

Original Article

Salvianolate lyophilized injection promotes post-stroke functional recovery via the activation of VEGF and BDNF-TrkB-CREB signaling pathway in T1DM-MCAO rats

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Abstract: Reports show that, while the mechanism remains unknown, salvianolate lyophilized injection (SLI) improves functional recovery after stroke in diabetic rats. In this study, we investigated the mechanism and effect of SLI on stroke outcome in type 1 diabetic (T1DM) rats. T1DM were induced in adult male Wistar rats by injecting streptozotocin. T1DM rats were then subjected to 90 minutes of middle cerebral artery occlusion (MCAO). SLI (10.5, 21, 42 mg/kg, respectively) was administered by tail vein injection at 24 hours after MCAO, and daily and last for 14 days. The neurological deficit score and brain infarct volume were assessed after 14 days. Also, VEGF, BDNF, TrkB, CREB and p-CREB levels in the ischemic brain tissue were analyzed with western blot at 14 days after MCAO. SLI significantly reduced neurological deficit scores and cerebral infarct volume, and reduced lesion volumes at all time points. SLI also increased the expression of VEGF, BDNF, TrkB, CREB and p-CREB protein levels in T1DM-MCAO rats. In summary, our results demonstrate that SLI can improve functional recovery after stroke in diabetic rats, and the mechanism of treating cerebral ischemic injury is related to the activation of the VEGF, BDNF-TrkB-CREB signaling pathway.

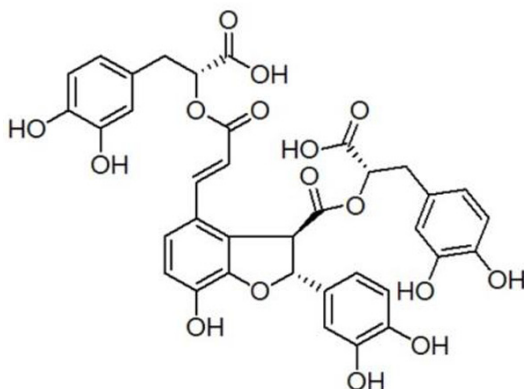
Keywords: Salvianolate lyophilized injection (SLI), stroke, type 1 diabetic, middle cerebral artery occlusion (MCAO), vascular endothelial growth factor (VEGF), BDNF-TrkB-CREB signaling pathway

Introduction

Stroke is the secondly most common cause of death and a major cause of disability worldwide [1]. Diabetes is a major risk factor in stroke patients [2]. About 30% of stroke patients are diabetics, and more than 50% of them develop towards post-stroke hyperglycemia. Clinically, post-stroke hyperglycemia and diabetes are related to worse neurological outcomes [3]. Diabetes-mediated microvascular disease of the brain is increasingly recognized as a risk factor for neurodegenerative diseases like stroke and vascular cognitive impairment [4]. Changes in cerebrovascular structure and function can lead to altered cerebral blood flow and

permeability of the blood-brain barrier (BBB) that not only contribute to the development of diabetes but also be benefit for recovery after stroke [5]. On the other hand, diabetes induces cytochrome c release from mitochondria into cytoplasm that may play a role in apoptosis of the CA1 pyramidal neurons [6] and cause a reduction in neurogenesis [7]. Thus, there is an urgent unmet medical need for an effective novel therapy for stroke in patients with diabetes.

Vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF) are two important neurotrophic factors that have multiple effects on sustaining and evoking ele-



Salvianolic acid B

Figure 1. The chemical structure of salvianolic acid B (Sal B). Molecular Formula: $C_{36}H_{30}O_{16}$. Molecular weight: 718.6138.

ments of brain plasticity [8, 9]. In experimental cell culture and animal models, activation of receptors for each of the latter trophic factors can protect neurons against ischemic injury [10, 11]. VEGF induces adult neurogenesis during exposure to an enriched environment or voluntary exercise [12] and reduces apoptosis after its infusion. The two functions of VEGF suggest a survival promoting effect of NSCs [13]. Likewise, BDNF regulates neuronal survival, cell migration and synaptic function [14, 15]. BDNF administration has also been shown to promote cell viability after insults [16]. The increase of BDNF production after exercise and caloric restriction is believed to play a major role in the neuroprotective effects of these treatments [17]. The production of VEGF and BDNF are increased in ischemic brain coupled with other neurorestorative treatments of stroke, such as bone marrow stromal cells [18, 19]. Therefore, it is reasonable to propose that VEGF and BDNF might be upregulated in the brain after treatment with a statin, and might orchestrate brain plasticity. Also, the BDNF-mediated effect is probably to act through activation of TrkB (a high-affinity tyrosine kinase receptor) [20, 21]. The full-length TrkB autophosphorylation regulate Erk/MAPK signaling on activation by BDNF, which may increase cAMP and activate cAMP response-element-binding protein (CREB)-regulated gene transcription. This mechanism further promotes transcription of BDNF [22]. This is a potential positive feedback mechanism that could produce a BDNF-induced synthesis of BDNF itself [23].

