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Potential cerebrovascular protective functions of lycium barbarum polysaccharide in alleviating hyperglycemia-aggravated cerebral ischemia/reperfusion injury in hyperglycemic rats

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Abstract. – OBJECTIVE: Lycium barbarum polysaccharide (LBP) is the efficient primary compound of Lycium barbarum and has been shown to alleviate hyperglycemia-aggravated cerebral ischemia/reperfusion (I/R) injury. However, the cerebrovascular changes related to diabetes mellitus (DM) and the potential cerebrovascular protective effects of LBP are still unknown. This study aimed to explore the cerebrovascular protective functions of LBP on cerebral I/R injury in diabetic rats and its potential mechanisms.

MATERIALS AND METHODS: Sprague Dawley (SD) rats were separated into three groups: the normoglycemic (NG), diabetic hyperglycemic (HG), and HG + LBP (50 mg/kg) treatment groups. A 30 min transient middle cerebral artery occlusion (tMCAO) with 24 h reperfusion was established. The neurological deficits, cerebral water content, infarct volume, and cerebrovascular permeability were assessed to evaluate the extent of cerebral injury. Histopathological alterations were assessed by hematoxylin and eosin, Nissl, immunohistochemical, and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining. A transmission electron microscope was used to detect ultrastructural alterations, and a western blot was used to examine protein expression.

RESULTS: The HG rats exhibited a significant increase in neurological deficits, cerebral water content, infarct volume, cerebrovascular permeability, neural cell death, and apoptosis compared with the NG rats, and the LBP treatment alleviated these effects. Cerebrovascular structure analysis showed that the cross-sectional area (CSA) and wall thickness were remarkably altered in the HG rats compared with the NG rats. The LBP treatment protected the cerebrovascular structure and vasoreactivity by decreasing the wall thickness and increasing the CSA, α-smooth muscle actin, and endothelial nitric oxide synthase expression of cerebral vessels.

CONCLUSIONS: The intake of LBP benefits the cerebrovascular structure and vasoreactivity in diabetic rats. Our research provides a possible new strategy for treating stroke in patients with DM.

Key Words:
Lycium barbarum polysaccharide, Diabetes, Cerebral ischemia and reperfusion, Vascular remodeling, Smooth muscle actin, Endothelial nitric oxide synthase.

Abbreviations

LBP: Lycium barbarum polysaccharide (LBP); I/R: Ischemia/Reperfusion; DM: Diabetes mellitus; NG: Normoglycemia (NG); HG: Hyperglycemia (HG); tMCAO: transient Middle Cerebral Artery Occlusion; CWC: Cerebral water constant; CBF: Cerebral blood flow; mNSS: modified Neurological Severity Score; TEM: Transmission electron microscopy; TTC: 2, 3, 5 – triphenyltetrazolium; STZ: Streptozotocin; CSA: Cross-sectional area; SMA: Smooth muscle actin; eNOS: endothelial Nitric Oxide Synthase; ROS: Reactive Oxygen Species.

Introduction

Epidemiological and clinical studies indicate that stroke is the second-highest cause of death

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and the third-highest cause of permanent disability globally[1,2]. Ischemic stroke accounts for approximately 80% of patients with stroke, which is caused by the occlusion of cerebral arteries and ultimately leads to irreversible brain damage[3]. Diabetes mellitus (DM), characterized by chronic hyperglycemia, is present in about 40% of patients with acute ischemic stroke and is related to increased cerebral vulnerability in ischemic injury and worsening clinical outcomes[4]. More clinical studies[5-7] and animal models have confirmed that metabolic diseases, especially DM and hyperglycemia, are the primary risk factors of stroke.

Many potential mechanisms are involved in hyperglycemia-aggravated cerebral ischemia/reperfusion (I/R) injury, including lactic acid accumulation, inflammatory response, reactive oxygen species (ROS) signaling, and so on[5,6]. In addition, diabetic cerebrovascular diseases, including macroangiopathy and microvascular reactivity injury, are essential mechanisms as well[6-8]. Diabetes mellitus is not only a chronic metabolic disease but also a vascular disease, leading to significant cerebral circulation dysfunction[6,9,10]. Although stroke is traditionally believed to be a vascular complication of DM caused by accelerating atherosclerosis or carotid artery disease, many studies suggest that the cerebral vasculature is also seriously influenced by a prolonged state of diabetic hyperglycemia[11,12]. Adverse cerebrovascular remodeling and altered cerebral vasoreactivity caused by DM are likely to result in hypoperfusion or hypoxia and further aggravate brain damage after stroke[13,14]. However, most previous studies have focused on cerebrovascular physiological dysfunctions such as endothelial cell damage, while the morphological changes of cerebrovascular architecture and related mechanisms are still unknown.

In recent years, in the field of traditional Chinese medicine, many mouse models have been established to study the effectiveness of traditional Chinese medicine, especially in the field of diabetes[15-17]. Lycium barbarum is a traditional Chinese medicine widely used to promote health and longevity or as an effective dietary supplement in many countries for more than 2,000 years[18]. Lycium barbarum polysaccharide (LBP), the major bioactive macromolecules and effective ingredients of Lycium barbarum, possess various beneficial functions in antioxidation, anti-inflammation, anti-tumor, neuroprotection, and immune regulation[18-20]. Furthermore, previous studies indicate the neuroprotective function of LBP is related to maintaining mitochondrial fission and fusion balance and decreasing the production of ROS in diabetic hyperglycemia[21-23]. In addition, increasing studies have revealed that LBP performs vascular protective effects by decreasing endothelial and smooth muscle cell dysfunction[23,24]. However, whether LBP could reduce ischemic brain damage through a cerebrovascular protective effect in diabetic hyperglycemia remains unclear. This research aimed to investigate the protective functions of LBP on hyperglycemia aggravated cerebral I/R injury and related cerebrovascular changes in hyperglycemic rats.

Materials and Methods

Animals and Reagents

Healthy male Sprague Dawley (SD) rats aged 8 weeks and weighing 210 to 230 g were supplied by the Experimental Animal Center of Ningxia Medical University. All animal interventions were conducted according to the NIH Guide for Care and Use of Laboratory Animals. The objective was to minimize the stress and usage of the rats involved in the research. The rats were kept in a standard laboratory with free access to water and food, a 12:12 hour light/dark cycle, controlled air humidity of 50%-60%, and room temperature of 22 °C ± 2°C. The reagents and products used included LBP (Ningxia Agricultural College), streptozotocin (STZ, Sigma-Aldrich, St Louis, MO, USA), Triphenyltetrazolium chloride (TTC, Sigma-Aldrich, St Louis, MO, USA), anti-endothelial nitric oxide synthase (anti-eNOS), anti-phosphorylated-eNOS (p-eNOS), anti-α-smooth muscle actin (anti-α-SMA, ProteinTech, Chicago, IL, USA), anti-β-actin (Cell Signaling Technology, Danvers, MA, USA), anti-CD34, horseradish peroxidase (HRP)-labeled goat anti-rat IgG and associated HRP-labeled secondary antibody (ZSGB-Bio, Beijing, China), hematoxylin and eosin (H&E) and Nissl staining kits (Beyotime, Shanghai, China), and a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) Assay Kit (Roche, Mannheim, Germany).

