ORIGINAL ARTICLE

Morin Attenuated Cerebral Ischemia/Reperfusion Injury Through Promoting Angiogenesis Mediated by Angiopoietin-1-Tie-2 Axis and Wnt/β-Catenin Pathway

Satchakorn Khamchai[1,2] · Wijitra Chumboatong[1] · Janejira Hata[3] · Chainarong Tocharus[4] · Apichart Suksamrarn[3] · Jiraporn Tocharus[1,5]

Abstract

Cerebral damage following cerebral ischemia/reperfusion injury affects the neurological deficits and motor impairment of stroke patients in the long-term period. Angiogenesis, the essential process for restoration of cerebral blood flow (CBF) in the ischemic brain, promotes the recovery of neurological function following ischemia. The aim of this study was to investigate the long-term effects of morin on angiogenesis and functional outcomes in a middle cerebral artery occlusion (MCAO) and reperfusion model. Male Wistar rats were subjected to MCAO, and they were administered 30 mg/kg of morin at reperfusion via *i.p.* injection daily for 14 days. Fourteen days after I/R injury, the rats were evaluated for the brain damage, and angiogenic factors involved in Ang1/Tie-2 and Wnt/β-catenin signaling. In addition, at 1, 7, and 14 days after reperfusion, rotarod and pole tests were performed to investigate the functional recovery. We found morin significantly reduced the infarct size, blood–brain barrier (BBB) leakage, and apoptotic cells at 14 days after I/R injury. It also promoted angiogenesis via boosting the expression of angiogenic proteins, such as angiopoietin 1 (Ang1), Tie-2, Wnt3α, β-catenin, and cyclin D1. Morin-mediated angiogenesis was confirmed by a significant increase in microvessel's density in the penumbra area and an increase in von Willebrand factor (vWF) protein expression of the morin-treated rats. Moreover, the rotarod and pole tests also demonstrated morin increased functional recovery in the morin-treated rats compared to the vehicle rats. Therefore, our data exposed that morin promotes angiogenesis and improves functional outcomes in MCAO and reperfusion rats.

Keywords Middle cerebral artery occlusion and reperfusion · Angiogenesis · Angiopoietin 1 · Wnt/β-catenin signaling · Morin

Abbreviations

Ang1	Angiopoietin 1
BBB	Blood-brain barrier
HIF-1α	Hypoxia-inducible factor 1-alpha
I/R	Ischemia/reperfusion
MCAO	Middle cerebral artery occlusion
NRP-1	Neuropilin-1
ROS	Reactive oxygen species
t-PA	Tissue-plasminogen activator
TTC	2,3,5-Triphenyltetrazolium chloride
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
vWF	Von Willebrand factor

✉ Jiraporn Tocharus
jtocharus@gmail.com

1 Department of Physiology, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand

2 Graduate School, Chiang Mai University, Chiang Mai 50200, Thailand

3 Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Ramkhamhaeng University, Bangkok 10240, Thailand

4 Department of Anatomy, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand

5 Center for Research and Development of Natural Products for Health, Chiang Mai University, Chiang Mai 50200, Thailand

Introduction

Stroke, the leading cause of death in adults worldwide, is the most frequent induction of permanent disability (Donnan et al. 2008; Johnston et al. 2009; Wong et al. 2007; Pundik et al. 2012). The most common type of stroke is

cerebral ischemia; cerebral ischemia–reperfusion (I/R) injury triggers the brain damage and long-term disability (Widgerow 2014). The recovery phase of the brain begins 2–14 days after cerebral I/R onset (Pettersson et al. 2000; Zhang and Chop 2002). There are several mechanisms for neurorestorative events and cell survival, including neurogenesis, synaptic plasticity, and angiogenesis. Angiogenesis is a crucial process and a natural defense mechanism helping restore oxygen and nutrient supply to the affected brain tissue (Krupinski et al. 2007; Hayashi et al. 2003). In response to cerebral I/R injury, hypoxic cells trigger the existing vasculature to release angiogenic factors for neurovascularization. Vascular endothelial growth factor (VEGF) is released to bind to vascular endothelial growth factor receptor (VEGFR) on the vascular endothelial cells (Talwar and Srivastava 2014; Marti et al. 2000). Moreover, neuropilin-1 (NRP-1), expressed in neurons, vessels, and astrocytes, also has angiogenic properties, identified as co-receptors for VEGF or to form complexes with VEGFR (Mey et al. 2013). These mechanisms lead to the initiation of an angiogenic response and new vessel formation. Subsequently, angiopoietin 1 (Ang1), an endogenous ligand for the vascular endothelial receptor tyrosine kinase Tie-2, promotes the stabilization and maintenance of vascular endothelial integrity (Chen et al. 2009; Moss 2013). Moreover, the Wnt/β-catenin pathway has an important role in the angiogenic activity by regulating cyclin D1 to promote endothelial cell proliferation for enhancing new vasculature (Martowicz et al. 2019; Xu et al. 2016). This establishes dynamic blood vessel structures and restores blood supply to the affected brain tissue, leading to improved functional outcomes after cerebral ischemia.