Salvianolate lyophilized injection (SLI) is composed of salvianolic extraction (commonly named “Danshen” in Chinese). Danshen, a very important component of Chinese medicine derived from the dried root or rhizome of *salvia miltiorrhiza* Bge (SM), has been widely used in China for the treatment of cerebrovascular conditions, such as ischemic stroke [24, 25]. Research has shown that the composition of *salvia miltiorrhiza* have protective effects against focal cerebral ischemia/reperfusion injury [26, 27], and salvianolic acids have neuroprotective effect [28, 29]. Salvianolic acids are the most abundant water-soluble compounds extracted from *salvia*. Among salvianolic acids, salvianolic acid B (Sal B) is one of the most abundant polyphenols, it is a condensate of three molecules danshennol and one molecule of caffeic acid (**Figure 1**). Polyphenols have been suggested to prevent post-angioplasty restenosis via the inhibition of VSMC migration and proliferation, but not constraining re-endothelialization. It has been found that VSMC proliferation and migration were inhibited by salvianolic B, which suppressing the expression level of CXCR4 receptor [30]. The greatest clinical impact of salvianolic acids is cardiovascular protection. However, its mechanism of action on diabetics with stroke is unknown. Therefore, we examined the neuroprotective and therapeutic effects of SLI in T1DM-MCAO rats. The therapeutic effects of SLI were evaluated by assessing infarct size, blood-brain barrier permeability, recovery of neurological function and production of neurotrophic factors, such as BDNF and VEGF, which may act in concert to induce neurological function recovery after stroke in diabetes rats.

Material and methods

Induction of type I diabetes in rats

All experiments were performed following an institutionally approved protocol in accordance with the Institutional Animal Care and Use Committee of Tianjin University of TCM. Eight-week-old male Wistar rats (Vital River Laboratory Animal Technology Co., Ltd) with an initial body weight of 200-220 g were used; type-I diabetes was induced by a standard intraperitoneal injection of streptozotocin (60 mg/kg; Sigma, St. Louis, MO). Seven days after streptozotocin administration, the blood glucose concentrations of rats were more than 15

mmol/L. These rats were retained as the diabetic rats as we previously described [31]. They were singly housed in a humidity-controlled room, maintained on a 12-hour light/dark cycle, with free access to food and water.

Focal cerebral ischemia-reperfusion injury model

Fourteen-weeks-old (Type 1 diabetes for 6 weeks) streptozotocin-induced diabetic (male, Wistar) rats with blood glucose concentration 15.4 to 32.1 mmol/L were subjected to focal cerebral ischemia-reperfusion injury [32]. Rats were fasted overnight with free access to water. The rat focal cerebral ischemia-reperfusion injury was induced by middle cerebral artery occlusion (MCAO) using a nylon suture method described previously [33-35]. All animals were anesthetized with 10% chloral hydrate (350 mg/kg ip). Briefly, after a midline neck incision, the right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were separated via a ventral midline incision. A 3-0 monofilament nylon suture (Beijing Shadong Biological Technology Co., Ltd., Beijing, China) was introduced into the ECA lumen and extended into the ICA (18.5 ± 0.5 mm) to block the origin of the MCA. Then, the exposed vessels were carefully ligated to prevent bleeding, and the incision was closed aseptically. Sham-operated animals were subjected to the same surgical procedure, but the suture was not advanced beyond the internal carotid bifurcation. A laser Doppler perfusion monitor (PeriFlux System 5010, Perimed, Stockholm, Sweden) was used to monitor rCBF throughout the study. The ischemic model was considered successful if about 75% reduction in CBF was induced immediately after placement of the suture [36], otherwise the animals were excluded. During MCAO and postconditioning period, body temperature was strictly maintained at $37 \pm 0.5^\circ\text{C}$ by a warming blanket. Sham-operated animals were not exposed to ischemia-reperfusion. After 1.5 h of ischemia, the nylon suture was removed to establish reperfusion. After arousal from anesthesia, the rats were returned to their cages with ad libitum access to food and water. Only animals that survived for 24 hours after stroke were included for outcome assessments; animals dead within 24 hours after stroke were counted for overall mortality rate of all groups.

Sample size calculation

A preliminary experiment was conducted with two groups (sham-operated and model-vehicle, $n = 6$) to determine the differences in mean lesion volumes between the two groups as measured on T2-weighted MR images (T2-LVs) obtained at 24 hours after reperfusion.

Treatment groups and drug administration

Salvianolate Lyophilized Injection (SLI, Tianjin Tasly Pride Pharmaceutical Co., Ltd, Tianjin, China) was dissolved in normal saline. One hundred male Wistar rats were divided randomly into five groups: sham-operated, model-vehicle, and SLI 10.5 mg/kg, 21 mg/kg and 42 mg/kg. SLI initially used intravenous injection (i.v.) 24 hours after MCAO and daily for 14 days. The sham-operated group and model-vehicle group were treated with isodose saline.

Neurological evaluation

Neurological function was evaluated at different time after stroke in T1DM-rats. Behavioral changes were assessed 24 hour after surgery using a five point scale [37, 38] as follows: 0, no neurological deficit (normal); 1, failure to extend right forepaw (mild); 2, decreased resistance to lateral push (mild to moderate); 3, circling or walking to the right (moderate); and 4, loss of walking or righting reflex (severe). An animal with no apparent deficits obtains a score of 0-3 is consistent with a middle cerebral artery occlusion. Only animals with a score of 3 prior to reperfusion were included in the analysis.

Magnetic resonance imaging

Quantification of Magnetic Resonance Imaging (MRI) evaluated cortical edema for before and after treatment of SLI in T1DM-MCAO rats. We used a Varian 7.0T (Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (CAMS), Beijing, China) horizontal scanner to exclude the animals without cortical damage. Based on MRI images, 6 animals were excluded from the study. The rats were anesthetized with 5% isoflurane in a gas mixture with 30% O_2 /70% N_2O . After induction, anesthesia was maintained throughout the imaging with 2.5% isoflurane inhaled through a nose mask. T2-weighted multislice images were acquired using a RARE sequence with the fol-

