

Original Article

SCM-198 protects ischemic brain injury via accelerating the recovery of brain glucose metabolism, ameliorating the damage of neurons and inhibiting the activation of microglia

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Abstract: SCM-198 was chemically synthesized based on the structure of Leonurine and have been found to be effective in prevention of ischemic stroke; However, its therapeutic effect on ischemic stroke was poorly understood. In this study, we used rats to build up permanent middle cerebral artery occlusion (MCAO) model to determine the therapeutic effects and potential mechanism of SCM-198 on ischemic injury. 2,3,5-triphenyltetrazolium chloride (TTC) staining, neurological deficit evaluation, ¹⁸F-FDG PET/CT scanning, transmission electron microscopy (TEM) and immunohistochemical staining were employed in the present study. TTC staining and neurological deficit evaluation results revealed that, in a 2 h window of opportunity, infarct volume and neurologic deficit scores were both decreased after SCM-198 treatment. In the PET/CT scanning study, we found the Ipsilateral-to-Contralateral ratio of all SCM-198 treatment groups were bigger than that of the MCAO group. In the TEM and immunohistochemical staining parts, we found the morphology of neurons was improved and the activation level of microglia was inhibited after SCM-198 treatment. Those results indicated that SCM-198 was a potential therapeutic option for ischemic injury in a 2 h window of opportunity, which may be related with its effects in recovering the brain glucose metabolism, ameliorating the damage of neurons and inhibiting the activation of microglia after ischemic injury happened.

Keywords: Ischemic stroke, SCM-198, MCAO

Introduction

Stroke is a main cause of morbidity and mortality throughout the world. There are two kinds of stroke, the more common kind, called ischemic stroke, accounts for approximately 85% of all strokes [1]. Ischemic stroke is mainly caused by a reduction or complete blockade of blood flow to regions of the brain, which leads to nutrition and oxygen delivery failure. Consequently, ischemic cascade is started, leading to a reduction in energy production associated with accumulation of toxic metabolites such as inflammatory mediators and free radicals that ultimately resulted in neurodegeneration.

Nowadays, thrombolysis is the only currently effective therapy for ischemic stroke; however, its application is limited due to the risk of intra-

cranial hemorrhagic transformation [2]. Recently, a lot of attention has been focused on SCM-198, which was chemically synthesized based on the structure of Leonurine, for its protective properties on prevention of ischemic stroke [3] and cardiovascular disease [4, 5] through anti-oxidation and regulation of mitochondrial function. In another study, Liu et al. demonstrated that Leonurine, a natural active ingredient of *Herba leonuri*, also had therapeutic effects on experimental stroke rats by increased activities of UCP4, SOD, CAT and Bcl-2, decreased levels of MDA and Bax, and ameliorated ultrastructure of mitochondria [6]. All those evidences indicated that SCM-198 may be a potential option for the treatment of ischemic stroke.

Clinically, stroke patients are often experience a significant temporal delay between ischemic

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onset and treatment initiation. Thus, therapeutic window of opportunity is of major consideration for all potential therapy of stroke. In this study, we used rats to build up permanent middle cerebral artery occlusion model and further investigated the therapeutic effects and potential mechanisms of SCM-198 on ischemic stroke in a 2 h window of opportunity.

Materials and methods

Chemical synthesis of SCM-198

SCM-198 was synthesized from syringic acid by carbonylation, reaction with thionyl chloride, and the Gabriel reaction, as previously described [7].

Animals and drug administration

This study was approved by the Ethical Committee of Animal Research of Fudan University; all experiments were performed strictly according to international guidelines on animal research. 8 weeks old male Sprague-Dawley (SD) rats, weighted 180-220 g, were supplied by the Laboratory Animal Research Center of Fudan University (Shanghai, China). All animals were fed rat chow and water *ad lib* and housed under a condition of 12-hour light/dark cycle. Rats were randomly divided into the Sham-operated group (Control group), MCAO model group (MCAO group), SCM-198 treatment group I (first treatment happened at 0.5 h after MCAO operation; Hereafter referred to as: 0.5 h group), SCM-198 treatment group II (first treatment happened at 1 h after MCAO operation; Referred to as: 1 h group) and SCM-198 treatment group III (first treatment happened at 2 h after MCAO operation; Referred to as: 2 h group) in accordance with particular situations. SCM-198 was administered by tail vein injection at dose of 15 mg/kg/d at predefined time after surgery, then once daily thereafter. Rats in the Control group and MCAO group were treated with equivalent volume of normal saline instead.

