

Intracarotid cold saline infusion contributes to neuroprotection in MCAO-induced ischemic stroke in rats via serum and glucocorticoid-regulated kinase 1

DAZHI WANG^{1,2*}, ZHI HUANG^{3*}, LEI LI⁴, YINGNAN YUAN⁵, LEI XIANG⁵,
XIAOWEN WU⁵, CAIFANG NI¹ and WENFENG YU²

¹Department of Interventional Radiology, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215006;

²Key Laboratory of Molecular Biology, Guizhou Medical University, Guiyang, Guizhou 550002;

³Department of Interventional Radiology, The Second Affiliated Hospital of Guizhou Medical University, Kaili, Guizhou 556000; ⁴Department of General Courses, People's Armed College of Guizhou University, Guiyang, Guizhou 550025; ⁵School of Medical Imaging, Guizhou Medical University, Guiyang, Guizhou 550002, P.R. China

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Abstract. Intracarotid cold saline infusion (ICSI) brings about neuroprotective effects in ischemic stroke. However, the involvement of serum and glucocorticoid-regulated kinase 1 (SGK1) in the underlying mechanism of ICSI is not fully understood; therefore, we used the rat middle cerebral artery occlusion (MCAO) model to investigate the neuroprotective effects of ICSI on ischemic stroke in rats, as well as the involvement of SGK1 in these effects. ICSI decreased infarct size and brain swelling, as determined by 2,3,5-triphenyltetrazolium chloride staining and the dry-wet weight method, respectively. The results of terminal deoxynucleotidyl transferase mediated nick end labeling (TUNEL) and Nissl staining showed that ICSI also suppressed apoptosis and increased the relative integral optical density (IOD) values of Nissl bodies in the rat MCAO model. Regarding the mechanism, the results of immunohistochemistry and western blotting revealed that ICSI upregulated SGK1 expression and downregulated beclin-1 and LC-3 expression in the rat MCAO model. In addition, SGK1 knockdown increased ICSI-mediated infarct size and brain

swelling, promoted apoptosis, and reduced the IOD values of Nissl bodies in the rat MCAO model. In addition, we found that SGK1 knockdown upregulated beclin-1 and LC-3 expression mediated by ICSI. Overall, ICSI had a neuroprotective effect on ischemic stroke after reperfusion by upregulating SGK1 and inhibiting autophagy.

Introduction

Therapeutic hypothermia is an effective method of neuroprotection (1) that is currently used to treat diseases such as hypoxic ischemic encephalopathy and severe craniocerebral trauma caused by cardiac arrest, with good results (2). Selective brain cooling (SBC) is a therapeutic, low-temperature method that has no effect on whole-body temperature (3). Since SBC avoids the side effects of low temperature, it has received increasing clinical and research attention. In particular, intracarotid cold saline infusion (ICSI) can cool the brain rapidly and uniformly; it is therefore considered an ideal SBC method (4). ICSI is the fastest and most effective way to lower brain temperature and protect the brain (5), with its protective effect being mediated through vascular flushing and low temperature (6,7). In previous studies, ICSI administered before reperfusion improved brain function after middle cerebral artery occlusion (MCAO) (8). The protective effect of ICSI on the brain results from hypothermia and cerebral artery flushing (5). However, the underlying mechanism of ICSI in the brain of patients with ischemic stroke is unclear.

The serum and glucocorticoid-regulated kinase 1 (SGK1) gene encodes a serine/threonine protein kinase that plays an important role in the cellular stress response (9). This kinase activates certain chloride, sodium and potassium channels, suggesting that it is involved in the regulation of cell survival, renal sodium excretion, and neuronal excitability (10). High expression of this gene may be associated with the development of several diseases, including hypertension and diabetic nephropathy (11). SGK1 is a member of the AGC protein kinase family and plays an important role in regulating ion

Correspondence to: Dr Caifang Ni, Department of Interventional Radiology, The First Affiliated Hospital of Soochow University, 889 Pinghai Road, Suzhou, Jiangsu 215006, P.R. China
E-mail: fangcaini66@outlook.com

Dr Wenfeng Yu, Key Laboratory of Molecular Biology, Guizhou Medical University, 9 Beijing Road, Guiyang, Guizhou 550002, P.R. China
E-mail: 2291860147@qq.com

*Contributed equally

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channel expression, as well as in promoting proliferation and survival in malignant epithelial cells (12). Recently, several studies have confirmed that SGK1 has a protective effect on ischemia-reperfusion injury. For instance, one investigation reported alterations in hippocampal *SGK1* gene expression in rats with transient cerebral ischemia (13). Furthermore, SGK1 was recently shown to play a protective role in the hippocampal neurons of rats with ischemia-reperfusion injury (14). In another study, the corticotropin-induced estrogen enhancement effect of SGK1 expression protected the heart from ischemia-reperfusion injury (15). However, no investigations have yet explored whether ICSI can affect SGK1 expression.

Autophagy is a cellular pathway that regulates the transportation of cytoplasmic macromolecules and organelles to lysosomes for degradation (16). Beclin-1 and microtubule-associated protein 1 light chain 3 (LC-3) are two important proteins involved in autophagic flux (17-19). Previous studies have shown that activation of autophagy plays an important role in cerebral ischemia and reperfusion injury (20,21). However, it remains unclear whether autophagic flux is related to the neuroprotective effects of ICSI and SGK1.

In the present study, we investigated the role of ICSI and clarified the mechanism of its neuroprotective effect.

Materials and methods

Animals. In total, 250 male 8 week old Sprague-Dawley rats weighing ~280 g were acquired from The First Affiliated Hospital of Soochow University (Jiangsu, China). The rats housed at a temperature of 22-25°C and 50-65% humidity, with a 12 h light/dark cycle and *ad libitum* access to food and water. The rats were raised individually in an animal facility. All procedures involving animals were approved by the Animal Research Committee of The First Affiliated Hospital of Soochow University.