Animal Groups

There were 86 male SD rats involved and randomized into three groups: (1) transient middle cerebral artery occlusion (tMCAO) in normoglycemic rats pretreated with saline (NG group), (2) tMCAO in STZ-induced hyperglycemic rats (HG group), (3) tMCAO in STZ-induced hyperglycemic rats pretreated with LBP (LBP group). Each

Table I. Summary of different groups.

Groups	Processing	TTC	CWC	His	WB	TEM
NG		7	5	8	7	2
Sham	Saline + sham-operation	3	2	3	3	1
24h I/R	Saline + tMCAO + reperfusion 24h	4	3	5	4	1
HG		7	6	8	8	2
Sham	STZ + sham-operation	3	3	3	4	1
24h I/R	STZ + tMCAO + reperfusion 24h	4	3	5	4	1
HG + LBP		7	6	8	8	2
Sham	STZ + LBP + sham-operation	3	3	3	4	1
24h I/R	STZ + LBP + tMCAO + reperfusion 24h	4	3	5	4	1

NG: normoglycemia; HG: hyperglycemia; TTC: 2, 3, 5-triphenyltetrazolium chloride; tMACO: transient middle cerebral artery occlusion; 24h I/R: tMCAO and reperfusion 24h. CWC: cerebral water constant; His: histology; WB: western blot; TEM: transmission electron microscopy.

group consisted of two subgroups: a sham-operation control and 30 min tMCAO plus 24 h reperfusion groups (Table I).

Induction of Diabetic Hyperglycemia Rats and Lycium Barbarum Polysaccharide Treatment

The rats fasted overnight with free access to water. Diabetic hyperglycemia in the rats was induced by an intraperitoneal injection of STZ (60 mg/kg). Age-matched rats given the same intraperitoneal injection volume of saline were used as the normoglycemic controls. The blood glucose levels were detected after three days of STZ injections with a OneTouch glucose monitor (Boshilong, Taiwan). According to our previous studies, caudal vein blood glucose over 16.8 mmol/L was the standard of diabetic hyperglycemia in rats[21,25]. The LBP (50 mg/kg) was administrated by gavage daily to the rats in the LBP group based on a previous study[26]. The LBP treatment was initiated three days after the induction of diabetic hyperglycemia and continued for four weeks prior to the tMCAO operation. Both the NG and HG groups' rats were treated with saline orally for four weeks.

Transient Middle Cerebral Artery Occlusion and Reperfusion

The focal brain ischemia was induced by the tMCAO method, which was processed following our previous study[21]. Anesthesia was induced with isoflurane via a face mask (Matrx VIP 3000). The rats' body temperatures were maintained with a temperature-controlled heating pad during the operation and recovery period. After a midline skin incision was made in the neck and under a dissection stereo microscope, the right external carotid artery (ECA), internal carotid artery (ICA), and common carotid artery (CCA) were carefully dissected from the soft tissue and vagus nerve. The right CCA and ICA were temporarily closed, and the right ECA was exposed and separated from the arterial branch. The ECA was ligated about 2 mm distal from the origin, and then a small incision was made to insert silicone-coated nylon filament (Doccol). The filament was inserted to the origin of the middle cerebral artery (MCA) for a 30 min occlusion. After that, the filament was removed, and the MCA achieved circulation again. Then, the incision was sutured, and the rats were placed in an animal intensive care unit (Lyon) to recover from anesthesia. In the sham rats, the ICA was surgically separated without occlusion of the MCA.

Assessing Neurological Deficits

In this study, two methods were used for assessment of the neurological deficits: (1) the neurological deficits in spontaneous activity were determined after the rats recovered from anesthesia with a Zea Longa score as follows: 0 = no neurological deficit, 1 = unable to extend the left paw, 2 = circling to the left, 3 = falling to the left, 4 = failure to walk spontaneously or unconsciousness. Rats with a Zea Longa score of 2 or more were regarded as successful tMCAO models. (2) A global neurological deficit was evaluated after 24 h reperfusion with a modified Neurological Severity Score (mNSS) consisting of five tests: motor, sensory, balance, reflex, and muscle tone. The scale of the mNSS was graded from 0-18, and a score of 13 to 18 was a serious injury, 7 to 12 was

a moderate injury, and 1 to 6 was a slight injury. In summary, a higher neurological score indicated more serious injury. The rats were given a code number, and the assessment of neurological deficits was performed and analyzed by two researchers blinded to the experimental conditions.

Assessing the Cerebral Infarction Volume

In this study, two methods were used for assessing the cerebral infarction volume: TTC staining and H&E staining and scanning[27]. (1) With TTC staining, the cerebral infarct volume was assessed 24 h after reperfusion following tMCAO. The rats were anesthetized and subsequently decapitated to dissect the whole brain. The brain was quickly collected, frozen at -20°C for 5 min, and then cut into four 2-3 mm slices coronally with a brain matrix (RWD, Shenzhen, China). The brain slices were quickly stained and incubated with a 1% TTC solution in a thermostatic box at 37°C for 30 min to assess the cerebral infarction's extent and size. The viable brain without infarction could be stained as deep red; however, the infarcted brain was white. The images were digitally photographed and analyzed by NIH ImageJ 1.53k software. To correct for brain swelling, the infarct area was calculated as (ipsilateral hemisphere area - contralateral hemisphere area) / (contralateral hemisphere area) × 100%. The infarct volume calculation integrated infarction areas for all slices of each brain. (2) The brain slices were embedded with paraffin, stained with H&E, and scanned (Motic, Xiamen, China). Five representative coronal sections of the brain were selected to accurately locate the cerebral infarction area and assess the infarction volume. The five coronal sections consisted of "1. Bregma 2.0 mm, 2. Bregma 1.0 mm, 3. Bregma 0.2 mm, 4. Bregma -1.0 mm, and 5. Bregma -3.0 mm (Paxinos and Watson, 1996)". The relative infarct volume was corrected and calculated as described above.

Detection of Cerebral Water Content and Cerebrovascular Permeability

The dry/wet weight method assessed the cerebral water content[28]. At 24 h after reperfusion, the rats were killed by decapitation under deep anesthesia, and their brains were removed quickly. The olfactory projections, cerebellum, and brainstems were discarded, and the brains were separated into halves. The right ischemic hemisphere was rapidly weighed to obtain the wet weight, and then the tissue sample was dried in an oven at 80°C for 48 h to get the dry weight. The cerebral water content was calculated as (wet weight - dry weight) / wet weight × 100%. Cerebrovascular permeability disruption was assessed with immunohistochemical detection of IgG[29]. After using the 10% goat serum to block endogenous peroxidase, the brain sections were incubated overnight with HRP conjugated goat anti-rat IgG (1:200, ZSGB-Bio, Beijing, China) at 4°C, and then stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB, ZSGB-Bio, Beijing, China). IgG extravasation was analyzed with the scanned section images.

Anatomy of the Circle of Willis and Pia Mater

The rats were deeply anesthetized and then transcardially perfused with saline and 4% PFA to flush the blood and fix the brain. The rats were decapitated, and the whole brain was collected with the circle of Willis and pia mater. The brain slices were fixed with 4% PFA and embedded with paraffin. The coronal section of "Bregma 0.2 mm (Paxinos and Watson, 1996)" was selected to calculate the wall thickness and cross-sectional area of the ICA within the circle of Willis.