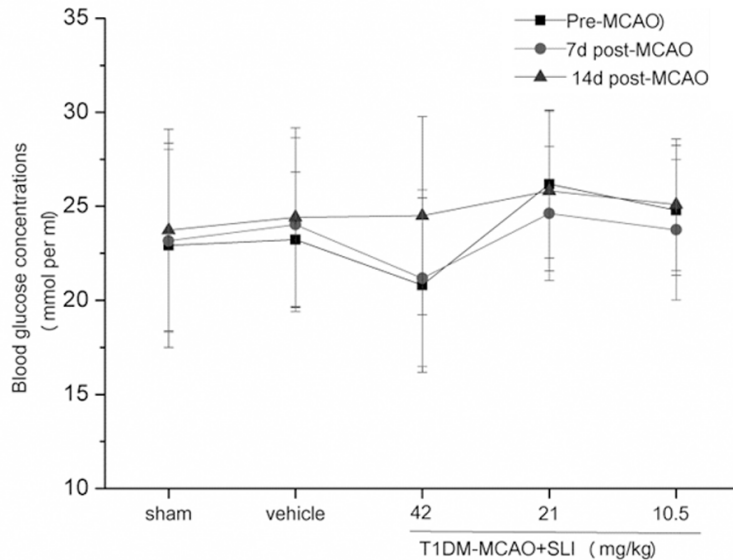


Figure 2. The blood glucose level is not changed in T1DM rats before and after stroke. The data are shown as mean \pm SEM. $^{**}P < 0.01$, vs. sham-operated group.

lowing parameters [39]: time-to-repetition TR = 3000 ms, effective time-to-echo = 72 ms, RARE factor 8, matrix size of 256×256 , field-of-view of $30 \text{ mm} \times 30 \text{ mm}$, and BR 12 slices with a slice thickness of 1 mm. Infarct volumes were analyzed by using VNMRJ (Applications Places System). Areas of surviving gray matter in the cortex and striatum were outlined for each hemisphere. The difference between the size of an intact area in the contralateral hemisphere and the respective residual area in the ipsilateral hemisphere was recorded as the infarcted area. The total infarct volume was calculated by multiplying the infarct area by the distance between the slices and summing together the volumes.

Brain damage evaluation

The brains were assessed using the 2, 3, 5-triphenyltetrazolium chloride (TTC, Sigma, St Louis, MO) staining method [40]. Animals were anesthetized with 10% chloral hydrate (350 mg/kg, i.p.). On the 14th day after T1DM-MCAO, their brains were removed and sectioned into consecutive 2 mm-thick coronal slices. The slices were immediately immersed into 2% TTC medium at 37°C for 20 minutes and then turned over for another 20 minutes. The stained slices were washed in phosphate-buffered saline (PBS) for 5 min and then fixed in 4% buff-

ered formaldehyde solution for 24 h. At the end of staining and fixation, color images of these slices were captured using a video camera (PowerShot S60, Canon, Tokyo, Japan). The infarct volume was analyzed using Image Pro Plus software. Percentage of infarct volume was calculated as follows: $[(\text{VL}-\text{VR})/\text{2VL}] \times 100$. (VL: volume of contralateral hemisphere; VR: volume of noninfarcted tissue in the lesioned ipsilateral hemisphere) [41].

Western blot analysis

Rats were sacrificed at 14 days after the administration of SLI in T1DM-MCAO. For protein extraction, brain tissues ($n = 4/\text{group}$) were extracted from the ischemic brain and placed on ice in 10 volumes of cold homogenization buffer (50 mM Tris, 120 mM NaCl, pH 7.4). Protease inhibitors (Wuhan Boster Biological Co., Ltd, China) were added, and then the tissue was homogenized. Briefly, the protein concentrations were determined with a bicinchoninic acid protein assay using bovine serum albumin as the standard. Equal amounts of protein (100 μg) were fractionated by SDS-PAGE and blotted to PVDF membrane (Millipore, Bedford, MA). The blots were blocked by 5% non-fat milk dissolved in PBS for 2 h, then probed overnight at 4°C with the following primary antibodies: VEGF (1:200 dilution, Santa Cruz Biotechnology), BDNF (1:1000 dilution, Cell signaling Technology), TrkB (1:1000 dilution, Cell signaling Technology), p-CREB and CREB (1:1000 dilution, Cell signaling Technology) and anti- β -actin (1:1000 dilution, Cell signaling Technology), all in 5% milk TBST. Membranes were washed three times, for 15 min each time, with PBS containing 0.5% Tween 20 (PBS-T) and incubated with secondary antibody (1:8000 dilution, Alexa Fluor[®] 800 goat anti-rabbit IgG, Invitrogen) in PBS at room temperature for 1 h. Signals were detected by enhanced chemiluminescence (Supersignal, Pierce, Rockford, IN, USA) using autoradiograms exposed from 10 to 30 min [42, 43]. These experiments were repeated independently in triplicate.

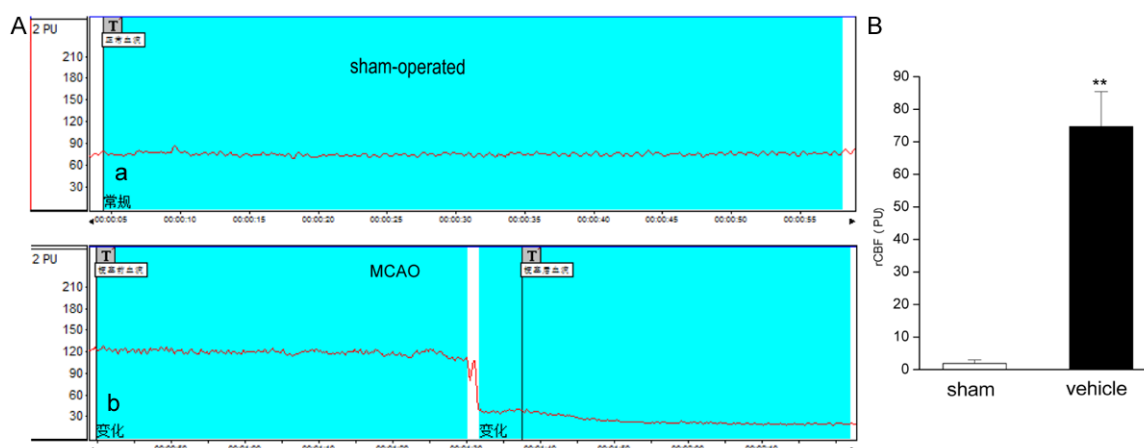


Figure 3. Regional cerebral blood flow (rCBF) changes after middle cerebral artery occlusion in rats. a: Sham-operated group; b: Model group. The signal of "T" in a, b indicates the onset time of different treatments. B. Showed the percentage of rCBF at Post-MCAO occupying the rCBF at before MCAO in sham group and MCAO group. Data are shown as mean \pm SEM. ** $P < 0.01$, vs. sham-operated group.