MCAO model establishment. To conduct surgery, the rats were anesthetized using an intraperitoneal injection of 10% chloral hydrate (400 mg/kg; cat. no. C8383, Sigma-Aldrich/Merck KGaA). Peritonitis was monitored and not observed during the study. Two temperature probes (Physitemp, Clifton, NJ, USA) were placed in the right cerebral cortex (3 mm lateral to bregma, 3 mm posterior, and 4 mm under the surface of the skull), as well as in the rectum, to continuously monitor temperature. An improved intraluminal filament was used to induce MCAO. After 2 h, reperfusion was established by filament shrinkage. To confirm the success rate of MCAO surgery and reperfusion, blood flow in the right cortex of the right MCA was measured using a laser Doppler flow meter 2 mm in the posterior fontanelle and 6 mm in the anterior fontanelle. After the filaments were inserted, 30% of the rats without blood flow reduction were lower than baseline. Reperfusion success was defined as an 80% increase over the baseline in blood flow after the filament was contracted. In groups treated with ICSI, the filament was contracted, and a modified PE50 canaliculus was inserted into the internal carotid artery about 5 mm from the external carotid artery. Next, by infusion with freshly made 10°C saline at a rate of 10-25 ml/h, the brain temperature was reduced to 33°C for 20 min. Normal infusion groups received intraperitoneal normal saline infusion (INSI)

at 37°C, while the other procedures were identical to those in the ICSI groups. A non-infusion group underwent insertion of a filtration membrane to a depth of 15-mm insufficient to induce MCAO and no infusion of renal tubules. After transfusion, the rats were subjected to rapid rewarming using a heating pad and bubble wrap; when their brain temperature had returned to baseline, they were considered reheated.

Grouping. The rats were randomly divided into five groups: i) non-infusion group (MCAO rats with no infusion; n=25; mortality rate: 4.0%); ii) ICSI group (MCAO rats treated with ICSI immediately after reperfusion; n=90; mortality rate: 15.6%); iii) INSI group (MCAO rats treated with INSI immediately after reperfusion; n=25; mortality rate: 8.0%); iv) ICSI+reperfusion group (MCAO rats treated with ICSI for 1 h immediately after reperfusion; n=85; mortality rate: 11.8%); and v) INSI+reperfusion group (MCAO rats treated with INSI for 1 h immediately after reperfusion; n=25; mortality rate: 8.0%). In addition, shRNAs for SGK1, as well as negative control (NC) shRNAs, were obtained from GenePharma Co., Ltd. (Shanghai, China). Rats in the ICSI and ICSI+reperfusion groups were treated using NC shRNAs and SGK1 shRNAs, respectively, while those in the ICSI group (n=50) were divided into the ICSI+NC subgroup (n=25; mortality rate: 4.0%) and ICSI+shSGK1 subgroup (n=25; mortality rate: 6.0%); the rats in the ICSI+reperfusion group (n=50) were divided into the ICSI+reperfusion+NC subgroup (n=25; mortality rate: 6.0%) and ICSI+reperfusion+shSGK1 subgroup (n=25, the mortality rate: 6.0%). After treatment for 50 h, the indicators were evaluated, the rats were euthanized, and their tissues were used in subsequent experiments. During the course of the experiment, the animals were also euthanized by cervical dislocation when they reached the standard of the humane endpoint. The mortality rate represents animals prematurely euthanized after reaching humane endpoints, rather than animals that were found dead. Humane endpoint is a refinement strategy to reduce the pain and suffering suffered by the animals during the experiment as much as possible. Humane endpoints mainly include weight loss of 20-25%, loss of appetite (anorexia more than 24 h), inability to stand, severe infection, tumors more than 10% of body weight, organ system failure, dyspnea, severe anemia, renal failure and peritoneal fluid. Animals were euthanized when two or more humane endpoints were observed.

Infarct size. As described in previous studies (22,23), after reperfusion for 48 h, the animals were anesthetized by intraperitoneal injection of 10% chloral hydrate (400 mg/kg) and then decapitated. The brain was rapidly removed, and the coronal section of brain was sliced in a special groove (thickness: 2 mm) after cooling in ice-cold saline for 10 min. The slices were immediately immersed in 4 ml of 1% 2,3,5-triphenyltetrazolium chloride (TTC) and incubated at 37°C for 10 min. Next, the TTC solution was replaced with 4% polyoxymethylene. After 8 h, a digital camera was used to photograph the sections, and the infarct size was measured using the UTHSCSA Image Tool 3.0 software (<https://uthscsa-image-tool.software.informer.com/>). To eliminate the effect of postoperative edema on injury volume, the infarcted volume (%) was calculated according to the following method:

(Volume of the left hemisphere-Non-infarct volume of right hemisphere)/(Volume of the left hemisphere) x100%.

Brain swelling. According to previous research (24), brain swelling was assessed using a 0.35-T Signa Ovation Excite MRI scanner (General Electric, Milwaukee, WI, USA).

Knockdown of *SGK1*. For *SGK1* knockdown, *SGK1* and control shRNA were acquired from RiboBio (Guangzhou, Guangdong, China). *SGK1* knockdown rats were obtained from the Shanghai Research Center for Model Organisms (Shanghai, China). In brief, after anesthesia, rats were placed on a stereotactic apparatus, and 50 μ g *SGK1* and control shRNA vectors were injected into the right ischemia region of rats, and the final volume was 10 μ l. The sequence of shSGK1 was as follows: Top strand, 5'-CACCGCCGGCTGTGCCTTCTCTCCATTCAAGAGATGGAGAGAAGGCACAGCCG GC-3'; bottom strand, 5'-AAAAGCCGGCTGTGCCTTCTCTCCATCTCTTGAATGGAGAGAAGGCACAGCCG GC-3'; Ds Oligo, 5'-CACCGCCTTCTCTCCATCCGC TGCTTTCAAGAGAAGCAGCGGATGGAGAGAAGGC-3' and 3'-CGGAAGAGAGGTAGGCGACGAAAGTTC TCTTCGTCGCCTACCTCTCTTCCGAAAA-5'.