Hematoxylin and Eosin, Nissl, Terminal Deoxynucleotidyl Transferase dUTP Nick-end Labeling, and Immunohistochemical Staining

According to the manufacturer's protocols, H&E and Nissl staining were performed (Beyotime, China). Neural cell apoptosis after the I/R injury was detected by TUNEL staining (Roche, Mannheim, Germany). According to the instructions protocol, the positive cells were counted in five high-power fields (400×). Immunohistochemical analysis of the brain was performed using the standard protocols provided by the antibody manufacturers. After antigen recovery, the brain sections were blocked with 10% goat serum. The sections were incubated overnight at 4°C with the primary antibodies: α-SMA (1:2,000, 14395-1-AP, ProteinTech), eNOS (1:200, 27120-1-AP, ProteinTech), and CD34 (ready to use, ZM-0046, ZSGB-Bio, Beijing, China). Next, the sections were briefly washed and incubated with appropriate secondary antibodies at 37°C for 45 mins. Then, DAB staining was used to visualize the reaction, and hematoxylin was used to counter-stain the nucleus. After that, the sections were photographed with a microscope (Olympus BX51), and ImageJ (1.53k, NIH) software was used to analyze the intensity of the immunolabeling[30].

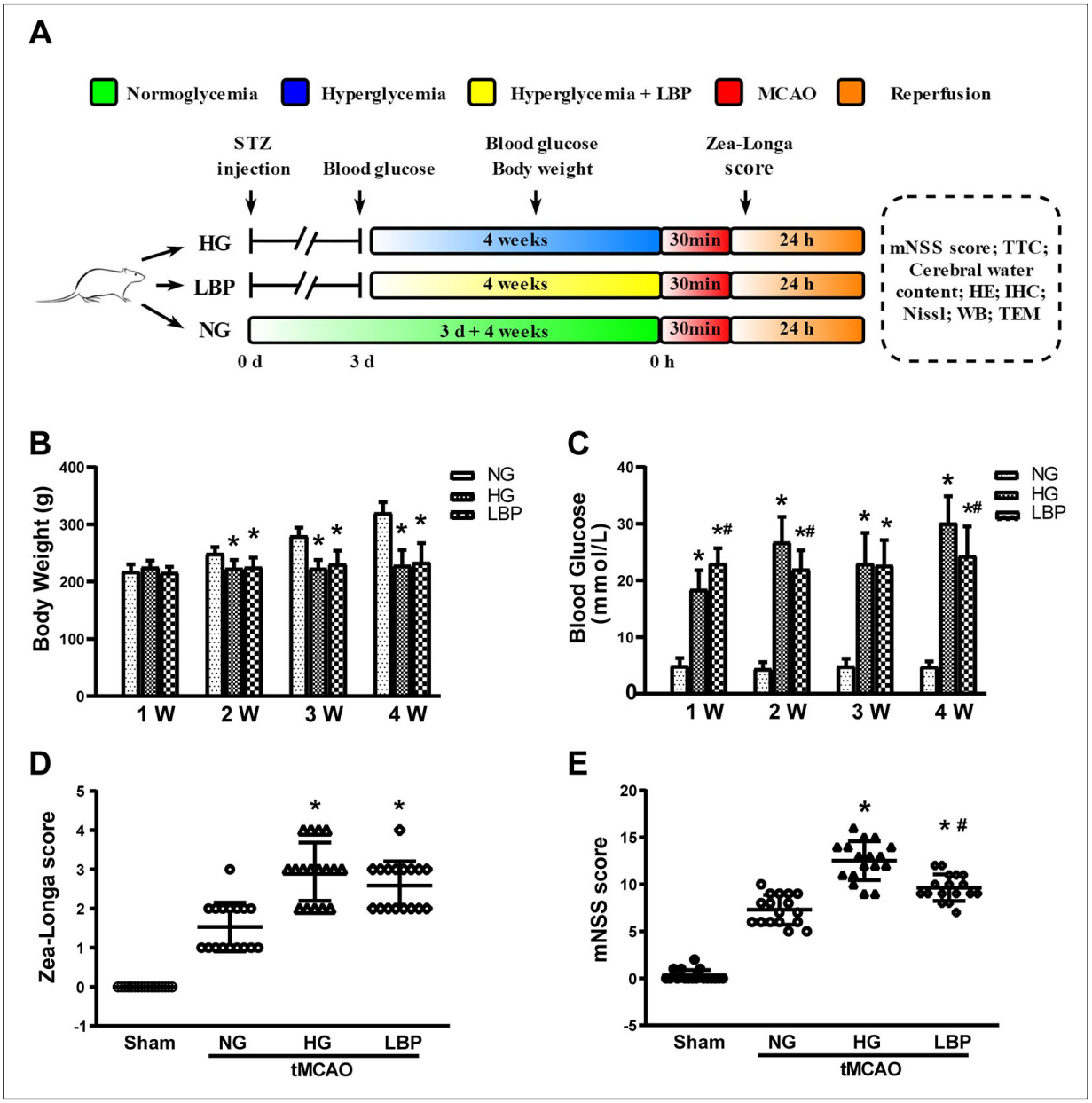

Figure 1. Effects of hyperglycemia and the Lycium barbarum polysaccharide treatment on the neurological deficits, body weight, and blood glucose following streptozotocin injection, transient middle cerebral artery occlusion, and ischemia/reperfusion injury. This flow diagram illustrates the experimental design (**A**). Summary of body weight (**B**) and blood glucose (**C**). Summary of neurological deficits assessed by the Zea Longa (**D**) and modified Neurological Severity Score (**E**) scales after 30 min transient middle cerebral artery occlusion. *$p < 0.05$ *vs.* the NG group; $^{\#}p < 0.05$ *vs.* the HG group.

Western Blot

The western blot analysis in this study was done using the circle of Willis arteries and part of the ICA. The rats were sacrificed, and tissues were collected and put on ice. Then, the tissues were homogenized in lysis buffer and broken up for collecting the supernatants after centrifugation. The concentration of each sample in the supernatant was detected with a BCA protein quantitation kit. Equal amounts of protein extracts were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After being blocked for 3 h with 5% BSA, the membranes were incubated at 4°C overnight using the primary antibodies anti-α-SMA, eNOS, p-eNOS, and β-actin. After washing with TBST, the membranes were incubated with

appropriate secondary antibodies for 1 h at room temperature. The ECL method was used for detecting signal protein bands, and images were performed by the Amersham Imager 600 system. The results of the densitometric values were expressed as ratios of the target proteins to β-actin.

Electron Microscopic Study

The penumbra of the ischemic cortex (1×1×2 mm^3) was fixed with 4% glutaraldehyde at 4 °C overnight, followed by washing three times with 0.1 M sodium dimethyl arsenate buffer then soaked in 1% osmium tetroxide at room temperature for 2 h. The tissue was dehydrated by a gradient series of alcohol, infiltrated with propylene oxide, and embedded in epoxy resin. Ultrathin sections (60 nm) were cut with a diamond knife and stained with uranyl acetate and lead citrate before examination by transmission electron microscope (HT7800, Hitachi, Tokyo, Japan).

Statistical Analysis

Data were expressed as mean ± standard deviation, and one-way analysis of variance for multiple comparisons was used to analyze the differences among the groups. Statistical analysis was performed by SPSS 20.0 (SPSS Corp., IBM, Armonk, NY, USA) and GraphPad Prism 8.0 software (La Jolla, CA, USA). $p < 0.05$ was considered statistically significant.