MCAO model

Ischemic stroke was induced to the rats by the method of permanent middle cerebral artery occlusion (MCAO) as previously described [3]. Briefly, rats were anesthetized with a ketamine/xylazine mixture (0.1 mL/100 g intraperitone-

ally), an incision of approximate 2 cm was cut between the right orbit and the external auditory canal. A hand-held drill was used to dig a small hole through the surface of skull. After removing the dura, left middle cerebral artery was exposed. Then, the middle cerebral artery was occluded by a cautery pencil (Advanced Meditech International, Inc, Flushing, New York, USA) from proximal to the olfactory tract to the inferior cerebral vein. To ensure a permanent occlusion, the branches of the MCA between these two points were occluded as well. The wound was closed with sutures.

Measurement of infarct volume after MCAO

Infarct volume was determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining. After three days treatment, animals (n=6 per group) were anesthetized intraperitoneally with 10% chloral hydrate and decapitated. Rat brains were immediately removed and sliced into 7 to 8 coronal slices (2 mm thick) using a brain slice matrix (RWD Life Science Co., Ltd, Shenzhen, China). Slices were then stained with 1% TTC solution at 37°C for 15 min, arranged and photographed. Infarct volume was quantified using Image J software (Windows version) as previously described [8].

Neurological deficit evaluation

During a period of seven days treatment, animals neurological deficit scores (n=6 per group) were evaluated daily by a neurological deficit grading system with a scale of 0 to 5 [9]. Standards were: 0, no obvious deficits; 1, difficulty in fully extending the contralateral forelimb (Refer to the surgery side) after rat's tail was raised; 2, freely walking around, whirled to the contralateral side while rat's tail was pressed slightly; 3, whirled to the contralateral side spontaneously; 4, no spontaneous activity; 5, death. The higher the neurological deficit score is, the severer the impairment becomes.

¹⁸F-FDG PET/CT scanning

¹⁸F-FDG PET/CT scanning was performed to determine the effects of SCM-198 on brain glucose metabolism. After seven days treatment, rats (n=3 per group) were anesthetized intraperitoneally with 2% pentobarbital sodium and injected intravenously with 0.2-0.3 mCi of ¹⁸F-deoxyglucose (¹⁸F-FDG). PET/CT scanning (Sie-

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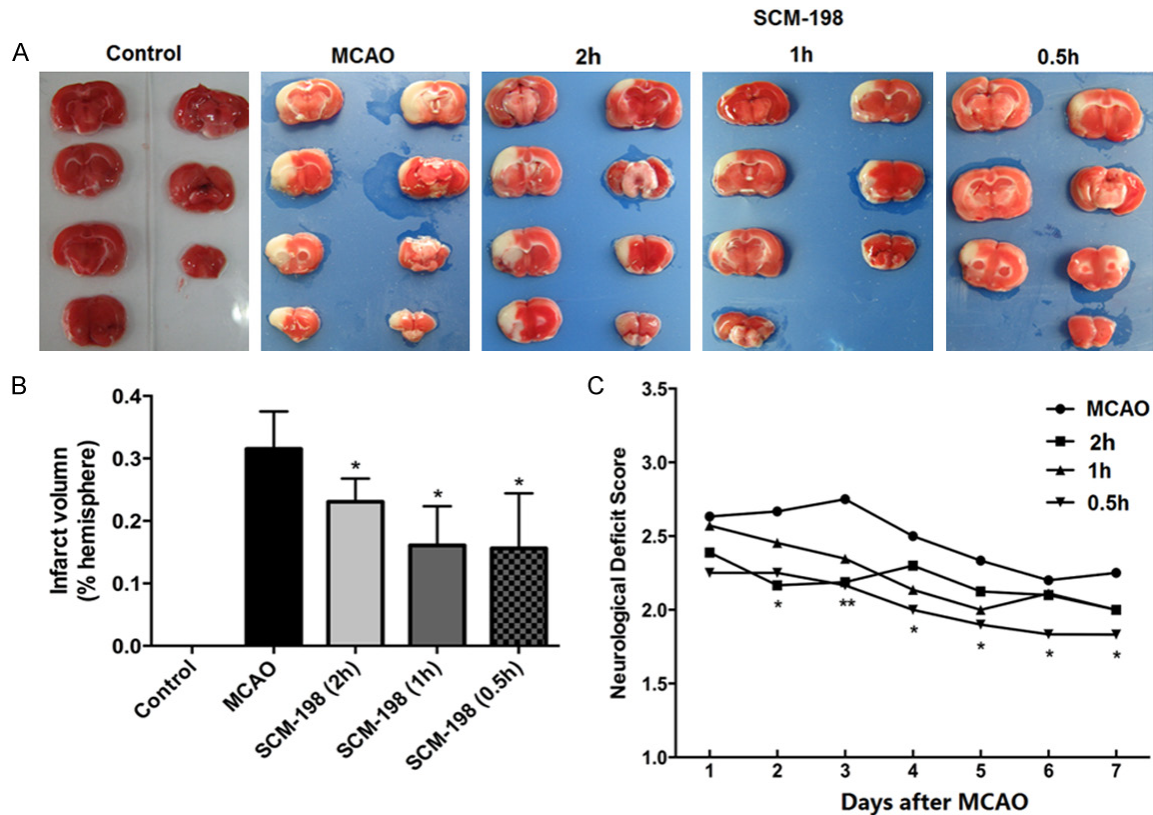