Terminal deoxynucleotidyl transferase mediated nick end labeling (TUNEL) staining. To identify apoptosis in the ipsilateral hippocampus, TUNEL staining was performed using the *In Situ* Cell Death Detection Kit (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. Briefly, the sections were washed for 30 min. They were then incubated in 0.1% Triton X-100 and 0.1% sodium citrate and rinsed three times using phosphate-buffered saline (PBS) for 10 min each time. The slices were then incubated in 0.3% PBS/0.1% Tween-20 for 30 min to inhibit endogenous peroxidase activity; they were then once again rinsed in PBS. Next, the sections and the 50- μ l TUNEL reaction mixture of the *in situ* Cell Death Detection Kit were incubated in a moist atmosphere for 60 min at 37°C in the dark. The slices were rinsed three times in PBS and observed under a Nikon TE300 microscope (Nikon, Inc., Tokyo, Japan). A negative control was achieved in each pore by incubating the slices in 50- μ l labeled solution instead of TUNEL reaction mixture. At least 10 randomly selected fields were used to count TUNEL-positive cells (x200 magnification). The cell count was performed by researchers who had been blinded to the rat conditions.

Nissl staining. Three days after the operation, the rats were anesthetized using 10% chloral hydrate (400 mg/kg body weight) by intraperitoneal injection, and their brains were extracted and fixed in 4% paraformaldehyde for 2 h. After continuous overnight incubation in 0.1 mol/l PBS containing 20 and 25% glucose, the brain was cut into 10-mm coronal sections using a cryogenic thermostat (Leica CM1950; Leica, Wetzlar, Germany) and stored at 20°C. Then, Nissl staining solution (Beyotime Institute of Biotechnology, Shanghai, China) was used, following the manufacturer's instructions. Briefly, the sections were soaked in 1% toluidine blue and dyed for 5 min at 50°C, washed with double distilled water, and then dehydrated with gradient ethanol; the sections were observed

under a Nikon Eclipse E400 microscope (x200 magnification; Nikon, Inc.).

Immunohistochemical analysis. Frozen brain sections in OCT compound (14- μ m thick) were formalin-fixed and subjected to immunohistochemical analysis to determine SGK1 expression. Endogenous peroxidase was blocked using 3% H₂O₂ for 5 min. The sections were then washed in PBS for a short time and cooled at room temperature for 20 min. They were then rinsed again in PBS. Non-specific protein was blocked by incubation in 5% horse serum for 40 min. The sections were incubated with primary antibodies (anti-SGK1; diluted 1:200; cat. no. YB-33275M; Suzhou Ard Biological Co., Ltd., Shanghai, China) for 1 h at room temperature. They were then incubated with fluorescein isothiocyanate/cyanine 3-conjugated IgG (1:500 dilution; cat. no. SC-2340; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at room temperature for 60 min. Ten microscopic fields per section were randomly photographed (x200; Nikon TE300; Nikon, Inc.) to allow the SGK-1-positive cells to be counted.

Western blotting. Treated tissues were lysed using RIPA buffer (Sigma-Aldrich/Merck KGaA). Total protein was separated using 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (cat. no. 88518; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The membranes were blocked with 5% non-fat milk for 1 h at room temperature and then subjected to incubation at 4°C overnight in primary antibodies against SGK1 (1:500; cat. no. ab59337), beclin-1 (1:2,000; cat. no. ab207612;), LC-3 (1:3,000; cat. no. ab51520) and GAPDH (1:2,500; cat. no. ab9485; all Abcam, Cambridge, UK). The next day, after washing, the membranes were incubated in secondary, peroxidase-conjugated anti-mouse (1:2,000, cat. No. 7076; Cell signaling Technology, Inc.) or anti-rabbit (1:2,000, cat. No. 5127; Cell signaling Technology, Inc.) antibodies for 1.5 h at room temperature. Finally, the results were determined using an enhanced chemiluminescence (ECL) substrate kit in an ECL system (Amersham Pharmacia). Quantity One software version 4.6.2 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for densitometric analysis.

Statistical data analysis. All data are presented as mean \pm standard error, and Graphpad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analysis. Student's t-test was used to evaluate the difference between two groups; one-way ANOVA with Tukey's post hoc was applied to assess the differences among more than two groups, and P-values <0.05 were considered as indicative of statistical significance.

Results

ICSI alleviates infarct size and brain swelling after MCAO. To induce ischemic stroke, MCAO was performed in Sprague-Dawley rats for 2 h by applying an intraluminal filament. The MCAO rats were treated with either ICSI or INSI after reperfusion (Fig. 1A). After reperfusion for 48 h, the infarct size was determined using TTC staining; brain swelling was also measured. The results indicated that the infarct size was significantly reduced in the ICSI and INSI groups compared with the non-infusion group; ICSI also alleviated