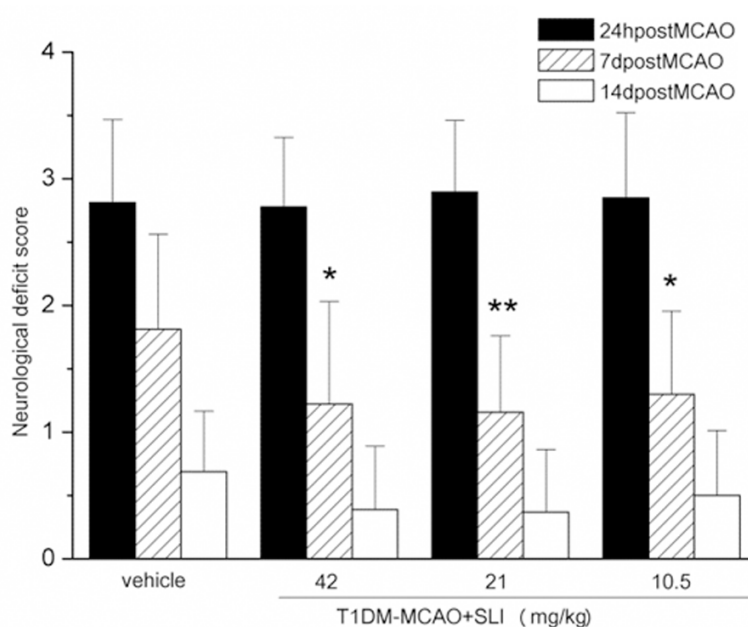


Figure 4. Neurological function was evaluated at 24 hours, and 7, 14 days after stroke in T1DM-rats. Data are shown as mean \pm SEM. ** $P < 0.01$, * $P < 0.05$, vs. model vehicle group.

Statistical analysis

The results were expressed as means \pm SEM. If appropriate, Student's t-test or one-way analysis of variance (ANOVA) for comparison versus values before adding sham, vehicle, SLI (10.5, 21 and 42 mg/kg) groups were performed. P values < 0.05 or 0.01 were considered to be statistically significant. Statistical analyses

were performed using SPSS 17.0 software.

Results

Blood glucose levels

At 6 weeks after STZ injection, the range of blood glucose concentration was about 15-30 mmol/L, which reflected that rats were affected by type I diabetes mellitus. Blood glucose concentrations were monitored at before MCAO, and at 7, 14 days after MCAO in T1DM-rats, which remained stable and had no significant difference at all of the time points (Figure 2).

Regional cerebral blood flow measurement

Cerebral ischemia-reperfusion injury model rats were subjected to reversible MCAO for 90 minutes followed by reperfusion for 14 days. The MCAO produced an occlusion visible by laser Doppler flowmetry as an abrupt 70-90% reduction in local cortical blood flow that normalized after removal of the occluding thread (that was not different from before occlusion in the operated rats) (Figure 3A, 3B). About 15% of animals in each group were excluded from data analysis due to failure