Figure 1. Infarct volume and neurologic deficit scores were decreased after SCM-198 treatment. A. Infarct volume of each treatment group was observed by TTC staining. B. Infarct volume was expressed by bar graphs. The percent hemispheric infarct volume was significantly lower in all SCM-198 treatment groups than that of the MCAO group ($P < 0.05$). C. Neurologic deficit scores of rats during seven days treatment. Rats in the MCAO group had higher scores than three SCM-198 treatment groups. The scores of 0.5 h group, not 1 h and 2 h groups, revealed a significant decrease than the MCAO group from day 2 to day 7 ($P < 0.05$). $n = 6$ each group.

mens Medical Systems, Erlangen, Germany) was conducted 45 min later. Rat was fixed on a plate to keep head in horizontal position during image acquisitions. CT scanning 10 min with parameters as 80 kV, 500 μ A, 600 s/r, 1100 ms exposure time, field of view (Fov): 768 mm \times 768 mm \times 512 mm. PET scanning 20 min with parameters as Fov: 128 mm \times 128 mm \times 159 mm. The axial, coronal, and sagittal planes images were acquired in 3D acquisition. Images were handled by Inveon Acquisition Workplace software (Siemens Medical Solutions, Erlangen, Germany). A specialist in nuclear medicine placed the regions of interest (ROI) in which covers middle cerebral artery area in ipsilateral side (i.e., surgery side) and contralateral side. Mean standardized uptake values (SUV), an established parameter for the quantification of 18 F-FDG uptake, were measured as described [10]. Considering of the individual variation, the Ipsilateral-to-Contralateral ratio

was used to evaluate the effects of treatment on brain glucose metabolism. Ipsilateral-to-Contralateral ratio = SUV of the Ipsilateral side/SUV of the Contralateral side $\times 100\%$ [11].

Transmission electron microscopy (TEM)

TEM was used to determine the effects of SCM-198 on hippocampal CA1 neuron ultrastructural changes. After seven days treatment, rats ($n = 3$ per group) were anesthetized intraperitoneally with 7% chloral hydrate, perfused with 0.9% saline and subsequently with 4% paraformaldehyde. The post-fixed brains, preparing 1 mm \times 1 mm \times 3 mm cubes, were processed as standard for TEM. All of the ultrathin sections were examined with a Jeol JEM 1200 EX transmission electron microscope (Jeol Ltd., Tokyo, Japan). The electron micrographs were taken by the same electron microscope. An investigator blinded to the study protocol examined the micrographs.