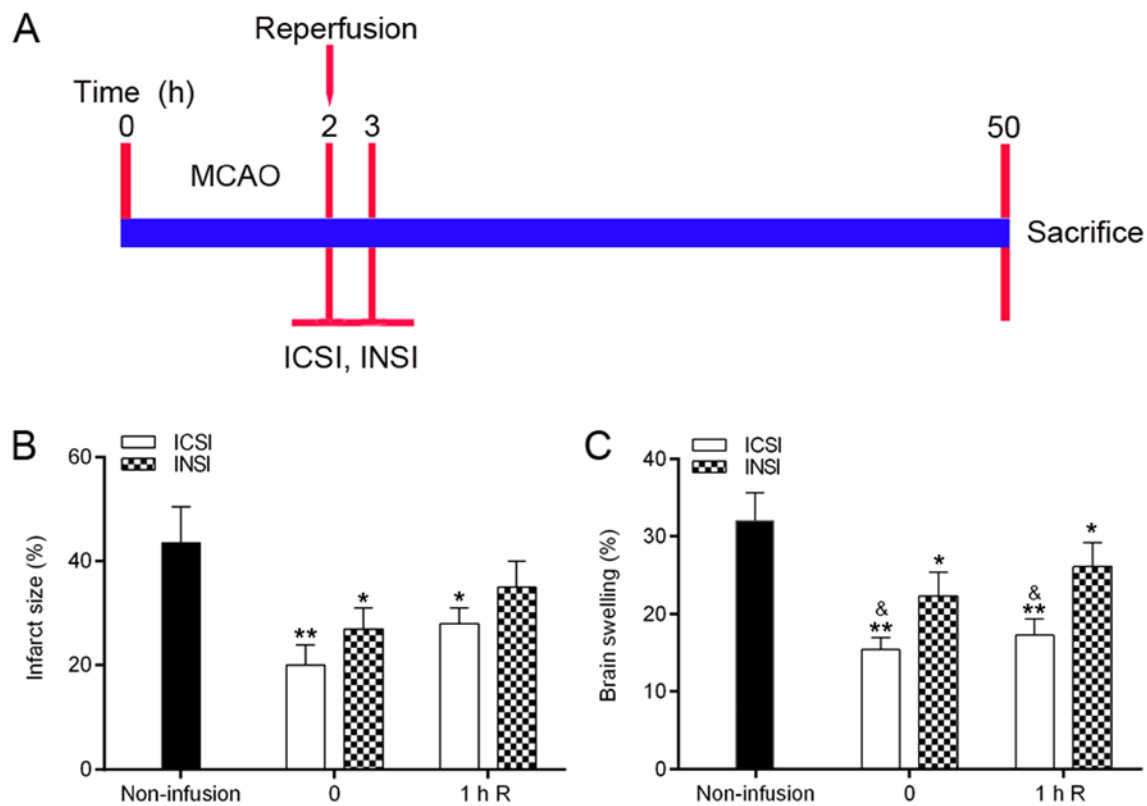


Figure 1. ICSI decreases infarct size and brain swelling in the rat MCAO model. Rats were randomly divided into the MCAO group (n=24), ICSI group (n=26), INSI group (n=23), ICSI+reperfusion group (n=25), and INSI+reperfusion group (n=23). (A) Experimental procedure in each group of rats is shown. (B) Infarct size and (C) brain swelling were detected in the rat MCAO models treated with reperfusion and either ICSI or INSI. * $P<0.05$, ** $P<0.01$ (infusion groups vs. non-infusion group); * $P<0.05$, ICSI vs. INSI. One-way ANOVA with Tukey's post hoc test was performed. All experiments were repeated three times. MCAO, middle cerebral artery occlusion; ICSI, intracarotid cold saline infusion; INSI, intracarotid normothermic saline infusion. 0, immediate infusion after reperfusion; 1 h R, 1 h after reperfusion.

the infarct size after reperfusion in the MCAO model ($P<0.05$, $P<0.01$; Fig. 1B). Brain swelling was significantly decreased in the ICSI and INSI groups relative to the non-infusion group. After reperfusion for 1 h, the brain swelling in the ICSI and INSI groups also showed significant reductions. In addition, brain swelling was significantly decreased in the ICSI group compared with the INSI group after reperfusion in the MCAO model ($P<0.05$, $P<0.01$; Fig. 1C).

ICSI alleviates cell apoptosis and nerve injury in the brain. To further explore the effects of ICSI on apoptosis and nerve injury in the brain after reperfusion in the MCAO model, TUNEL and Nissl staining were performed. There were significantly fewer TUNEL-positive cells in the ICSI and ICSI-1h-R groups than that noted in the non-infusion, INSI and INSI-1h groups ($P<0.05$, $P<0.01$, $P<0.001$; Fig. 2A and B). We also found that the relative integral optical density (IOD) values of Nissl bodies were higher in the ICSI and ICSI-1h-R groups than in the non-infusion group, that they were higher in the INSI and INSI-1h groups than in the non-infusion group, and that they were higher in the ICSI and ICSI-1h-R groups than in the INSI and INSI-1h groups ($P<0.01$, $P<0.001$; Fig. 2A and C).

ICSI increases the expression of SGK1 and decreases the expression of the autophagy markers beclin-1 and LC-3. To determine the expression of SGK1, immunohistochemistry was performed. The results showed that SGK1 expression was

higher in the infusion groups than that in the non-infusion group, that it was higher in the ICSI group than in the INSI group, and that it was higher in the ICSI-1h-R group than in the INSI-1h-R group (Fig. 3A). In addition, we confirmed that SGK1 expression was higher in the infusion groups than in the non-infusion group, that it was significantly higher in the ICSI and ICSI-1h-R groups than in the INSI and INSI-1h-R groups. The expression of both beclin-1 and LC-3 was significantly downregulated in the infusion groups compared with that in the non-infusion group, and it was significantly decreased in the ICSI and ICSI-1h-R groups compared with that in the INSI and INSI-1h-R groups ($P<0.05$, $P<0.01$, $P<0.001$; Fig. 3B).

Knockdown of SGK1 decreases the protective effect of ICSI. To investigate whether ICSI contributes to SGK1-mediated neuroprotection in rats with ischemic stroke, animals in the ICSI and ICSI-1h-R groups were transfected with SGK1 shRNA. Four groups of rats were then subjected to cold saline infusion: Rats given immediate ICSI (ICSI group), SGK1-knockdown rats given immediate ICSI (ICSI+shSGK1), rats given ICSI starting 1 h after reperfusion (ICSI-1h-R), and SGK1-knockdown rats given ICSI starting 1 h after reperfusion (ICSI-1h-R+shSGK1; Fig. 4A). A western blot assay was used to verify the knockdown effect of SGK1, and the results revealed that SGK1 expression was significantly suppressed after transfection with SGK1 shRNAs ($P<0.01$, $P<0.001$; Fig. 4B). TTC staining and the dry-wet weight method were