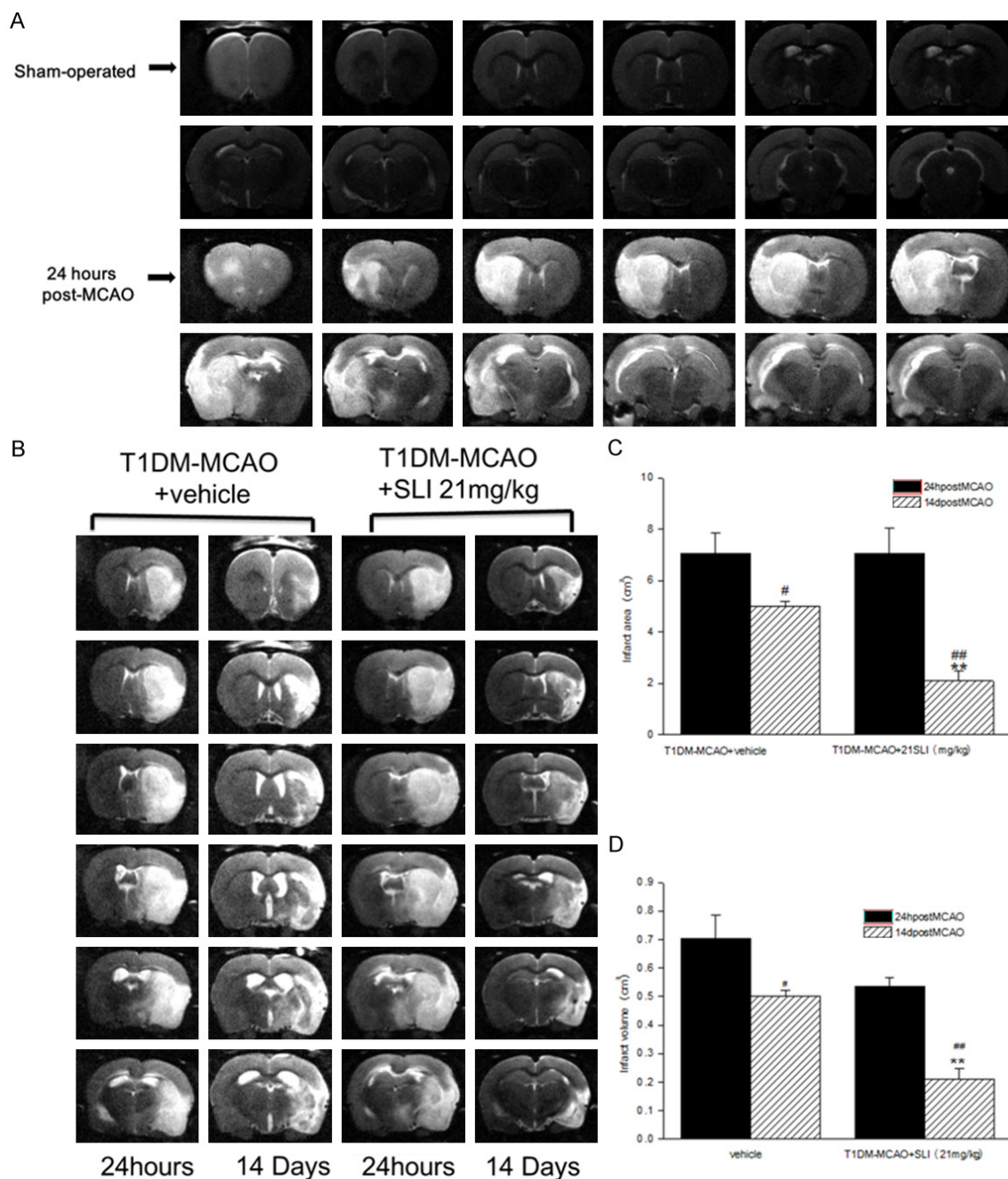
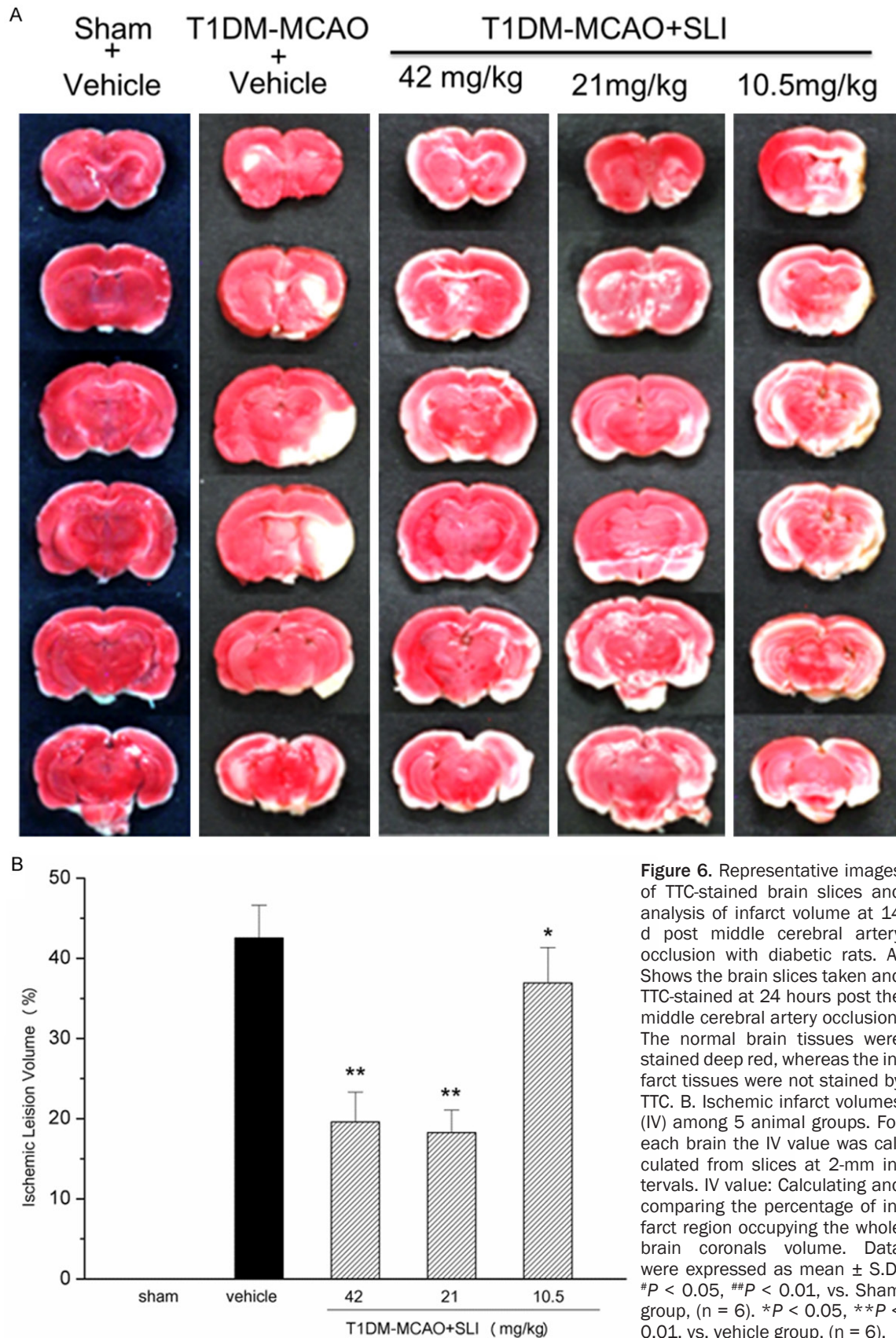


Figure 5. Temporal changes in the infarct in the T1DM-MCAO rats. A. Cerebral Infarction MRI Images, which showed the infarct volume at 24 hours after stroke in T1DM rats. In this picture the normal brain tissues were described as grey regions whereas the infarct tissues were white. B. Cerebral Infarction MRI Images, showed the infarct volume before and after treatment in T1DM-MCAO rats. C. Showed the values of infarct area before and after treatment. D. Showed the values of infarct volume before and after treatment. Data are shown as mean \pm SEM. $^{**}P < 0.01$, vs. model-vehicle group; $^{##}P < 0.01$ vs. 24 h post-MCAO.

during the surgical procedure. Before MCAO, physiological parameters (temperature, plasma glucose, and body weight) were measured and there were no significant differences between the treatment groups.

