermia and glycyrrhizin on TNF-α gene expression. Hypothermia attenuated elevated expressions of TNF-α in peri-ischemic regions of ischemic brain, but glycyrrhizin did not. In previous study, mRNA expression of TNF-α was more sensitive to HMGB1 treatment than that of

IL-1β, therefore was reached to a maximal level with lower level of HMGB1.[25] Difference between the sensitivities of these two cytokine expressions in response to HMGB1 may explain the discrepancy described above. The concentration of glycyrrhizin that was used in this study may have been insufficient for complete blocking of HMGB1 signaling, as shown by hypothermia.

In this study, hypothermia was induced 15 min after MCA occlusion and glycyrrhizin and neutralizing antibody were injected 30 min before MCA occlusion. However, in clinical situation, hypothermia or therapeutics cannot be treated in this time phase for stroke patients. To evaluate the possibility of clinical application of hypothermia and glycyrrhizin as neuroprotectant in ischemic stroke, hypothermia treatment and injection of neutralizing antibody or glycyrrhizin should be started, more than 1 hr after MCA occlusion. Further study will be needed to verify the effects of hypothermia and glycyrrhizin or neutralizing antibody injection in more clinically relevant time phases of stroke model.

Ⅴ. CONCLUSIONS

I designed this study to investigate the importance of HMGB1 action in the neuroprotection of therapeutic hypothermia and the inflammatory role of HMGB1 in the propagation of ischemic damage.

1. TTC staining result showed that after 4 hr of mild hypothermia (33°C) treatment, the increase of infarct size in MCAO rat was reduced.
2. The immunohistochemistry results demonstrated that hypothermia attenuated the reduction of HMGB1-positive cells in the ischemic cortex.
3. The ELISA results showed that hypothermia reduced the increase in serum HMGB1 levels in MCAO rats.
4. The TTC staining results showed that intraperitoneal injection of glycyrrhizin inhibited the increase of infarct size in the ischemic cortex.
5. The immunofluorescence analysis showed that glycyrrhizin reduced depletion of HMGB1 in the ischemic cortex.
6. The TTC staining results showed that HMGB1 neutralizing antibody inhibited the increase of infarct size in the MCAO-treated rat cortex.
7. The real-time PCR results showed that hypothermia and glycyrrhizin reduced the elevation of inflammatory cytokine gene expression (IL-1β, IL-6, TNF-α) in peri-ischemic regions of the MCAO-treated rat cortex.

The current study presented that therapeutic hypothermia attenuates ischemic damage by inhibition of extracellular HMGB1 release and HMGB1-induced inflammatory cytokine expressions in the post-ischemic cortex.

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ABSSTRACT (IN KOREAN)

뇌경색에 대한 저체온요법의 치료효과와 관련된 염증기전의 규명

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이 정 호

허혈성 뇌경색에서 염증반응은 뇌경색에 의해 손상받는 부위가 시간이 지남에 따라 확장되는 현상에 관여하며, 뇌경색의 중증도를 결정하는데 있어 중요하다. 치료적 저체온요법은 현재 신경손상을 막는 가장 유망한 치료법으로 알려져 있지만, 신경보호를 일으키는 기전은 확실하게 알려져 있지 못하다. 이 연구에서는, 치료적 저체온증이 허혈성 세포 손상시 분비되는 염증성 사이토카인인 high mobility group box 1 (HMGB1)의 분비를 줄이고, 허혈성 뇌손상에 대한 염증반응의 활성화를 막는다는 가설을 증명하고자 하였다. 연구결과에 의하면, 저체온은 중뇌동맥 폐쇄를 통해 쥐에서 뇌경색과 유사한 상황을 유도한

동물모델에서 뇌경색에 의한 손상부분의 크기를 줄였다. 면역염색화학 분석법과 효소면역측정법(ELISA)을 사용하여 실험한 결과에서, 저체온증은 high mobility group box 1이 뇌경색에 의해 손상받은 뇌부위에서 순환계로 분비되는 과정을 억제하였다. 또한 real-time PCR로 실험한 결과, 저체온증은 뇌경색이 일어난 뇌부위, 그 중에서도 특별히 peri-ischemic region에서 뇌경색 부위가 시간이 지남에 따라 커지는 데에 중요한 기전으로 알려진 염증성 사이토카인의 유전자발현을 줄였다. High mobility group box 1에 직접적으로 결합하여 그 작용을 억제하는 약물인 glycyrrhizin를 뇌경색 동물모델에 투여했을 때에도 저체온증과 유사한 염증성 사이토카인에 대한 효과를 보였다. 결론적으로 이 연구는 저체온증이 high mobility group box 1의 분비를 줄이고, 이에 의해 염증성 사이토카인 발현의 활성화를 억제하는 것을 보였다. 이 연구를 통해 저체온증이 뇌경색에서 보이는 신경보호에서 high mobility group box 1이 중요하게 관련되어 있음을 보였다.

핵심되는 말: 뇌경색, 저체온증, 염증, 사이토카인

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