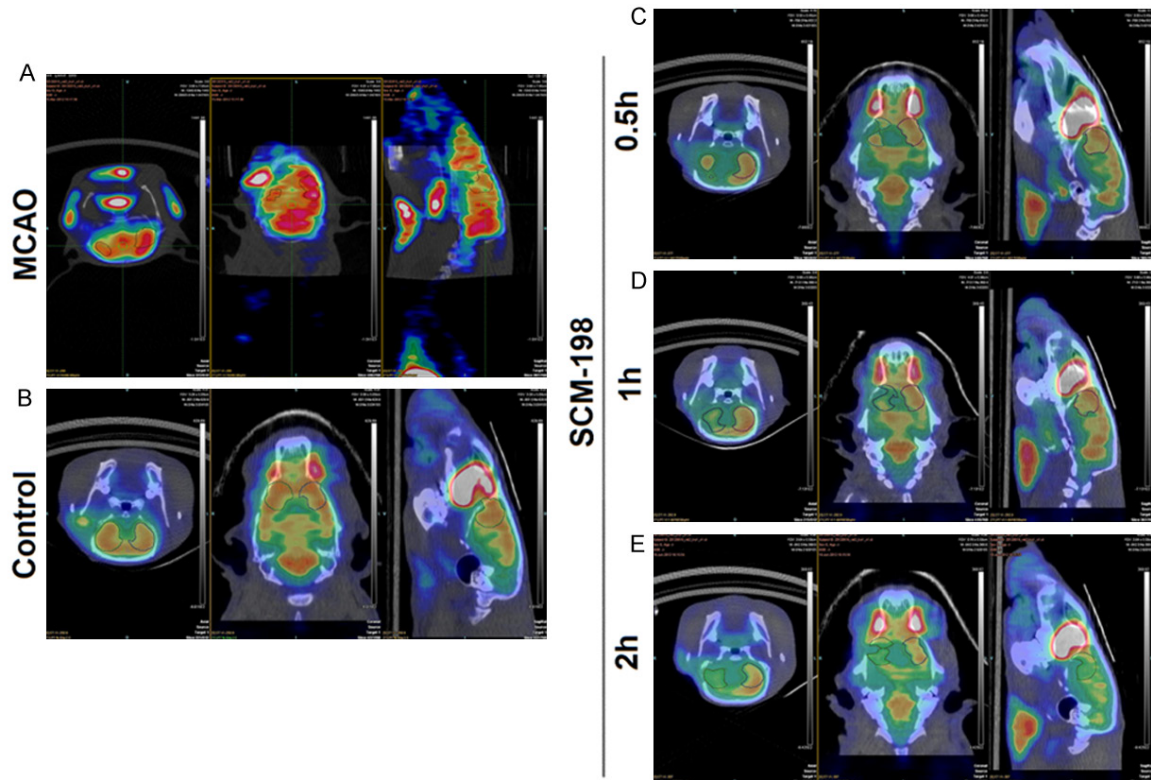


Figure 2. Representative ^{18}F -FDG PET/CT pictures of rats brain 7 days after treatment. From the left to the right were axial plane, coronal plane and sagittal plane images, respectively. Coronal plane images were used to calculate mean SUV value. ROIs were marked with solid lines, left are the ipsilateral side (MCAO surgery side), right are the contralateral normal side. A. MCAO group. B. Control group. C. 0.5 h group. D. 1 h group. E. 2 h group. N=3 each group.

Immunohistochemical staining

Immunohistochemical staining was performed to determine the effects of SCM-198 on iba-1 immunoreactivity in the hippocampal CA1 region. After seven days treatment, rats (n=5 per group) were anesthetized intraperitoneally with 7% chloral hydrate, perfused with 0.9% saline and subsequently with 4% paraformaldehyde. Rats brains were then removed and post fixed over 12 h in 4% paraformaldehyde fixative solution, immersed in 15% and 30% sucrose solution over 6 days at 4°C. After that, brains were embedded by paraffin and sliced into 20 μm . Sections were treated with 0.3% hydrogen peroxide (H_2O_2) for 30 min and washed in PBS. Background was blocked in PBS containing 4% bovine serum albumin (Sigma Chemical Co., MO, USA) for 30 min at room temperature and then incubated with a 1:1000 dilution of rabbit anti-Iba1 polyclonal antibody (Abcam, Cambridge, UK) overnight at 4°C. Following further washed with PBS, immunohistochemical staining was performed by a MaxVision antibody complex method using the MaxVision kit (Fu-

jianMaixin Biological Technology Ltd, Fujian, China). Sections were washed in PBS, visualized using diaminobenzidine tetrahydrochloride (FujianMaixin Biological Technology Ltd, Fujian, China) and mounted on the gelatin-coated slides. The slides were viewed and quantified under a DMLA microscope (Leica Microsystems, Wetzlar, Germany).