SLI improves functional outcome

To test whether SLI treatment improves functional outcome after stroke in T1DM rats, neural deficit scores was assessed. **Figure 4** shows



that T1DM-MCAO rats significantly attenuated functional outcome compared with sham-operation rats ($P < 0.05$). However, SLI treatment starting at 7, 14 days after MCAO significantly improved functional outcome after stroke in T1DM-MCAO rats compared to model-vehicle group rats ($P < 0.05$, $P < 0.01$).

SLI reduces the damaged area of the infarct volume

We had further analyzed, qualitatively and quantitatively, the infarct volume of the cortex using MRI. Cortical edema became obviously in the infarct cortex at 24 hours after MCAO in ischemia-reperfusion injury affected rats (**Figure 5A**). Starting from 24 hours after MCAO, however, one group executed SLI treatment, other one accepted vehicle. The infarct cortex became apparently in the control group at 14 days after MCAO whereas it became significantly less severe in the SLI treatment groups relative to vehicle group (**Figure 5B**). After 14 days of SLI administration, the infarct area and volume were reduced significantly, (**Figure 5C, 5D**). These results demonstrate that SLI has a protective role in reducing the infarct volume after MCAO.

In present study, effects of SLI on T1DM-MCAO rats brain infarct area were observed by TTC staining method (**Figure 6A**). The infarct volume (36.6 ± 6.9 , 19.3 ± 5.8 , 18.6 ± 3.9 mm³) in the SLI (10.5 mg/kg, 21 mg/kg and 42 mg/kg) treatment groups were significantly smaller than that of the model-vehicle group (42.8 ± 7.8 mm³). Comparison tests indicated a statistically significant decrease between the SLI (42 mg/kg, 21 mg/kg) groups relative to model-vehicle group ($n = 6$). Also, the infarct size in the model and SLI groups show a significant difference relative to the sham group ($n = 6$) (**Figure 6B**), which prove the success of MCAO model.

Effects of SLI on proteins expressions in ischemic brain tissue

The protein levels of BDNF and VEGF were detected by western blot. The level of VEGF in the ischemic brain tissue of model group (0.127 ± 0.032) was significantly lower as compared with the sham group (0.460 ± 0.041) (**Figure 7B**). The level of VEGF (0.762 ± 0.052 , 0.657 ± 0.061 , 0.661 ± 0.048) in SLI treatment (42, 21, 10.5 mg/kg, respectively) groups were shown

in **Figure 7B**. As compared with sham group (0.446 ± 0.051), the BDNF expression level in the ischemic brain tissue was significantly lower in model group (0.269 ± 0.042). SLI treatment (42, 21, 10.5 mg/kg) groups (0.615 ± 0.072 , 0.574 ± 0.061 , 0.613 ± 0.058) were described in **Figure 7D**. In **Figure 7F**, the value of TrkB in model group (0.2269 ± 0.027) was significantly lower as compared with the sham group (0.37 ± 0.0521). The expression level of TrkB (0.41 ± 0.071 , 0.391 ± 0.068 , 0.2613 ± 0.036) in SLI treatment (42, 21, 10.5 mg/kg) groups were higher than model group obviously. In **Figure 7H**, the value of CREB in model group (0.132 ± 0.023) was significantly lower as compared with the sham group (0.287 ± 0.053). The level of CREB (0.283 ± 0.047 , 0.252 ± 0.056 , 0.155 ± 0.028) in SLI treatment (42, 21, 10.5 mg/kg) groups were higher than model group. As compared with sham group (0.283 ± 0.0151), the P-CREB level in the ischemic brain tissue was significantly lower in model group (0.074 ± 0.0042). SLI treatment (42, 21, 10.5 mg/kg) groups (0.271 ± 0.03171 , 0.187 ± 0.065 , 0.085 ± 0.043) were described in **Figure 7J**.

Discussion

Salvianolate Lyophilized Injection (SLI) is a kind of water-soluble component of Danshen which is a priority to the modern Chinese native medicine preparation. Danshen, a very important traditional Chinese medicinal herb, can be used to promote blood flow and resolve blood stasis. It has been widely used in the treatment of coronary artery diseases and cerebrovascular diseases including stroke for over a thousand years [44-46]. In clinical studies, the therapeutic efficacy of Danshen in stroke has been confirmed and no adverse effects have been reported [47]. In TNF- α -treated HAECs, Sal B is found to attenuate VCAM-1 and ICAM-1, whose effects are associated with its anti-inflammatory property by inhibiting the activation of NF- κ B pathway triggered by TNF- α [48]. Sal B can protect from TNF- α -mediated disorganization of endothelial cell junctions by attenuating tyrosine phosphorylation of cell junction proteins, such as VE-cadherin and b-catenin. According to results of immuno-precipitation studies, Sal B can prevent b-catenin disassociation from the cytoskeleton in TNF- α -treated HUVECs [49]. Sal B inhibited not only MMP-2 activation induced by LPS, but also TNF- α , angiotension II