Results

Lycium Barbarum Polysaccharide Treatment Decreased Neurological Deficits Aggravated by Diabetic Hyperglycemia After Cerebral Ischemia/Reperfusion Injury

The experiment workflow is illustrated in Figure 1A. The rats' body weight and blood glucose were continuously measured over four weeks in the different groups. The average body weight of the rats in the NG group showed a gradually increasing trend over time, and they were heavier than the other two groups from the second week (Figure 1B, $p < 0.05$). The average body weight of the HG and LBP groups' rats remained unchanged or even reduced. The LBP group had a heavier average body weight than the HG group in the third and fourth weeks, whereas no significant difference was found. As expected, the average blood glucose of the NG group was maintained at a low level, and that of the HG and LBP groups was significantly increased after STZ injection (Figure 1C, $p < 0.05$). Compared with the HG group, the LBP group showed an obvious decreased average blood glucose in the second and fourth weeks, but it was still higher than the NG group and above 16.8 mmol/L. The neurological deficits of spontaneous activity were evaluated using the Zea Longa score (Figure 1D), and global neurological deficits were evaluated using the mNSS (Figure 1E). The data showed that the sham rats had no neurological deficits. Both the HG and LBP groups had more serious neurological deficit scores compared with the NG group (Figure 1D and E, $p < 0.05$). Compared with the HG group, the LBP treatment significantly decreased the neurological deficits.

Lycium Barbarum Polysaccharide Treatment Decreased Ischemic Brain Injury and Protected Cerebrovascular Permeability After Cerebral Ischemia/Reperfusion Injury

Representative TTC staining images of each group (Figure 2A) showed that the NG group had limited cerebral infarction volumes, including the striatum and fractional involvement of the overlying cortex of the forebrain. Compared with the NG group, the infarct volumes were notably enlarged in the HG group, which covered most parts of the striatum and cortex of the right brain regions. The LBP treatment prior to tMCAO significantly decreased the infarction volumes compared with the HG group (Figure 2D, $p < 0.05$). Brain section scanning (Figure 2B) and analysis (Figure 2E) after H&E staining enabled us to locate and precisely define the cerebral infarction regions. Compared with the HG group, the LBP group had decreased infarction regions of the motor, somatosensory, and auditory cortexes (Figure 2E, $p < 0.05$).

IgG extravasation was used to assess cerebrovascular permeability and detect capillary leakage by immunostaining. Compared with the NG group, the extent and size of IgG staining remarkably increased in the HG and LBP groups (Figure 2C). Compared with the HG group, IgG leakage was reduced in the LBP group (Figure 2F, $p < 0.05$).

The cerebral water content reflected the severity of brain edema after injury. The CWC was obviously increased in the HG group compared with the NG group (80.87 ± 0.68% *vs.* 87.13 ± 0.41%, Figure 2G, $p < 0.05$), while with the LBP treatment, the CWC was decreased compared with the HG group (84.56 ± 0.69% *vs.* 87.13 ± 0.41%, Figure 2G, $p < 0.05$).

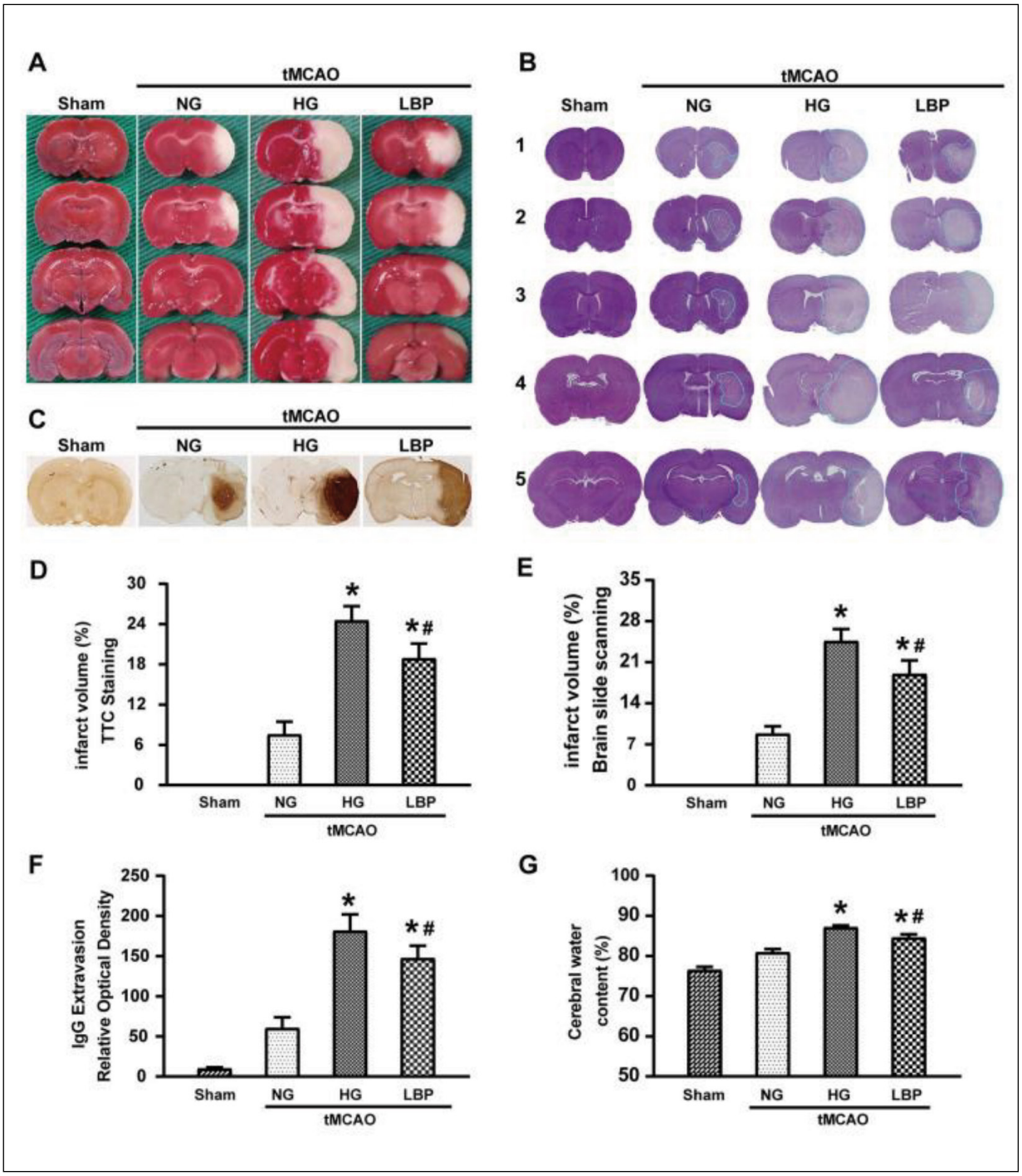

Figure 2. Effects of hyperglycemia and the Lycium barbarum polysaccharide treatment on the cerebral infarct volumes, cerebrovascular permeability, and cerebral water content after 24 h of reperfusion with 30 min transient middle cerebral artery occlusion. The representative triphenyltetrazolium chloride-stained brain section images show the infarct volumes (pale area) (**A**). Representative scanning image of the infarct area stained with hematoxylin and eosin 24 h of ischemia/reperfusion in the different groups (**B**). Representative scanning image of the IgG-stained brain sections showing the cerebrovascular permeability of the infarct area 24 h of ischemia/reperfusion in the different groups (**C**). Quantitative summary of the infarct volumes of triphenyltetrazolium chloride staining (**D**). Quantitative summary of infarct the volumes of the brain sections scanning (**E**). Quantitative summary of the IgG extravasation of the brain infarct area (**F**). Quantitative analysis of the cerebral water content detected by the wet/dry weight method (**G**). $^{*}p < 0.05$ *vs.* the NG group; $^{\#}p < 0.05$ *vs.* the HG group.