Statistical analysis

Prism 5.0 software (GraphPad Prism Software Inc., CA, USA) was used for analysis. Every two groups were compared by 1-way ANOVA with Tukey as post hoc test for *P* values. All values are expressed as mean \pm SEM or SD. Values of *P*<0.05 were considered statistically significant.

Results

Infarct volume and neurologic deficit scores were decreased after SCM-198 treatment

Brain infarct volume was examined by TTC staining. The infarct volume of each group was

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Table 1. Glucose utilization in ROI regions treated with or without SCM-198 (n=3, $\bar{x} \pm SE$)

Groups	SUV value		Ipsilateral-to-Contralateral ratio
	Ipsilateral side	Contralateral side	
Control group	5.714 \pm 0.11	5.609 \pm 0.06	101.9% \pm 3.6%
MCAO group	3.719 \pm 0.07	4.747 \pm 0.11	78.3% \pm 4.9%*
2 h group	3.906 \pm 0.07	4.441 \pm 0.12	88.1% \pm 5.2%*
1 h group	5.339 \pm 0.08	5.736 \pm 0.07	93.1% \pm 2.8%* [☆]
0.5 h group	4.246 \pm 0.06	4.657 \pm 0.06	91.2% \pm 2.5%* [☆]

*vs. Control group, P<0.05; [☆]vs. MCAO group, P<0.05.

showed in **Figure 1A** and **1B**. As we can see, there was no infarct area seen in the Control group which was stained deep red. Infarct area which was stained pale gray was noticed in other four groups (**Figure 1A**). The percent hemispheric infarct volume of all SCM-198 treatment groups was significantly lower compared with the MCAO group (P<0.05) (**Figure 1B**). 0.5 h group and 1 h group have lower infarct volume than 2 h group; However, this difference has no significant meaning (P>0.05).

Neurological deficit scores can reflect the degree of neurological deficit caused by ischemic injury. The higher the score is, the severer the impairment becomes. Apparently, rats in the Control group had no neurological injury; therefore, the neurological deficit score of this group was considered to be 0. Rats in the MCAO group had higher scores than other three SCM-198 treatment groups during the whole treatment period. The scores of 0.5 h group, not 1 h and 2 h groups, revealed a significant decrease than the MCAO group from day 2 to day 7 (P<0.05) (**Figure 1C**).

The recovery of glucose metabolism was accelerated by SCM-198 treatment

¹⁸F-FDG PET/CT scanning was performed after seven days treatment. Glucose utilization of each group, reflected by SUV values, was showed in **Figure 2**. It was found that, in the Control group, the glucose utilization of the ipsilateral side ROI region was almost same as that of the contralateral side (**Figure 2B**); While in the MCAO group, the glucose utilization of the ipsilateral side was decreased significantly compared with that of the contralateral side (**Figure 2A**). After administration of SCM-198, the glucose utilization was improved compared with the MCAO group (**Figure 2C-E**). At the end of

treatment, the Ipsilateral-to-Contralateral ratio of the Control group, MCAO group, 2 h group, 1 h group and 0.5 h group was 101.9% \pm 3.6%, 78.3% \pm 4.9%, 88.1% \pm 5.2%, 91.2% \pm 2.5% and 93.1% \pm 2.8%, respectively (**Table 1**). These results suggested that SCM-198 had the potential to accelerate the recovery of glucose metabolism after ischemic injury.

SCM-198 showed protection effects on neurons after ischemic injury

As we know, blood-brain barrier (BBB) disruption during MCAO can cause secondary brain injury, including irreversible neuron losses, injury and degeneration. According to our previously study, SCM-198 could lessen BBB damage (data not show); Therefore, in this study, we tried to explore that whether SCM-198 could maintaining the structural completeness of hippocampal CA1 neurons to improve ischemic brain injury. As we can see from **Figure 3A**, large vacuoles and lysosomes were noticed in the cytoplasm in the MCAO group after seven days treatment. Nearly all of the mitochondria in the MCAO group showed ultra-structural pathological changes and most of them were swollen. We could hardly found normal neurons in this group. SCM-198 treated groups revealed less intercellular edema, better neuron ultra-structure and better mitochondrial protection than the MCAO group. In all SCM-198 treatment groups, neurons were swelling and with less dense cytoplasm compared with normal neurons (**Figure 3B, 3D**). Well-protected neurons demonstrated great amelioration after SCM-198 treatment.