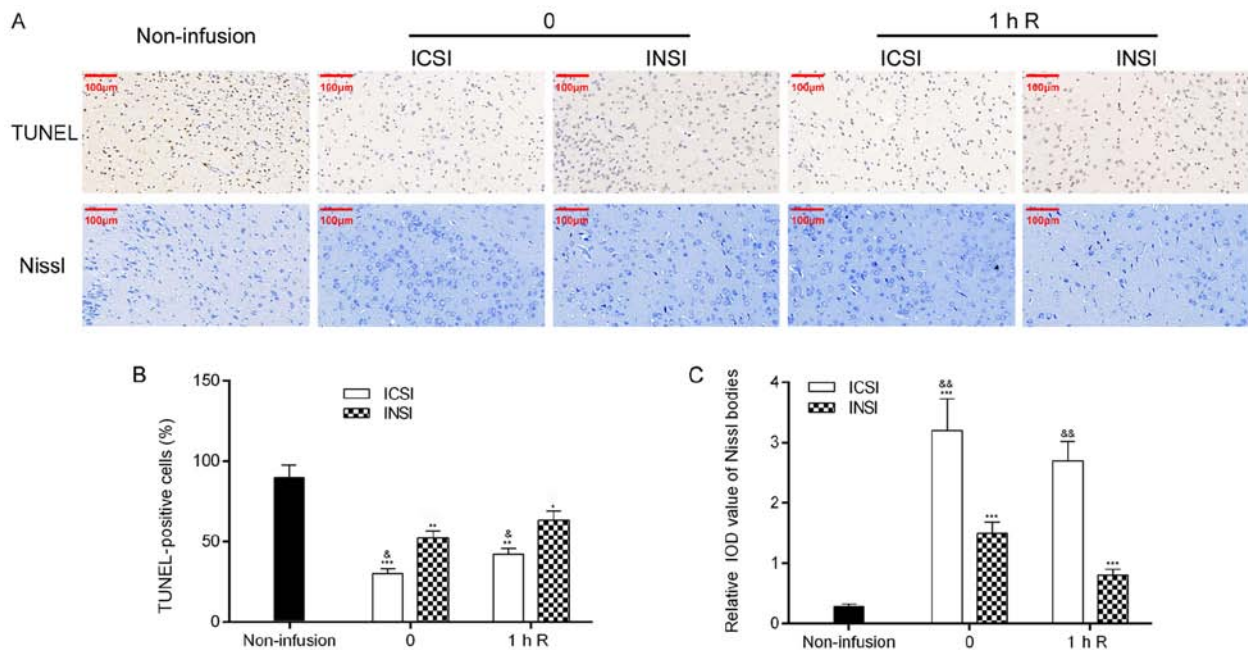


Figure 2. ICSI inhibits apoptosis and increases the relative IOD value of Nissl bodies in the rat MCAO model. (A) Apoptosis and the relative IOD value of Nissl bodies were measured using TUNEL staining and Nissl staining, respectively. Magnification, x100; scale bar, 100 μ m. (B) TUNEL-positive cells were analyzed. (C) Relative IOD value of Nissl bodies was determined. * P <0.05, ** P <0.01, *** P <0.001, infusion groups vs. non-infusion group. & P <0.05, &< P <0.01, ICSI vs. INSI group; comparisons were carried out at the same time point. Data were evaluated using one-way ANOVA with Tukey's post hoc test. All experiments were repeated three times. IOD, integral optical density; MCAO, middle cerebral artery occlusion; ICSI, intracarotid cold saline infusion; INSI, intracarotid normothermic saline infusion. 0, immediate infusion after reperfusion; 1 h R, 1 h after reperfusion.

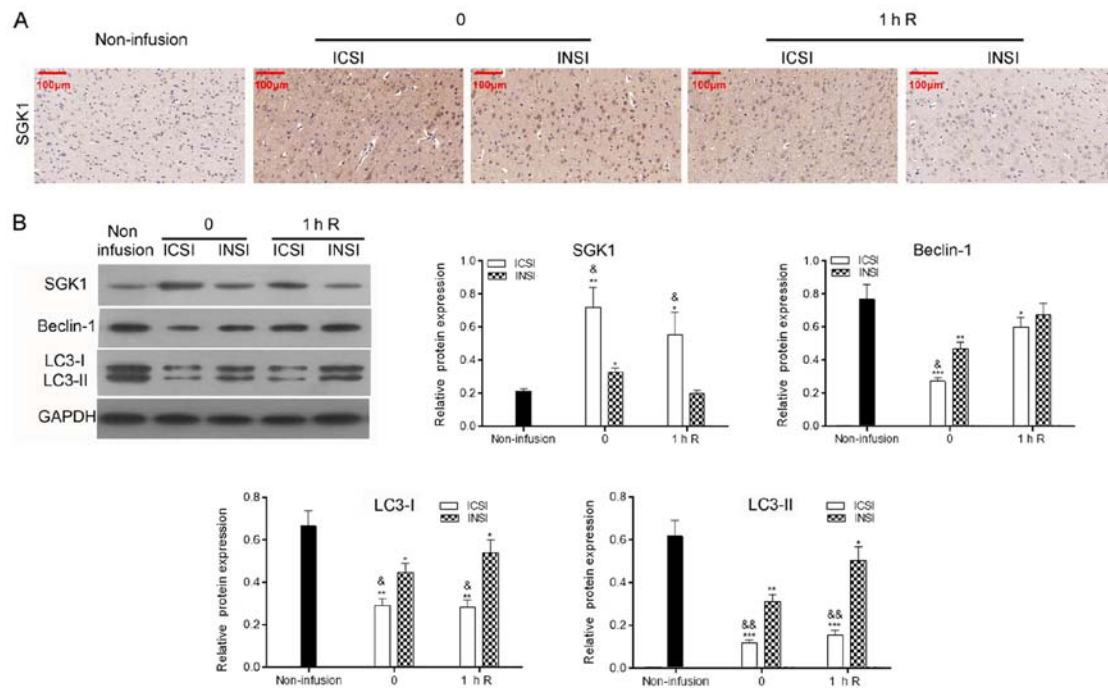


Figure 3. ICSI upregulates SGK1 expression and downregulates beclin-1 and LC-3 expression in the rat MCAO model. (A) SGK1 expression was assessed using immunohistochemistry. Magnification, x100; scale bar, 100 μ m. (B) Western blot analysis of SGK1, beclin-1 and LC-3 expression. * P <0.05, ** P <0.01, *** P <0.001, infusion groups vs. non-infusion group; & P <0.05, &< P <0.01, ICSI vs. INSI; comparisons were carried out using one-way ANOVA with Tukey's post hoc test at the same time point. All experiments were repeated three times. SGK1, serum and glucocorticoid-regulated kinase 1; MCAO, middle cerebral artery occlusion; ICSI, intracarotid cold saline infusion; INSI, intracarotid normothermic saline infusion. 0, immediate infusion after reperfusion; 1 h R, 1 h after reperfusion.

applied to measure infarct size and brain swelling. The results revealed that knockdown of SGK1 using shRNAs significantly increased infarct size and brain swelling, as compared to the

ICSI group (P <0.05, P <0.01; Fig. 4C and D). Therefore, we suggest that SGK1 knockdown aggravated the process of ischemic stroke treatment using ICSI.