Lycium Barbarum Polysaccharide Treatment Attenuated Diabetic Cerebrovascular Remodeling and Cerebral Vasoreactivity Represented by the Internal Carotid Artery in the Circle of Willis

We assessed the morphological changes in the cerebrovascular architecture by measuring the wall thickness and cross-sectional area (CSA) of the ICA within the circle of Willis four weeks after STZ injections with or without LBP treatment (Figure 3A). The representative images indicated that the wall thickness of the ICA was remarkably greater in the HG group compared with the NG group, while the CSA of the ICA from the HG group was significantly decreased. The LBP treatment prevented the ICA morphological changes, significantly decreased the wall thickness, and increased the CSA in the LBP group compared with the HG group (Figure 3B and C, $p < 0.05$).

Next, eNOS and α-SMA expressions of the ICA within the circle of Willis were detected to investigate cerebral vasoreactivity and pathological cerebrovascular remodeling. The IHC staining findings showed that eNOS expression was downregulated in endothelial cells and restored by the LBP treatment (Figure 3D, $p < 0.05$), and the α-SMA expression in the smooth muscle cells of the ICA media was the same as eNOS (Figure 3E, $p < 0.05$).

Then, we used western blot to further investigate eNOS, p-eNOS, and α-SMA expressions in the cerebral arteries (Figure 3F). The results showed that total eNOS, p-eNOS, and α-SMA expressions were significantly reduced in the HG group, which could be restored by the LBP treatment (Figure 3G, H, and I, $p < 0.05$).

Thus, the results revealed that diabetic hyperglycemia was associated with morphological changes of cerebrovascular architecture and cerebral vasoreactivity. The most important characteristic alterations were morphological changes, including increased wall thickness and decreased CSA, accompanied by decreased expressions of eNOS, p-eNOS, and α-SMA. The LBP treatment could attenuate diabetic cerebrovascular remodeling and protect cerebral vasoreactivity.

Lycium Barbarum Polysaccharide Treatment Attenuated Diabetic Cerebrovascular Alterations Represented by Small Vessels in the Pial Collaterals and Capillaries of the Brain Parenchyma

To further investigate the morphological and histopathological alterations of diabetic microangiopathy, we examined the arterioles in pial collaterals and capillaries within the parenchyma. From the representative H&E pictures, we could see that the rats in the HG group exhibited increased wall thickness and decreased CSA in the arterioles of the pial collaterals compared with the NG group; the LBP treatment attenuated the alterations (Figure 4A). However, statistical analyses were not done due to the variation of arterioles in different coronal sections. The IHC staining findings of α-SMA and eNOS revealed that both expressions were decreased and restored by the LBP treatment (Figure 4B and C, $p < 0.05$).

The morphology of the endothelial cells and capillary density were measured by CD34 IHC staining (Figure 4A). The capillaries of the brain parenchyma in the NG group rats showed linear or branched patterns as the main modes, and punctate capillaries were fewer, while the capillaries of the brain parenchyma in the HG groups' rats showed a punctate pattern as the main mode and linear or branched capillaries were fewer than in the NG group. The LBP treatment attenuated capillary alterations and made linear or branched capillaries more than the HG group. The data showed that the capillary density was significantly increased in the HG group rats and restored by the LBP treatment (Figure 4D, $p < 0.05$).

The ultrastructural alterations of the capillaries after I/R injury were demarcated by transmission electron microscope analysis (Figure 4E). The morphology of the capillaries was normal in the non-ischemic sham rats. The capillary luminal membrane was smooth, and the basal membrane was intact. Morphological deformities of the capillaries were observed in the rats 24 h after I/R injury. In the NG group, the capillary luminal membranes were not smooth, the endothelial retraction was mild, and the basal membranes were partly dissolved. Compared with the NG group, morphological deformities of the capillaries were more evident in the HG group, accompanied by rough capillary luminal membranes and increased space between the endothelial cells and basal membranes. The LBP treatment could attenuate capillary morphological deformities compared with the HG group.

Lycium Barbarum Polysaccharide Treatment Decreased Cerebral Injury, Neuron Death, and Neural Cell Apoptosis After Cerebral Ischemia/Reperfusion Injury

Both H&E and Nissl staining were utilized to evaluate the effect of diabetic hyperglycemia and