Microglial activation was attenuated by SCM-198

Iba-1 is an established marker of microglia. Immunohistochemical staining of brain slices against iba-1 showed that MCAO surgery induced excessive microglial activation, lots of iba-1-immunoreactive microglia became ramified (**Figure 4A**); While less iba-1-immunoreactive microglia were noticed in all SCM-198 treatment groups (**Figure 4B-D**); IOD values of SCM-198 treatment groups were decreased when compared with the MCAO group (**Figure 4E**, P<0.01), which suggested that microglial

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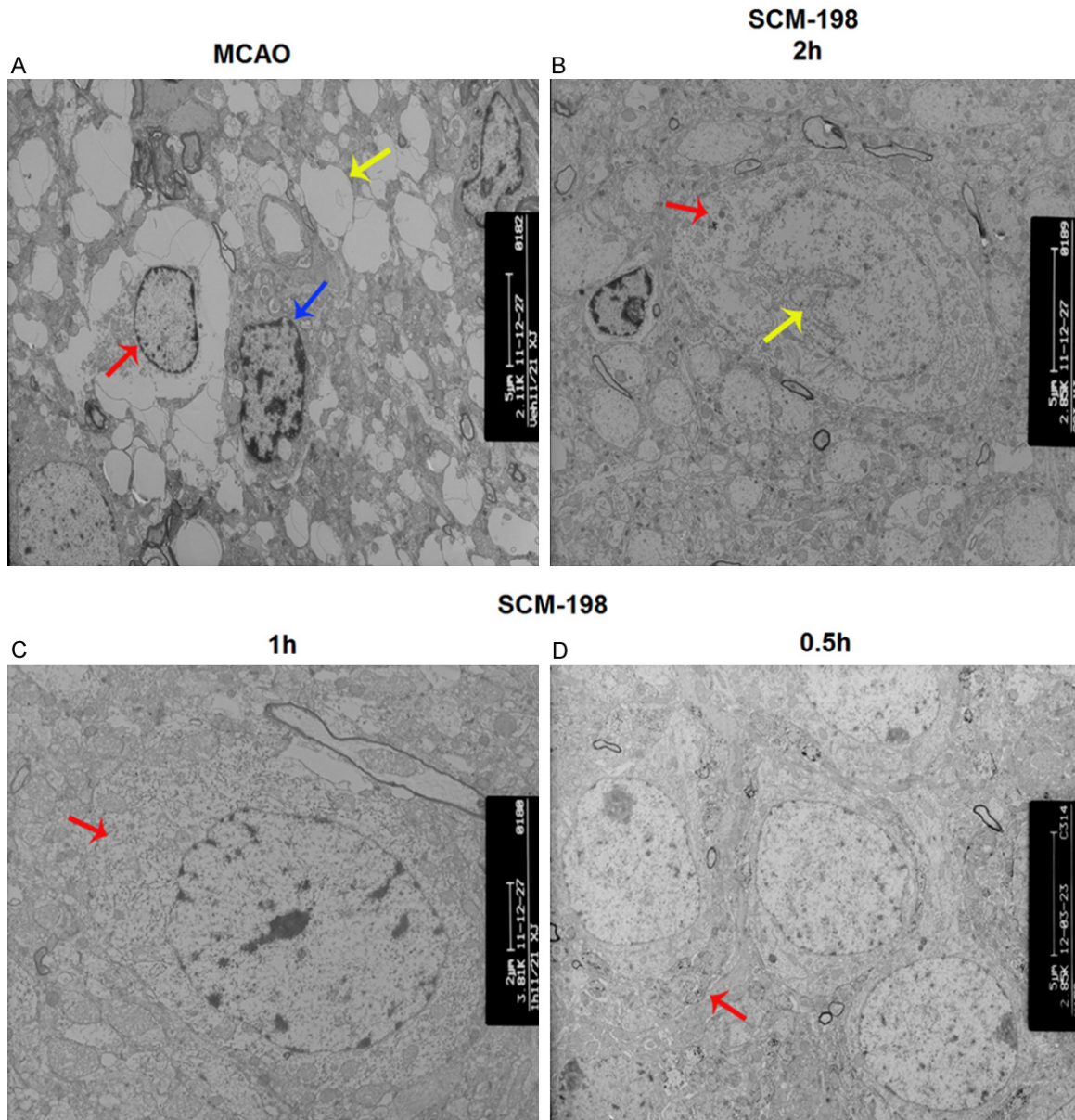