Morin (3,5,7,2′,4′-pentahydroxyflavone) is a flavonol isolated from *Maclura cochinchinensis* ("kae lae" in Thai), belonging to the Moraceae family (Fig. 1). Many studies have reported that morin exhibits multiple pharmacological and physiological effects, including anti-oxidant, anti-inflammatory, anti-apoptotic, and neuroprotective activity (Kim et al. 1999; Lee et al. 2008; Oh et al. 2004; Uyar
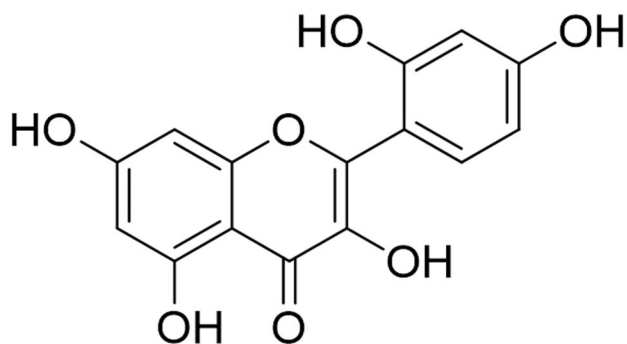
et al. 2006; Zhang et al. 2008). Our previous study provided evidence that morin attenuated oxidative stress, apoptosis, inflammation, and blood–brain barrier (BBB) disruption during the acute phase of a middle cerebral artery occlusion (MCAO) model (Khamchai et al. 2020). However, its benefits in improving cerebral damage and neurological outcomes via the angiogenesis pathway during the recovery phase of cerebral I/R have not been reported. In the present study, we investigated the neuroprotective effects of morin during the recovery phase of MCAO and reperfusion rats via promotion of angiogenesis and brain recovery.

Materials and Methods

Reagents

Anti-Ang1, anti-Tie-2, and anti-NRP-1 were purchased from Abcam (Abcam, Cambridge, UK). Anti-cyclin D1 and anti-HIF-1α were purchased from Cell Signaling (Cell Signaling, MA, USA). Anti-VEGF, anti-wnt3α, anti-β-catenin, anti-vWF, and anti-actin were purchased from Millipore (Millipore, MA, USA). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Extraction and Isolation of Morin

Morin was obtained from *M. cochinchinensis* by the method described previously (Khamchai et al. 2020) with slight modification. Briefly, the dried, pulverized heartwood of *M. cochinchinensis* (5.0 kg) was extracted successively with *n*-hexane, ethyl acetate, and methanol at room temperature. To the ethyl acetate extract containing morin was added methanol–water (1:1) with stirring. The solid which was separated out was collected by filtration and recrystallized from methanol–water (1:1) to give pure morin (5.0 g). The spectroscopic (infrared [IR], ^{1}H and ^{13}C nuclear magnetic resonance [NMR] and mass spectra) data were in agreement with those of morin previously isolated by our group (Khamchai et al. 2020).

Animals and Drug Administration

Male Wistar rats (weighing 300–350 g) were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakorn Pathom, Thailand. All were accommodated on a 12/12 h light/dark cycle with a constant ambient temperature (25 ± 1 °C) and fed a standard pellet rat diet and water ad libitum. All experiments in this study were approved by the Institutional Animal Care and Use Committee at the Faculty of Medicine, Chiang Mai University (Permit number: 33/2559), in compliance with National Institutes of Health (NIH) guidelines. Briefly, we randomly

Fig. 1 The chemical structure of morin

divided the 54 rats into three groups ($n = 18$): (1) Sham group, (2) MCAO group (MCAO rats treated with normal saline at reperfusion), and (3) Morin group (MCAO rats treated with morin at reperfusion). The administration time point and the morin dosage (30 mg/kg BW) were chosen based on our previous study (Khamchai et al. 2020). The vehicle and morin groups were given the treatment via daily intraperitoneal (i.p.) injections for 14 days.