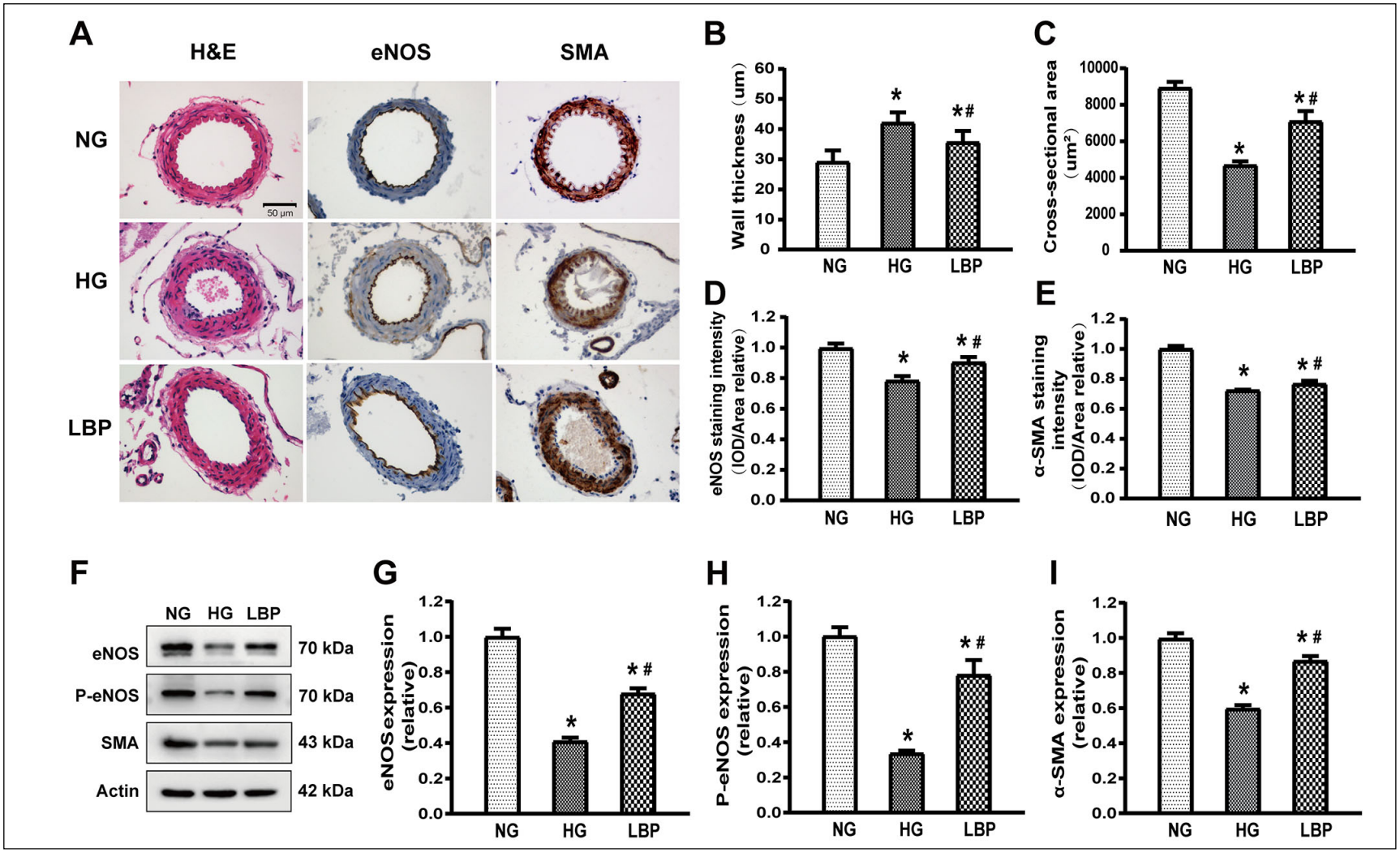

Figure 3. Effects of hyperglycemia and the Lycium barbarum polysaccharide treatment on changes of the cerebrovascular structure in the internal carotid artery within the circle of Willis. Representative cross-section images of hematoxylin and eosin and immunohistochemical staining of endothelial nitric oxide synthase and α-smooth muscle actin (200×, scale bar = 50 μm) (**A**). Measurements of the wall thickness (**B**) and cross-sectional areas (**C**) in the internal carotid artery, respectively. The protein expression intensities of endothelial nitric oxide synthase (**D**) and α-smooth muscle actin (**E**). Western blot analyses of endothelial nitric oxide synthase, anti-phosphorylated-eNOS, and α-smooth muscle actin protein expressions were normalized to housekeeping protein β-actin (**F**). Summary of the relative values of the above proteins (**G, H,** and **I**). *$p < 0.05$ *vs.* the NG group; $^{\#}p < 0.05$ *vs.* the HG group.

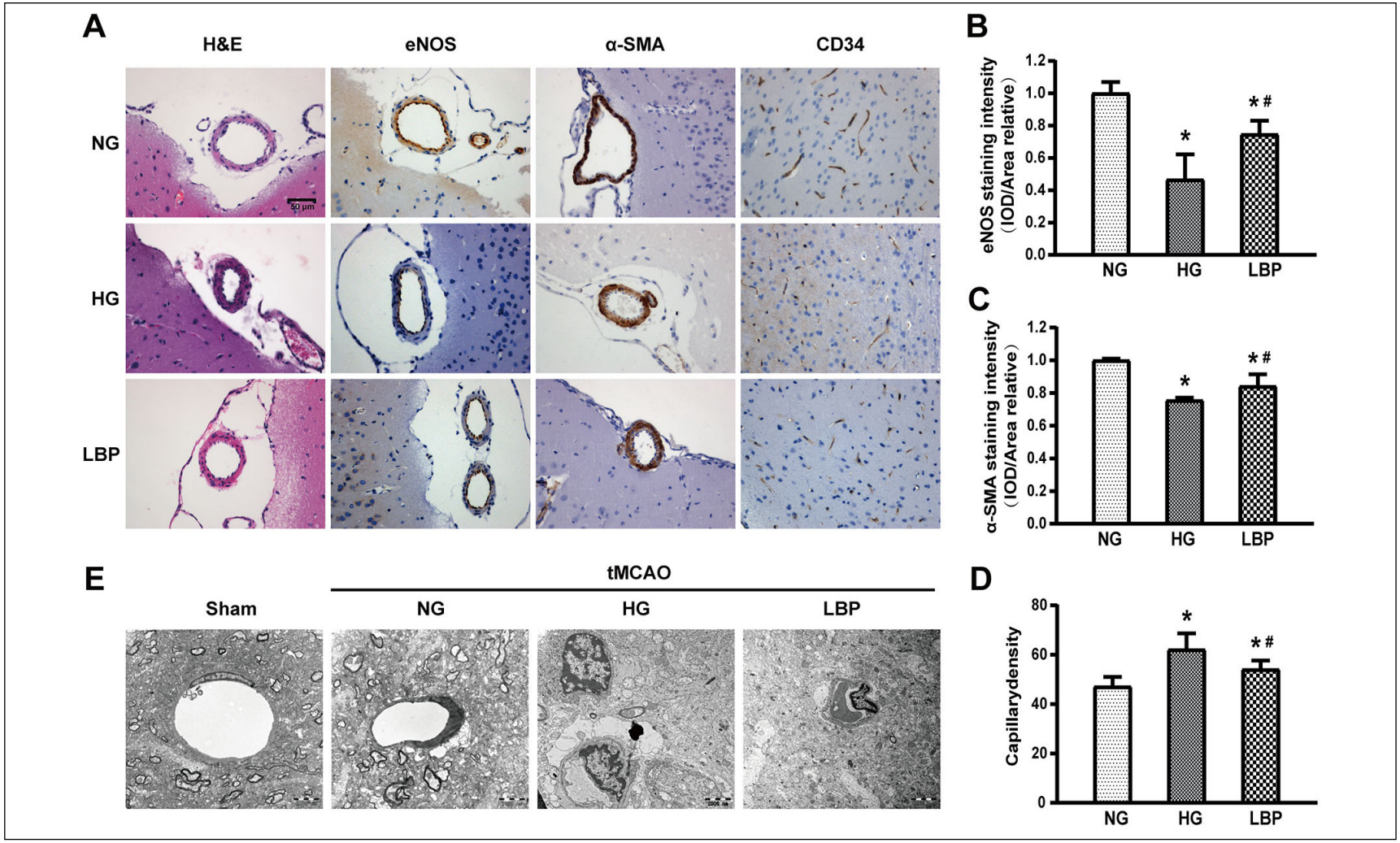

Figure 4. Effects of hyperglycemia and the Lycium barbarum polysaccharide treatment on changes of the small vessels in the pial collaterals, brain parenchyma capillaries, and penumbra of the ischemic cortex. Representative images of hematoxylin and eosin and immunohistochemical staining of endothelial nitric oxide synthase, α-smooth muscle actin, and CD34 (200×, scale bar = 50 μm) (**A**). The mean values of normalized protein expressions of endothelial nitric oxide synthase (**B**) and α-smooth muscle actin (**C**). Measurement of capillary density (**D**). $^{*}p < 0.05$ *vs.* the NG group; $^{\#}p < 0.05$ *vs.* the HG group. The ultrastructural alterations of the capillaries after ischemia/reperfusion injury in the different groups (scale bar = 2,000 nm) (**E**), respectively.