Figure 3. Transmission electron micrographs of the hippocampal CA1 region of rats brain. A. MCAO group. Red arrow represented destroyed neuron; yellow arrow represented cavitation; blue arrow represented activated microglia. B. 2 h group. Red arrow represented irregular dense mitochondria; yellow arrow represented neuron swelling. C. 1 h group. Red arrow represented distorted mitochondria. D. 0.5 h group. Red arrow represented slight dendritic swelling. N=3 each group.

activation during ischemic injury could be attenuated by SCM-198 treatment.

Discussion

SCM-198 was chemically synthesized based on the structure of Leonurine and have been found to be effective in ischemic stroke [3, 6]. In this study, we confirmed treatment of SCM-198 at dose of 15 mg/kg/d could significantly alleviate ischemic injury in a 2 h window of op-

portunity, which may be related with its effects on brain glucose metabolism and Neuronal protection.

Brain is a highly energy-consuming organ and vulnerable to glucose and oxygen deprivation. Glucose metabolic activity of ischemic core was found reduction after ischemic [11]. ^{18}F -FDG is a glucose analog which has been widely used in experimental and clinical stroke research for its potential in predicting final tis-

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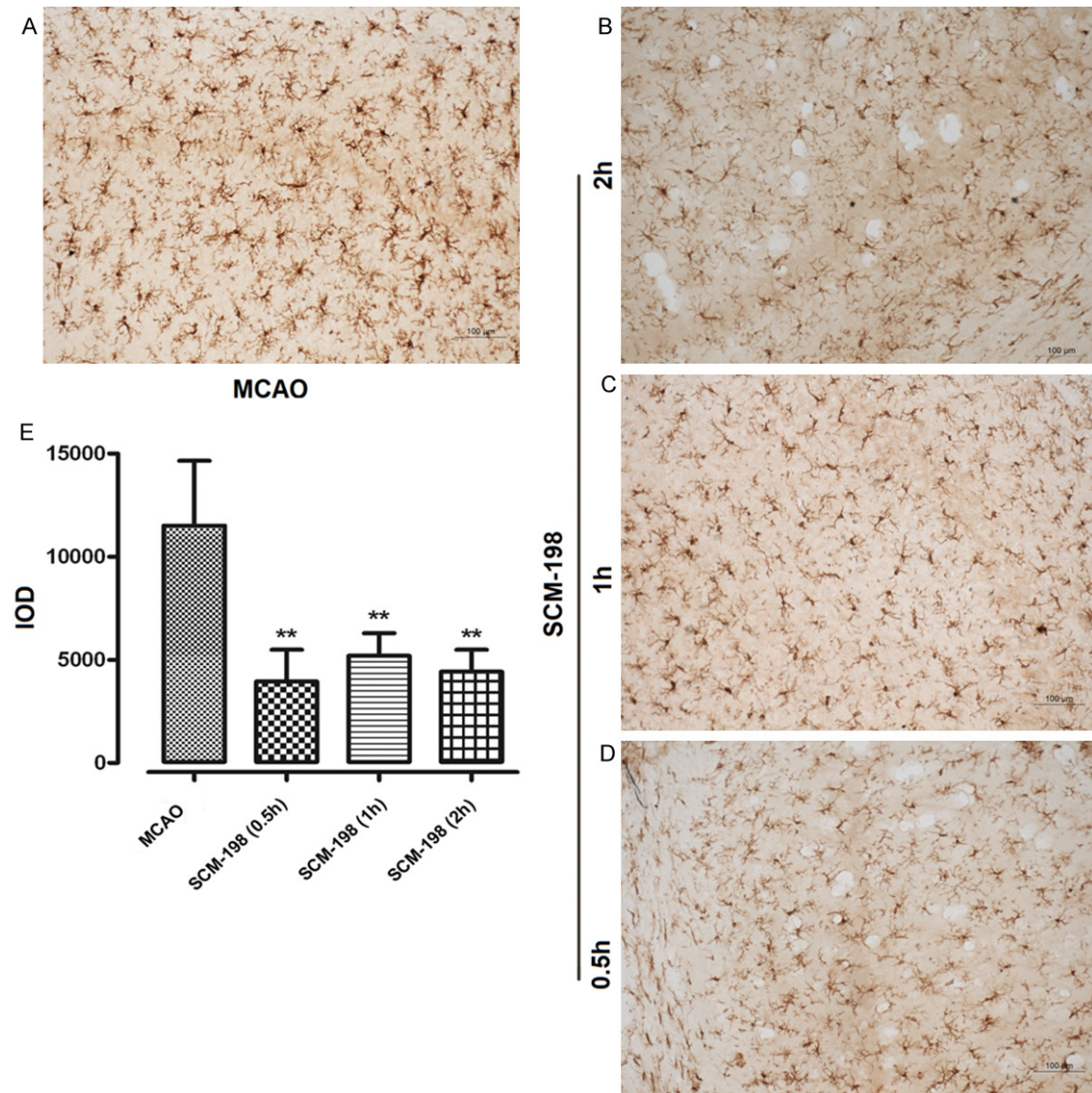