Surgical Preparation of MCAO Model in Rats

All rats were anesthetized with an i.p. injection of zoletil (30 mg/kg) and xylazine (10 mg/kg). An intraluminal mono-filament technique was used to conduct MCAO on the rats, as previously described (Longa et al. 1989). Briefly, the right common carotid artery (CCA) and external carotid artery (ECA) were identified, and the 4–0 filament (Doccol Corp., Sharon, MA, USA) was inserted into the internal carotid artery and advanced until we felt a slight resistance. Crucially, we monitored the effective occlusion in cortical perfusion ($< 25\%$ of the baseline value) by laser Doppler flowmetry (AD Instruments, Dunedin, New Zealand) (Ansari et al. 2011). After 120 min of MCAO, we carefully withdrew the filament to allow the reperfusion. After surgery, the rats were transferred to a room-temperature environment (25 ± 1 °C) until sacrifice.

Rotarod Test

Six rats from each group were trained for three days before MCAO surgery. They were placed on the apparatus for 30 s without rotation and then after 2 min with a constant low speed (4 rpm). The rats were tested until they successfully stayed on the rotarod spindle for 1 min. This procedure was performed only on the first day of training. After resting for 10 min, the rats underwent a single baseline trial on the accelerating rotarod where the spindle increased in speed from 4 to 40 rpm for 6 min. The animals were evaluated on days 1, 7, and 14 after reperfusion. The maximum duration on the rotarod before falling was recorded for each animal. The rats were tested three rounds per day. The data were presented as the mean of duration times.

Pole Test

The rats were located head-upward on the top of the vertical rough-surfaced pole (diameter 2.5 cm, height 100 cm), and the time until they descended to the floor was recorded with a maximum duration of 120 s. They were trained on a day before MCAO surgery, allowed to descend three times in a single session. The total time until the rat reached the floor with its four paws (time to come down) and the time needed to turn completely head downward (time to turn) were recorded. Could they not turn downward and instead dropped from the pole, the time is recorded as 120 s. The results were shown as the mean of three trials.

Analysis of Infarct Volume

Six whole brains from each group were collected 14 days after surgery and cut into 2 mm-thick slices. The slices were incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC) at 37 °C for 20 min and fixed overnight in 4% buffered formaldehyde solution. We determined the infarct size by analyzing the area of the ischemic lesion in each section by using ImageJ software. The infarct volume (%) was calculated as follows: $100\% \times$ [(contralateral hemisphere volume – non-infarct ipsilateral hemisphere volume)/contralateral hemisphere volume] (Ashwal et al. 1999).

TUNEL Assay

At 14 days after reperfusion, the brains were fixed in 4% paraformaldehyde (PFA) and cut into 4-µm-thick slices, which, after that, were deparaffinized and rehydrated. Then, TUNEL assay kit (Roche Diagnostics Corp., Indianapolis, IN, USA) was used with nuclei stained with brown particles. Finally, the total cells and TUNEL-positive cells were observed under a light microscope (Olympus AX70, Tokyo, Japan). We randomly chose five fields ($\times 20$ and $\times 40$ magnification) of a coronal section of the cerebral cortex at the same level. Then, we counted the number of apoptotic cells and the total cells of five adjacent sections (Janyou et al. 2017). The average value was used to compute the apoptotic index (Apoptosis index (AI) = number of positive cells/total cells).

Investigation of BBB Disruption

BBB leakage was determined by Evans blue injection, as previously described (Yang and Rosenberg 2011). At 14 days after reperfusion, we anesthetized the rats and administered 2% Evans blue solution (4 ml/kg) via intravenous injection into the jugular vein. Thirty minutes after Evans blue injection, we perfused the rats intracardially with cold phosphate-buffered saline (pH 7.4). The brain tissues were then collected and homogenized in DMSO, and the homogenates were incubated at 50 °C. The samples were centrifuged at 12,000 g at 4 °C for 30 min, and the supernatants were collected. The absorbance was measured at 620 nm using a spectrophotometer (BioTek Instruments Inc, Winooski, VT, USA).