Neuroprotective effect of Salvianolate lyophilized injection

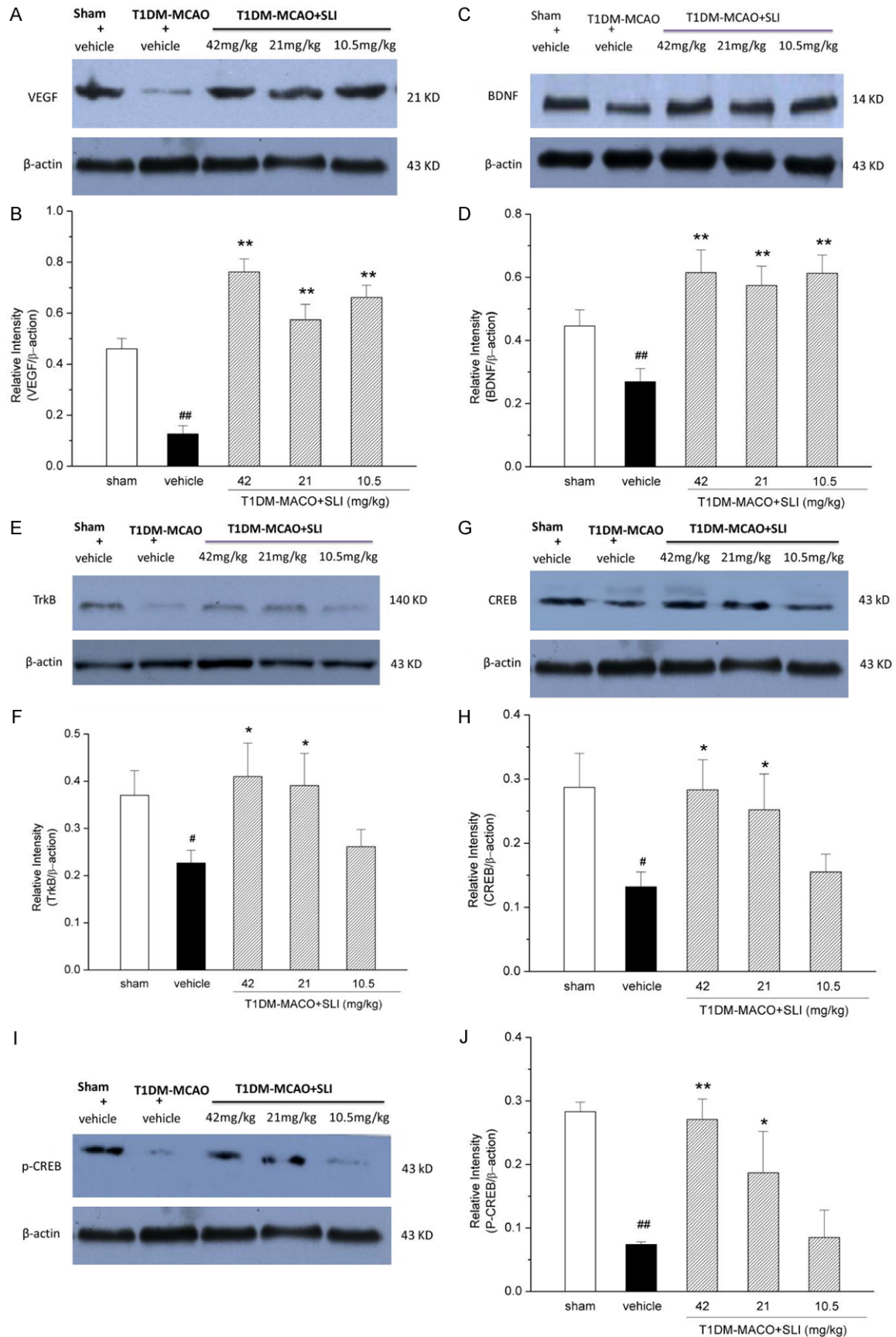


Figure 7. Effects of Salvianolate lyophilized injection on activated expression of VEGF and BDNF-TrkB-CREB signaling pathway. Proteins are extracted from the ischemic hemisphere of T1DM rats undergoing ischemia 90 min followed by 14 days reperfusion separately. The samples were subjected to Western blot analysis with antibodies as indicated. (A, C, E, G and I) represent western blot which show protein levels of VEGF, BDNF, TrkB, CREB and CREB phosphorylation respectively. In (B, D, F, H and J) densitometric analysis was used to quantify VEGF, BDNF, TrkB, CREB and p-CREB protein levels versus β -actin in 4 independent western blots respectively, and the data were expressed as the normalized folds with respect to sham and vehicle. In (B, D, F, H and J) SLI administration groups showed a significant effect on elevating the levels of VEGF, BDNF, TrkB, CREB and p-CREB comparing with vehicle group. Values represent mean \pm SD, $^{##}P < 0.01$, $^{\#}P < 0.05$, vs. Sham; $^{**}P < 0.01$, $^{*}P < 0.05$, vs. model.

and H_2O_2 . It has been demonstrated that Sal B inhibited protein expression and gelatinolytic activity in HASMCs by the inhibition of NADPH oxidase-dependent ROS generation [50]. In the first 18 hours after administration, Sal B significantly inhibited PAI-1 gene expression when HUVECs were exposed to TNF- α , and targets of Sal B in regulating TNF- α -stimulated PAI-1 production in HUVECs are possibly NF- κ B and ERK-AP-1 pathways [51]. The effect resulted from the reduced expression of VEGF protein, which modulated the ERK pathway. The endothelial permeability is also increased by loss of cell-cell adhesion junctions [44].