LBP on the histopathological alterations of the ischemic penumbra. As shown in Figure 5A, the cortex structure was normal in the non-ischemic control rats, with no brain edema and noticeable swelling of the stroma; only a few dispersed dead neurons were discovered. After 24 h of reperfusion, the ischemic penumbra showed apparent histopathological alterations characterized by brain edema, swelling of the stroma, and neuronal pyknosis. As expected, hyperglycemia further increased the percentage of pyknotic cells of the ischemic penumbra after reperfusion compared with the NG group. The LBP treatment alleviated brain edema and neuronal pyknosis (Figure 5B, $p < 0.05$). Similarly, Nissl staining indicated hyperglycemia reduced the proportion of Nissl-positive cells, and the LBP treatment increased them (Figure 5C, $p < 0.05$). The results showed that hyperglycemia increased neural cell injury, and the LBP treatment could save them. Then, we used TUNEL staining to detect apoptotic cells in the penumbra of the ischemic cortex. As described in Figure 5A, no apoptotic cells were observed in the sham group, and I/R injury created many TUNEL-positive cells. Compared with the NG group, hyperglycemia further increased TUNEL-positive cells. However, the LBP group showed a significant decrease in TUNEL-positive cells after reperfusion (Figure 5D, $p < 0.05$).

Discussion

This study demonstrated that the LBP treatment significantly decreased the neurological deficit scores, cerebral infarct volume, brain edema, and cerebral I/R injury aggravated by diabetic hyperglycemia. The LBP treatment attenuated diabetic cerebrovascular remodeling and impaired vasoreactivity, represented by the ICA within the circle of Willis and small vessels of the pial collaterals, accompanied by decreased α-SMA and eNOS expressions. In addition, the LBP treatment reduced the capillary density of the brain parenchyma in diabetic rats and alleviated ultrastructural damage after injury. Finally, the protective effect of LBP was also related to a decrease in brain neural cell death by inhibiting apoptosis after injury.

Ischemic stroke is a common cause of mortality and disability worldwide[1,2]. Both clinical studies and animal experiments have implicated that DM is a significant risk factor of ischemic stroke because it may induce and worsen brain injury and result in a series of adverse effects[5-7]. Patients with DM are more likely to suffer a stroke, have a larger cerebral infarction, have more severe brain edema, and have worse neurological outcomes afterward than patients without DM. The present study successfully established an STZ-induced diabetic animal model with tMCAO-induced ischemic stroke in rats. After STZ induction, the diabetic rats had typical symptoms of DM (with increased food and water intake, urine output, and decreased body weight). At the same time, the blood glucose of the caudal vein remained above 16.8 mmol/L. In our study, the time for tMCAO was 30 min, which was shorter than most previous studies with an embolism in excess of 60 min. Our previous studies indicated that a 30 min interruption of cerebral blood flow (CBF) in diabetic rats might cause an obvious brain infarction[21,25,31]. If the embolism period was extended, the mortality of the diabetic rats was too high to be accepted for the study. Moreover, our previous studies revealed that the severity of brain injury was most serious after 24 h reperfusion, so we chose this time point to assess the cerebral infarct volume, brain edema, neurologic deficit scores, and so on. In addition, to quantify the cerebral infarct volume more precisely, we selected two assessment methods: TTC staining and scanning of the brain slices. As expected, the scanning of the brain slices made it possible to locate more precisely the brain infarction and explain the reasons for the different neurological deficits compared with TTC staining. The present study results show that diabetic hyperglycemia rats exhibited a larger cerebral infarct volume, more severe brain edema, deficient neurological scores, and more pyknotic neurons than normoglycemic rats. All the findings confirm that diabetic hyperglycemia may aggravate cerebral impairment after cerebral I/R injury. The mechanisms involved in diabetic hyperglycemia aggravated cerebral I/R injury are complex and still under study[5,6,32,33], and potentially effective intervention drugs have made little advances. Therefore, further research is required to explore new effective mechanisms and medications for treating stroke.

Lycium barbarum polysaccharide, the main bioactive compound and effective ingredient of Lycium barbarum, has been extensively investigated for its potential health properties. In previous studies, LBP has been reported to have a hypoglycemic effect, which is more significant in individuals who did not take any oral hypoglycemic agents[34,35]. Accumulating evidence also suggests that LBP provides a neuroprotective ef-