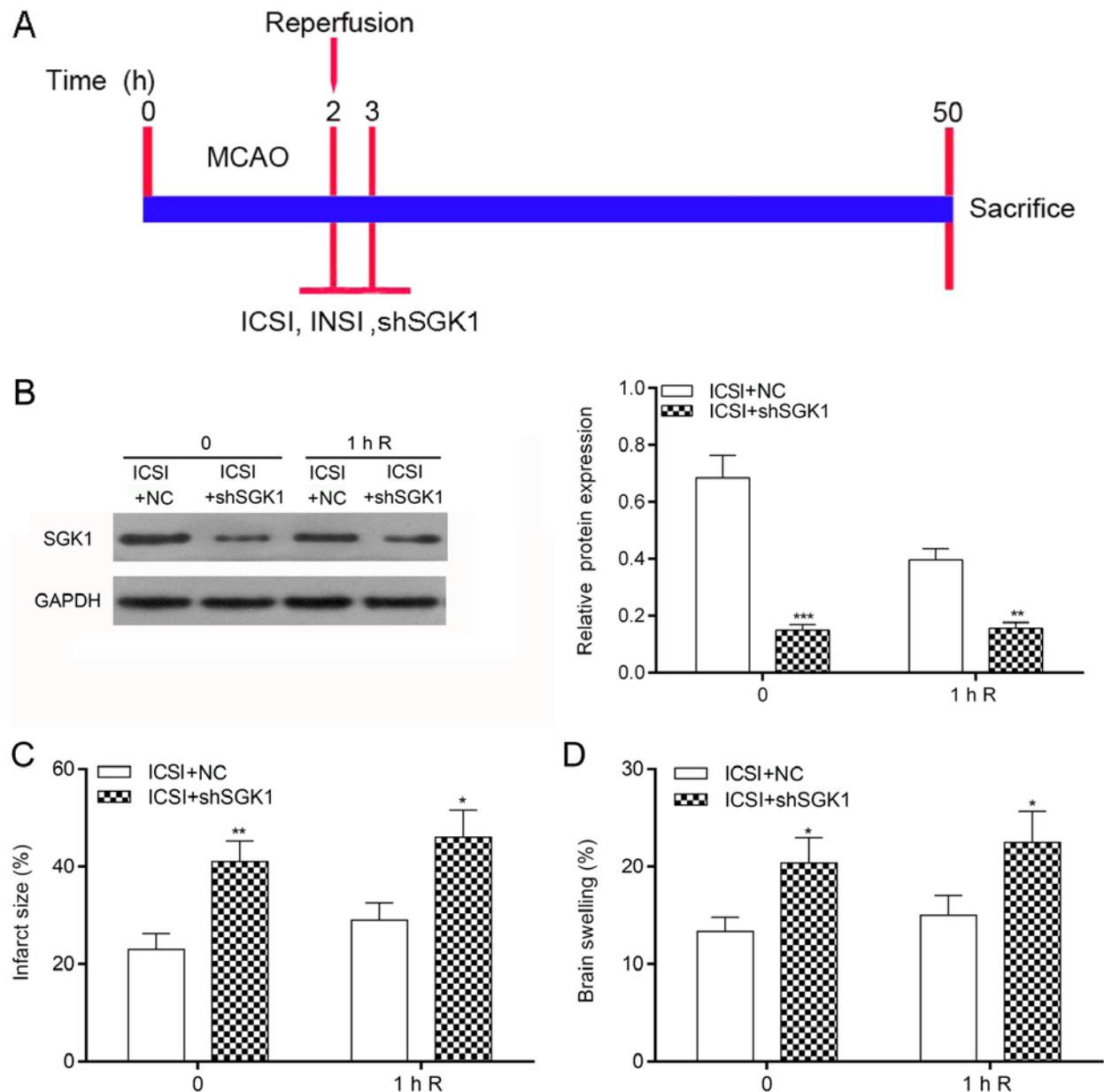


Figure 4. SGK1 knockdown increases infarct size and brain swelling mediated by ICSI. NC shRNAs or SGK1 shRNAs were transfected into the rats in the ICSI and ICSI+reperfusion groups. The rats were divided into the ICSI+NC group (n=24), ICSI+shSGK1 group (n=23), ICSI+reperfusion+NC group (n=23), and ICSI+reperfusion+shSGK1 group (n=23). (A) Protocol of the experiments. (B) SGK1 expression was examined by western blot analysis in each group. (C) Infarct size was measured using TTC staining after SGK1 knockdown in the ICSI group. (D) The brain swelling of the ICSI group after SGK1 knockdown. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. ICSI group by Student's t-test. All experiments were repeated three times. SGK1, serum and glucocorticoid-regulated kinase 1; MCAO, middle cerebral artery occlusion; ICSI, intracarotid cold saline infusion; INSI, intracarotid normothermic saline infusion. 0, immediate infusion after reperfusion; 1 h R, 1 h after reperfusion.

Knockdown of SGK1 decreases the protection of neurons from apoptosis and injury. To explore whether SGK1 affects apoptosis and ischemic stroke injury in rats treated using ICSI, TUNEL staining and Nissl staining were performed. The results showed that SGK1 knockdown using shRNAs promoted ICSI-mediated apoptosis in the MCAO model ($P<0.05$, $P<0.01$; Fig. 5A and B). Meanwhile, we demonstrated that SGK1 knockdown significantly increased the relative IOD value of Nissl bodies mediated by ICSI in the MCAO model ($P<0.01$, Fig. 5A and C). Therefore, we confirmed that SGK1 knockdown significantly accelerated apoptosis and increased the relative IOD value of ICSI-mediated Nissl bodies in the rat MCAO model.