Figure 4. Immunohistochemical staining for Iba-1 in the hippocampus CA1 region of rats brain. A. MCAO group. Microglial activation was noticed. Lots of ramified Iba-1-immunoreactive microglia were shown in the CA1 region. B-D. SCM-198 treatment groups. In all SCM-198 treatment groups, microglial activation was also existed, while the number of microglia was less than that of the MCAO group. E. IOD value of Iba-1 positive cell at the hippocampus CA1 region. Values are expressed as mean \pm SD, ** $P < 0.01$ vs. MCAO group. $N = 5$ each group.

sue fate [12]. In our study, 7 days after surgery, significant decrease of mean SUV value in ROI region of the surgery side was observed in the MCAO group indicating poor glucose utilization. In all SCM-198 treatment groups, the glucose utilization was improved as indicated by bigger Ipsilateral-to-Contralateral ratio. These results indicated that SCM-198 had the potential to improve the outcome of acute ischemic stroke through recovering the brain glucose metabolism.

Neuron, also called nerve cell, is the fundamental component of nervous system and plays important role in maintaining nervous system function. Plenty studies have demonstrated that neurons were damaged after ischemia and this damage would be relieved after effective treatment [13-15], which makes neuron a perfect target to evaluate the therapeutic effects of potential stroke therapy. In our current study, we found ischemia induced abundant neuron damage; while SCM-198 treatment ameliorat-

ed the damage of neurons in ischemic stroke. This observation was also consistent with the results from Liu et al. [6].

Microglia cells are resident tissue macrophages located in the central nervous system and are thought to modulate degenerative and regenerative functions in brain [16]. Neuronal death induced by cerebral ischemic causes the activation of microglia [17]. Microglia activation may reflect the extent of severity of ischemic injury [18]. Iba-1 is an established marker of microglia, which has been used to examine the levels of microglia activation [19, 20]. In this study, microglia was strongly activated in the CA1 region of the MCAO group 7 days after ischemic injury. While, in the SCM-198 treatment groups, the activated microglia was significantly decreased after treatment. This result suggested that inhibition of microglia activation may result to the neuronal protection of SCM-198.

To summarize, our study confirmed that SCM-198 was a potential therapeutic option for ischemic injury in a 2 h window of opportunity, which may be related with its effects in recovering the brain glucose metabolism, ameliorating the morphology of neurons and inhibiting the activation of microglia after ischemic injury happened; however, there were some limitations in the present study. Firstly, we mainly explored the potential therapeutic effects of SCM-198 on ischemic stroke; while mechanism exploration was limited. Secondly, in the TEM and immunohistochemistry part, the Control group was absent which should be set up to observe the normal status of neuron and microglia. Thirdly, our study confirmed that SCM-198 was effective for ischemic stroke within a 2 h window of opportunity, this time point should be prolonged to determine whether SCM-198 has a longer therapeutic window of opportunity.

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Disclosure of conflict of interest

None.

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