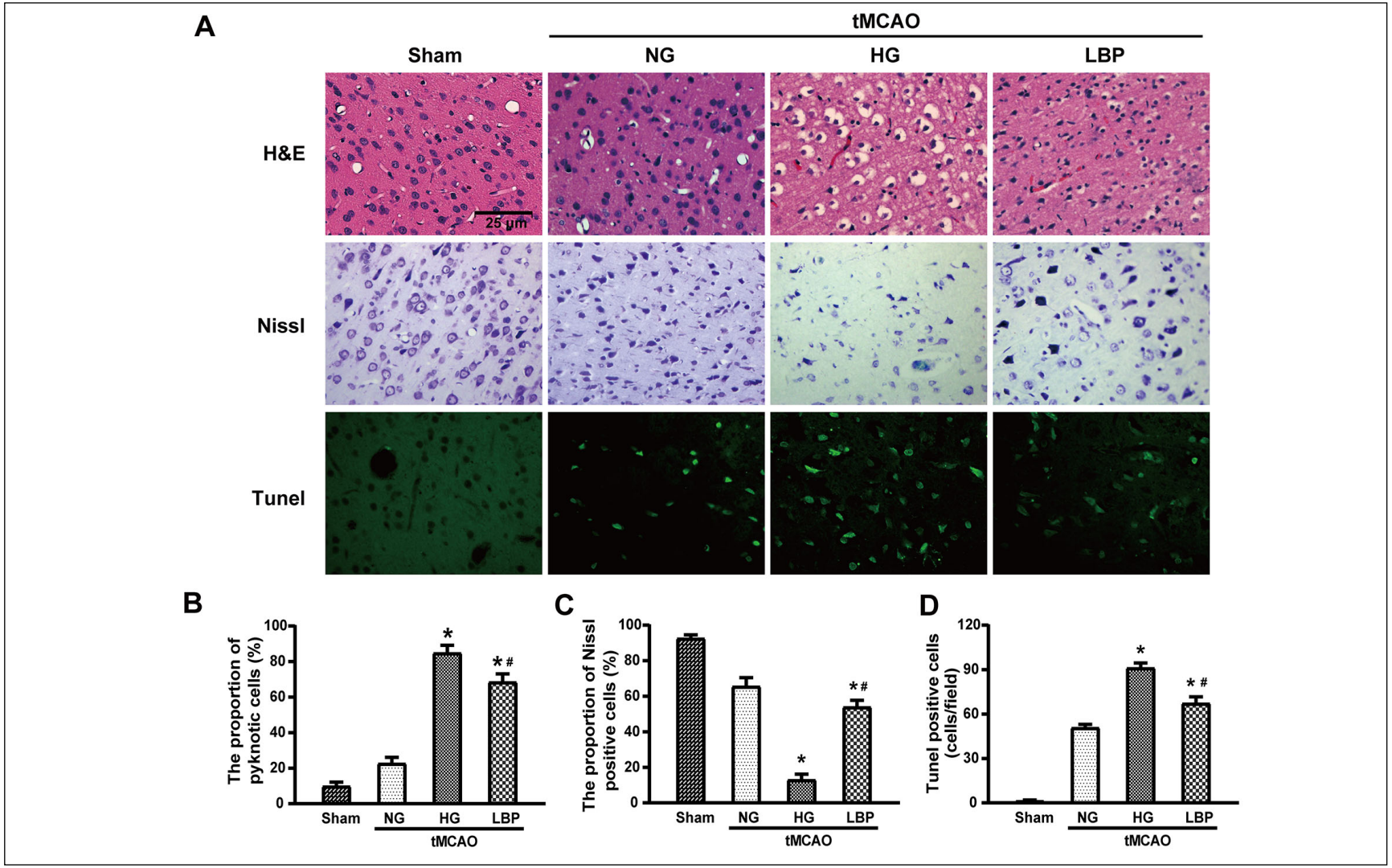

Figure 5. Effects of hyperglycemia and the Lycium barbarum polysaccharide treatment on histopathological changes and neural cell apoptosis assessed by hematoxylin and eosin, Nissl, and terminal deoxynucleotidyl transferase dUTP nick-end labeling staining after 24 h reperfusion with 30 min of transient middle cerebral artery occlusion. The representative pictures of hematoxylin and eosin, Nissl, and terminal deoxynucleotidyl transferase dUTP nick-end labeling staining in the penumbra of the ischemic cortex (400×, scale bar = 25 μm) (**A**). Quantification of pyknotic cells (**B**), Nissl bodies (**C**), and apoptotic cells (**D**) in the ischemic penumbra of the cortex. *$p < 0.05$ *vs.* the NG group; $^{\#}p < 0.05$ *vs.* the HG group.

fect after experimental ischemia *in vivo* and *in vitro*[36,37]. Our current and previous studies provided additional evidence of LBP in neuroprotection with diabetes. However, the hypoglycemic effect of LBP is not significant in this study, and this discrepancy is likely attributed to the difference in LBP dosage and experimental conditions. In this study, the diabetic hyperglycemia rats treated with LBP had significantly decreased cerebral infarct volume, brain edema, neurologic deficit scores, and neural cell apoptosis compared with the hyperglycemia ischemic rats 24 h after I/R injury. Lycium barbarum polysaccharide has been shown to increase cell viability, decrease ROS, and promote anti-autophagic effects mediated by the phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin signaling pathway[37]. Lycium barbarum polysaccharide has also been demonstrated to improve hyperglycemia-enhanced ischemic brain damage by improving mitochondrial neurogenesis and maintaining a dynamic balance[21,38]. Therefore, the neuroprotective effect following experimental I/R injury with diabetic hyperglycemia seems not to be related to the hypoglycemic effects.

Diabetes mellitus is not only a chronic metabolic disease but also a vascular disease, leading to diabetic cerebrovascular diseases, causing intracranial diseases of both the large and small vessels[6,9,39]. On the one hand, ischemic stroke may be the greatest known end organ effect. On the other hand, diabetic macrovascular diseases, such as cerebral atherosclerosis and microvascular diseases, are major drivers of ischemic stroke and exacerbate post-stroke injuries[40,41]. The morphological and functional alterations of the cerebral vessels significantly affect the maintenance of stable CBF in the different processes. Previous studies have observed reductions of CBF in patients with DM compared with healthy controls[42], and there were temporal and spatial changes in the STZ-induced animal models[43]. The adverse cerebrovascular remodeling and alteration of the cerebral vasoreactivity are caused by DM, leading to significant cerebral circulation dysfunction, probably causing hypoperfusion or hypoxia and greater brain damage. A paper by Liu et al[44] reported adverse vascular remodeling of the carotid arteries accompanied by high expression of forkhead box O1 in diabetic rats for eight weeks. Kelly-Cobbs et al[45] and Yasir et al[46] reported that diabetes might cause cerebrovascular remodeling by activating the endothelin A receptors of the middle cerebral arteries. In the present study, we assessed the morphological alterations of different cerebral vessels represented by the ICA in the circle of Willis, small vessels in the pial collaterals, and capillaries of the brain parenchyma. The results demonstrated that diabetes profoundly affected the cerebrovascular architecture and caused adverse cerebrovascular remodeling, accompanied by increased wall thickness, decreased CSA, and α-SMA expression. The LBP treatment attenuated diabetic cerebrovascular remodeling and protected the cerebrovascular structure. According to Poiseuille's law, the size of the vessel is the most potent determinant of blood flow. Therefore, a change in the radius or cross-section will dramatically impact it[47]. The myogenic response mediated by small arteries is another essential intrinsic property of vessels and contributes considerably to CBF regulation[48,49]. Unlike most other vascular beds, the pial artery contributes significant resistance in the cerebral circulation[48,50]. In this study, IHC and western blot were applied to analyze α-SMA expressions and evaluate the smooth muscle cells of the cerebral vessels. The results suggested that diabetes may affect the myogenic response by inducing smooth muscle cell dysfunction, and LBP treatment may alleviate it.

NO-dependent cerebral vasoreactivity significantly contributes to the generation of a dilatory response in the cerebral circulation. Nitric oxide synthases are the rate-limiting enzyme for NO synthesis, and eNOS is usually assessed for evaluating cerebral vasoreactivity. Monteiro et al[51] reported that patients with diabetes without symptomatic cerebrovascular disease showed a significant deficiency in cerebral vasoreactivity and neurovascular coupling. Poittevin et al[14] showed impaired cerebral vasoreactivity in the diabetic mouse model and delayed angiogenesis after stroke. In this study, we analyzed the eNOS and p-eNOS expressions of vessels. The findings suggested that DM may affect cerebral vasoreactivity by reducing eNOS expression of vascular endothelial cells, and the LBP treatment may increase it. The results of the capillary density of the brain parenchyma reveal that DM leads to increased but dysfunctional neovascularization in the cerebrovasculature. However, this augmented angiogenesis is associated with weak vessel wall maturity as indicated by increased permeability and nonperfused vessels[52,53]. The results from the transmission electron microscope revealed that vascular endothelial cells showed more serious injury and had more myelin sheaths of axons in diabetic rats. The LBP treatment reduced injury and edema following stroke. In conclusion, mor-

phological alterations are the basis for functional changes. Alterations in cerebrovascular structure induced by diabetes affect cerebral vasoreactivity and CBF in a way that stimulates hypoperfusion underlies ischemic events and are determining factors of ischemia-induced injury.

Conclusions

In summary, this study successfully developed an STZ-induced diabetic hyperglycemia rat model with a tMCAO induced I/R injury. Diabetic hyperglycemia may increase cerebral infarction, brain edema, neurological outcomes, and neural cell apoptosis 24 h after I/R injury. The LBP treatment may decrease diabetic cerebrovascular injury and protect the cerebrovascular structure and vasoreactivity. In addition, it has been demonstrated that LBP has an excellent safety profile and does not have any documented harmful effects associated with its use. Although the underlying pathological mechanisms have not been completely explored, this research provides a possible new strategy for treating stroke in patients with DM.

Conflicts of Interests

The authors declare that they have no conflicts of interests.

Ethics Approval

The present study was approved by the Institutional Animal Care of Ningxia Medical University, the Ethics Review Committee of Ningxia Medical University and the General Hospital of Ningxia Medical University (2020-558). All animals were treated in compliance with the National Research Council' s Guide for the Care and Use of Laboratory Animals (1996).

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Data Availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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