Knockdown of SGK1 upregulates ICSI-mediated downregulation of beclin-1 and LC-3 expression. Furthermore, western blotting was performed to analyze the influence of SGK1 knockdown on the expression of beclin-1 and LC-3 downregulated by ICSI in the rat MCAO model. These results indicated that the ICSI-mediated decreases in the expression of Beclin1 and LC3 could be significantly upregulated by the knockdown of SGK-1 ($P<0.05$, $P<0.01$, $P<0.001$; Fig. 6).

Discussion

Ischemic stroke involves hemiplegia and disturbance of consciousness caused by cerebral thrombosis, cerebral

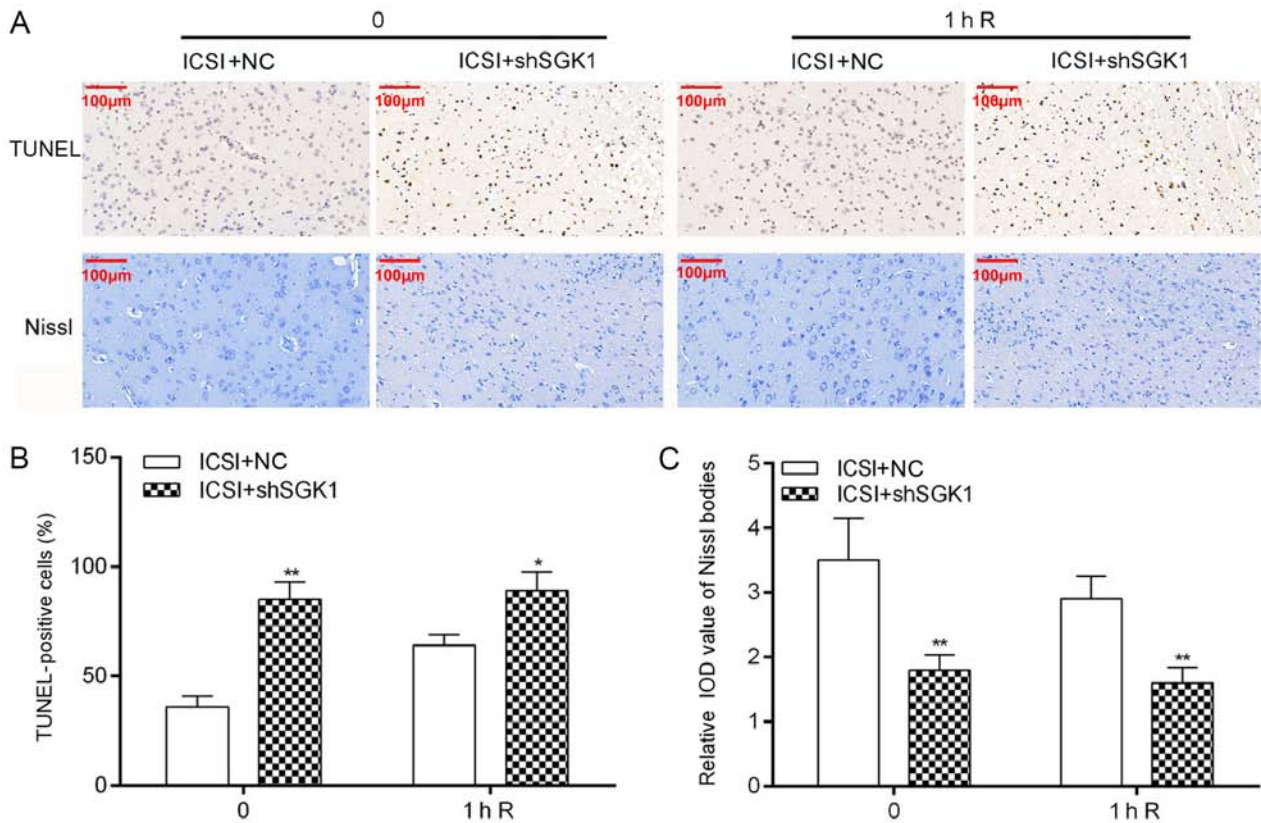


Figure 5. SGK1 knockdown promotes apoptosis and decreases the related IOD values in ICSI-mediated Nissl bodies in the rat MCAO model. (A) TUNEL staining and Nissl staining in the ICSI group after SGK1 knockdown (shSGK1). Magnification, $\times 100$; scale bar, $100\ \mu\text{m}$. (B) TUNEL staining in the ICSI groups after SGK1 knockdown. (C) Nissl staining in the ICSI groups after SGK1 knockdown. * $P < 0.05$, ** $P < 0.01$ vs. ICSI+NC group by Student's t-test. All experiments were repeated three times. SGK1, serum and glucocorticoid-regulated kinase 1; MCAO, middle cerebral artery occlusion; ICSI, intracarotid cold saline infusion; 0, immediate infusion after reperfusion; 1 h R, 1 h after reperfusion.