In this study, we examined rats that not only suffered diabetes but also sustained cerebral ischemic stroke to observe intervention effect and potential mechanism of SLI in treating stroke. In present study, we evaluated neurological and motor functions to observe the effect of SLI on functional disabilities in T1DM-MCAO rats. SLI administration could promote the ability in improving the neurological function and motor deficits, comparing with vehicle group. In addition, TTC staining results revealed that SLI alleviated the T1DM-MCAO cerebral infarct formation when compared to the vehicle. Besides the brain tissue was subjected to MRI evaluation for further confirmation on cerebral injury. At 14 days after stroke, MRI results showed the brain infarct volumes of model groups were increased significantly in T1DM-MCAO animals compared with sham-operated. These findings further witnessed that SLI reduced the infarct size and improved neurological function after stroke in T1DM rats.

VEGF and BDNF are two important neurotrophic factors that have multiple effects on neurogenesis. BDNF and VEGF stimulate adult neurogenesis and enhance the appearance and migration of new neurons in the SVZ and dentate gyrus [52-54]. Neurogenesis occurs close to blood vessels, where VEGF expression is

high and angiogenesis is ongoing [55]. The production and release of BDNF were increased substantially by the newly activated and expanded vasculature, whose induction is both spatially and temporally associated with recruitment of new neurons [56]. More than inducing angiogenesis, VEGF also plays a role in stimulating neurogenesis and axonal outgrowth and improves the survival of mesencephalic neurons [57], and VEGF is mitogenic for astrocytes and promotes growth/survival of neurons [58]. Our data have shown that SLI promotes angiogenesis, neuronal plasticity as well as increases VEGF expression. We propose that the increase of VEGF induced by SLI might not only cause angiogenesis, but also provides a supportive microenvironment, which could promote neural functional recovery after T1DM-MCAO.

Previous studies have demonstrated that BDNF could protect neurons against cerebral ischemic damage [59-61], glucose deprivation and oxidative stressors, which were more specific insults relevant to ischemic stroke [62-64]. BDNF promote the plasticity and survival of neurons and take effect in adaptive responses of the brain to environmental challenges [65]. The administration of exogenous BDNF intravenous treatment at post-ischemic improves long-term functional neurological outcome for induction of neurogenesis [13]. Studies reflected that cerebral ischemia can differently affect BDNF levels decreased in the core and increased in the penumbra areas [66], supporting a role for protection by BDNF. Indeed researchers have demonstrated that intravenous treatment of BDNF can reduce the infarct volume and promote functional recovery after cerebral ischemia [67]. Equally, basal BDNF levels enriched environment or exercise were up regulated by intravenous treatment of BDNF, which have shown decreased infarct volumes following MCAO [68-70]. Conversely decreasing BDNF levels or attenuating its effects leading

to cerebral ischemia diminishes recovery of function [71, 72]. BDNF has been shown to exert anti-apoptotic and neuroplastic properties, also to enhance nerve and angiogenesis. Our data show that SLI treatment after T1DM-MCAO induces the expression of BDNF, which is consistent with other reported studies that it can increase synaptic activity and elicits compensatory angiogenesis [73].

BDNF has been identified as bind to TrkB receptors and thus reveal its signaling regulation [20, 21, 74]. Accordingly, we found that secretion of TrkB was also enhanced by SLI treatment. Increased truncated TrkB receptors may play to the reduced BDNF-TrkB signaling and may lead to neuronal injury [75]. In fact, being a functional unit, only full-length TrkB receptor can be phosphorylated by BDNF and signal the downstream pathways, which could be a crucial factor limiting the ability of the brain to overcome the ischemic stress although the level of BDNF remains at the normal level as mentioned above [21, 76]. Fortunately, as we demonstrated in this experiment, SLI could largely prevent the decrease of BDNF induced by ischemia, which might be an important mechanism behind the SLI-induced brain to overcome cerebral ischemia. BDNF not only activates intracellular signaling cascades through full-length activation, but also cuts the loss of full-length TrkB under ischemic conditions. Based on the evidence built up the ground, we conclude that SLI has an effect on TrkB receptors. Therefore, the BDNF-TrkB pathway is likely to be a novel signaling mechanism for the SLI-mediated neuroprotection against ischemia stress.

There is abundant evidence to support that CREB plays an important role of the transcriptional factor in mediating opioid-induced signaling [77], while phosphorylated CREB is a constitutive transcriptional factor and possibly mediates neuroprotection [78]. CREB may be regulated by the administration of opioid drugs and the subsequent signal changes in gene expression. CREB phosphorylated at Ser133 of p-CREB protein suffer a rapid and transient increase within the ischemic core area, following transient focal ischemia and a marked decrease in p-CREB positive nuclei at 12 and 24 h reperfusion [79]. In the present study, we thoroughly examined SLI's effect on total and phosphorylated CREB proteins in ischemic

brain tissue. We found that the total CREB and p-CREB were observably reduced in T1DM-MCAO group, while SLI treatment groups increased the expression of total CREB and phosphorylated CREB protein, implying that SLI might play an important role in CREB signaling.

In summary, SLI treatment not only promotes recovery of ischemic damage but also improves neurological function through multiple mechanisms of action, including increasing VEGF and BDNF production, up-regulating BDNF-TrkB-CREB signaling together with promoting recovery of brain tissue injury. These effects pertain to not only stroke, but also diabetes in T1DM-MCAO rats. We expect our study to provide a translational clue for a potential application in clinical settings. However, the present results are limited to treatment with SLI at 24 hours after MCAO and monitoring for 14 days after Stroke with diabetes rats. There is a need to further illuminate the effects of SLI on different models of ischemia. Also, it is equally important to estimate a long-term outcome of SLI in T1DM-MCAO. All these issues should be carefully addressed in future investigations.

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Disclosure of conflict of interest

None.

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