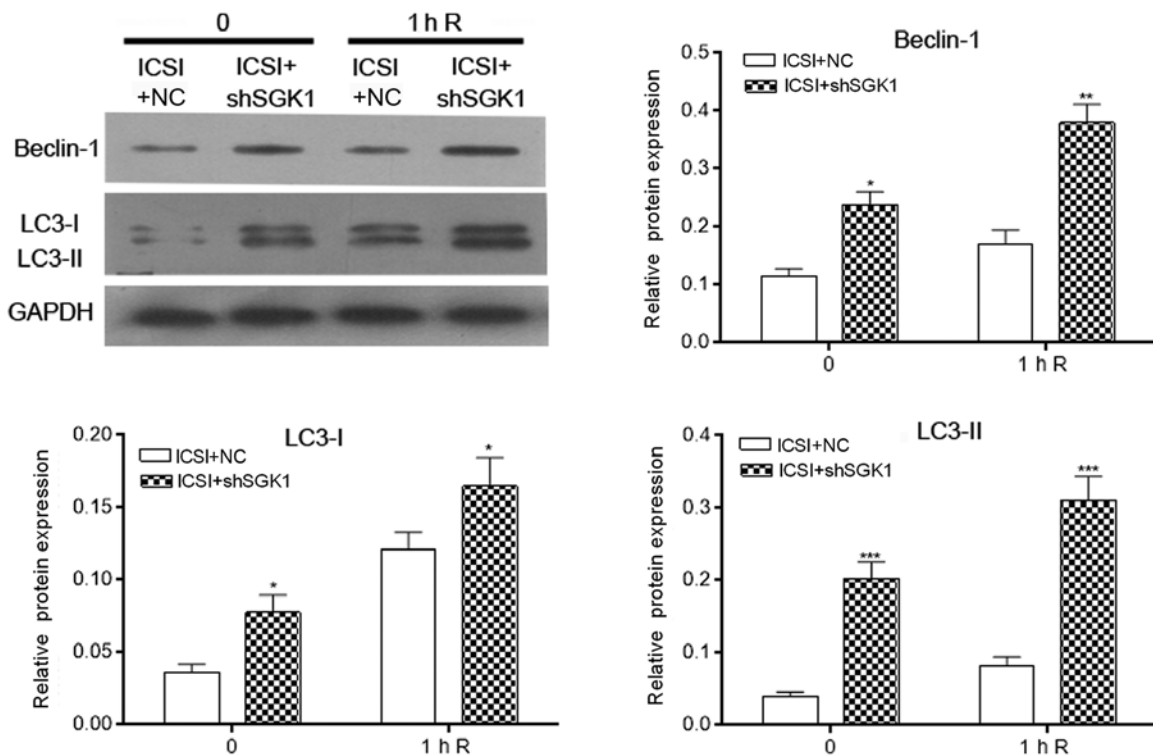


Figure 6. SGK1 knockdown upregulates ICSI-mediated decreased beclin-1 and LC-3 expression in the rat MCAO model. Western blot analysis of beclin-1 and LC-3 in the ICSI groups after SGK1 knockdown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. ICSI+NC group by Student's t-test. All experiments were repeated three times. SGK1, serum and glucocorticoid-regulated kinase 1; MCAO, middle cerebral artery occlusion; ICSI, intracarotid cold saline infusion; 0, immediate infusion after reperfusion; 1 h R, 1 h after reperfusion.

artery occlusion, and consequent cerebral infarction (25). At present, ischemic stroke is the most common cerebrovascular disease (26). It is characterized by its high incidence, high disability rate, high recurrence rate, and high fatality rate, and it has become a major threat to human health (27). Therefore, it is crucial that researchers and clinicians discover preventative and treatment strategies.

Therapeutic hypothermia is an effective method of protecting brain cells, and it is widely used to treat hypoxic-ischemic encephalopathy (28). The neuroprotective mechanisms of low temperature include i) reducing the metabolism of nerve cells and reducing acidosis; ii) inhibiting the production and release of endogenous harmful substances, such as glutamic acid, aspartic acid and serotonin; iii) protecting the blood-brain barrier and reducing cerebral edema and intracranial pressure; iv) preventing the proliferation of polymorphonuclear leukocytes and reducing the intracellular inflammatory response; v) altering the transmission of genetic information and promoting protein synthesis and recovery; and vi) inhibiting nerve cell apoptosis (29). Intracarotid cold saline infusion (ICSI) is a novel and selective cryogenic method (30,31). A large number of clinical studies have shown that ICSI can rapidly reduce brain temperature in rats, significantly protecting brain function and ensuring clinical safety (25,32,33).

In the present study, the rat middle cerebral artery occlusion (MCAO) model was established and treated using ICSI and intracarotid normothermic saline infusion (INSI). TTC staining and brain swelling measurement were used to evaluate the effects of ICSI on local brain tissues. In the present study, ICSI was found to alleviate infarct size and brain swelling in the MCAO model after reperfusion. In addition, it was confirmed that ICSI inhibited cell apoptosis and nerve injury in the brain. Therefore, it was further demonstrated that ICSI is a fast, efficient, and safe method of implementing selective hypothermia in the brain. Selective cerebral hypothermia during acute ischemia has therapeutic effects on ischemic reperfusion, including reducing infarct volume, reducing brain swelling, and improving neurological deficits.

Previous research has revealed that serum and glucocorticoid-regulated kinase 1 (SGK1) protects against ischemia-reperfusion injury. For instance, dexamethasone, which is associated with SGK1, can protect against renal ischemia-reperfusion injury (34). SGK1 is involved in renal ischemia-reperfusion injury (35), while glucocorticoid affects SGK1 expression and protects against ischemia-reperfusion injury after heart transplantation (36). SGK1 inhibits cell death and inflammation in the ischemic-reperfused heart (37). In the present study, it was demonstrated that ICSI increased SGK1 expression and that SGK1 silencing increased infarct size and brain swelling, accelerated apoptosis, and decreased the relative IOD value of ICSI-mediated Nissl bodies. Therefore, we suggest that ICSI protected against brain injury in MCAO-induced ischemic stroke rats.

Autophagy is an evolutionarily conserved process in eukaryotes that processes the turnover of intracellular substances (16,38,39). Many studies have shown that maladjusted autophagy regulation is related to various diseases, such as malignant tumors, autoimmune disease, neurodegenerative disease and pathogenic microorganism infection (40-43). Beclin-1 and LC-3 serve as specific markers of autophagy and have been applied in

both clinical and basic research (44,45). In the present study, it was revealed that ICSI downregulated beclin-1 and LC-3 expression and that knockdown of SGK1 upregulated beclin-1 and LC-3 expression. ICSI inhibited autophagy by regulating SGK1 expression in the rat MCAO model.

ICSI was demonstrated to have neuroprotective potential in MCAO-induced ischemic stroke. In addition, ICSI significantly upregulated SGK1 expression, and SGK1 silencing promoted cerebral injury in MCAO-induced ischemic stroke. Furthermore, it was confirmed that ICSI was conducive to the neuroprotective efficacy of MCAO-induced ischemic stroke in rats by SGK1 and autophagy. However, additional studies are needed to validate the function of SGK1 and the potential mechanism of ICSI in ischemic stroke, and we must optimize our experimental grouping.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

DW, ZH, CN and WY conceived and designed the study. DW and ZH performed the experiments. LL, YY, LX and XW analyzed the data and were the primary contributors to the writing of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The First Affiliated Hospital of Soochow University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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