Western Blot Analysis

Fourteen days after reperfusion, the cerebral penumbra of each group was collected and kept at –80 °C until use. To extract total proteins, the brains were homogenized, and the total protein concentrations were determined using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin (BSA) as the standard. Twenty-five µg of total protein in each sample was loaded into the lanes of 10–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels. Then, we transferred isolated proteins to polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA, USA), and they were blocked in fresh blocking buffer (containing 5% skim milk in 0.1% Tween-20 in Tris-buffered saline, pH 7.4) for 3 h at room temperature. Subsequently, we incubated the membranes with the primary antibodies (anti-Ang1, anti-Tie-2, anti-wnt3α, anti-β-catenin, anti-HIF-1α, anti-VEGF, anti-NRP-1, anti-cyclin D1, or anti-vWF) overnight at 4 °C. Next, the membranes were washed with Tris-buffer saline and Tween-20 (TBST) and incubated with anti-rabbit IgG or anti-mouse peroxidase-conjugated secondary antibody. The membranes were incubated with Immobilon Western (Millipore, MA, USA), and they were exposed to X-ray film. Densitometric analysis was performed by scanning films with a densitometer. The results were normalized using β actin by Image J analysis.

Determination of the Vascular Element Within Neurovascular

Immunohistochemistry staining was used to visualize von Willebrand factor (vWF)-positive vessels. Fourteen days after reperfusion, the rats from each group were perfused with 4% paraformaldehyde in PBS. The rat brains were harvested and post-fixed overnight. The brain sections were prepared using standard protocols. After incubation with a blocking solution, all sections were incubated with anti-vWF (1:1000) for 48 h at 4 °C. Then, the sections were incubated with secondary antibody for 30 min. Afterward, 3,3-diaminobenzidine tetrahydrochloride (DAB) reagent was added, and the sections were incubated for 10 min at room temperature and then counterstained with hematoxylin for 2 min. The image was observed under light microscopy (AX70 Olympus, Tokyo, Japan). For semi-quantitative measurements of vWF-labeled vessels, only the vWF$^+$ vessels with a clear lumen and those vessels that formed ring-like or tubular structures were counted. Three sections per brain from the same levels were chosen, and the number of vessels in each field was counted.

Histology Analysis

Brains from each group were collected and placed in 4% PFA for 48 h. The brain tissues were then embedded in paraffin, processed to 4 µm-thick slices, and stained with hematoxylin & eosin (H&E) and Nissl staining. Morphological changes were detected in the cerebral cortex at the same level in each group. They were observed using a light microscope (Olympus AX70, Tokyo, Japan).

Statistical Analysis

The data are presented as mean ± standard error of the mean (SEM). Statistical differences between the two groups were determined using the Student's t-test. Other data were analyzed using a one-way analysis of variance (ANOVA), followed by Dunnett's post-hoc test. A p-value < 0.05 was considered a statistically significant difference between experimental and control groups.

Results

Morin Attenuates Cerebral Damage at 14 days After Cerebral I/R

The neuroprotective effect of morin on cerebral I/R at 14 days after onset was first examined by TTC staining. Morin treatment significantly decreased cerebral infarction, as shown by the reduced infarct area and the percentage of infarct volume ($p < 0.001$ and $p < 0.05$; Fig. 2A, C). In addition, the Evans blue extravasation assay was performed to evaluate BBB disruption. The dark blue dye color in the brain tissue represented the leakage of the Evans blue extravasation as BBB leakage. The severity of the BBB leakage is shown as OD_{620nm}/g. The result showed the sham group did not have leakage of Evans blue. The extravasation of Evans blue was significantly increased in the vehicle-treated group and significantly decreased with the morin treatment ($p < 0.001$ and $p < 0.01$; Fig. 2B, D).

Next, the number of apoptotic cell deaths in the penumbra area in the cerebral cortex was investigated at 14 days after reperfusion via TUNEL staining. The apoptotic cells are presented as dark brown particles. We found that the apoptotic index was significantly reduced in the morin-treated group compared with the vehicle. There were no TUNEL-positive cells in the sham group ($p < 0.001$ and $p < 0.01$; Fig. 2E, F).

The histological changes in the cerebral cortex were also assessed by hematoxylin and eosin and Nissl staining. There was a difference in morphological changes between each group. Shrinkage in neuronal cells with pyknotic nucleus and vacuoles were usually seen in the vehicle-treated group. Moreover, there was also loose connective tissue in the
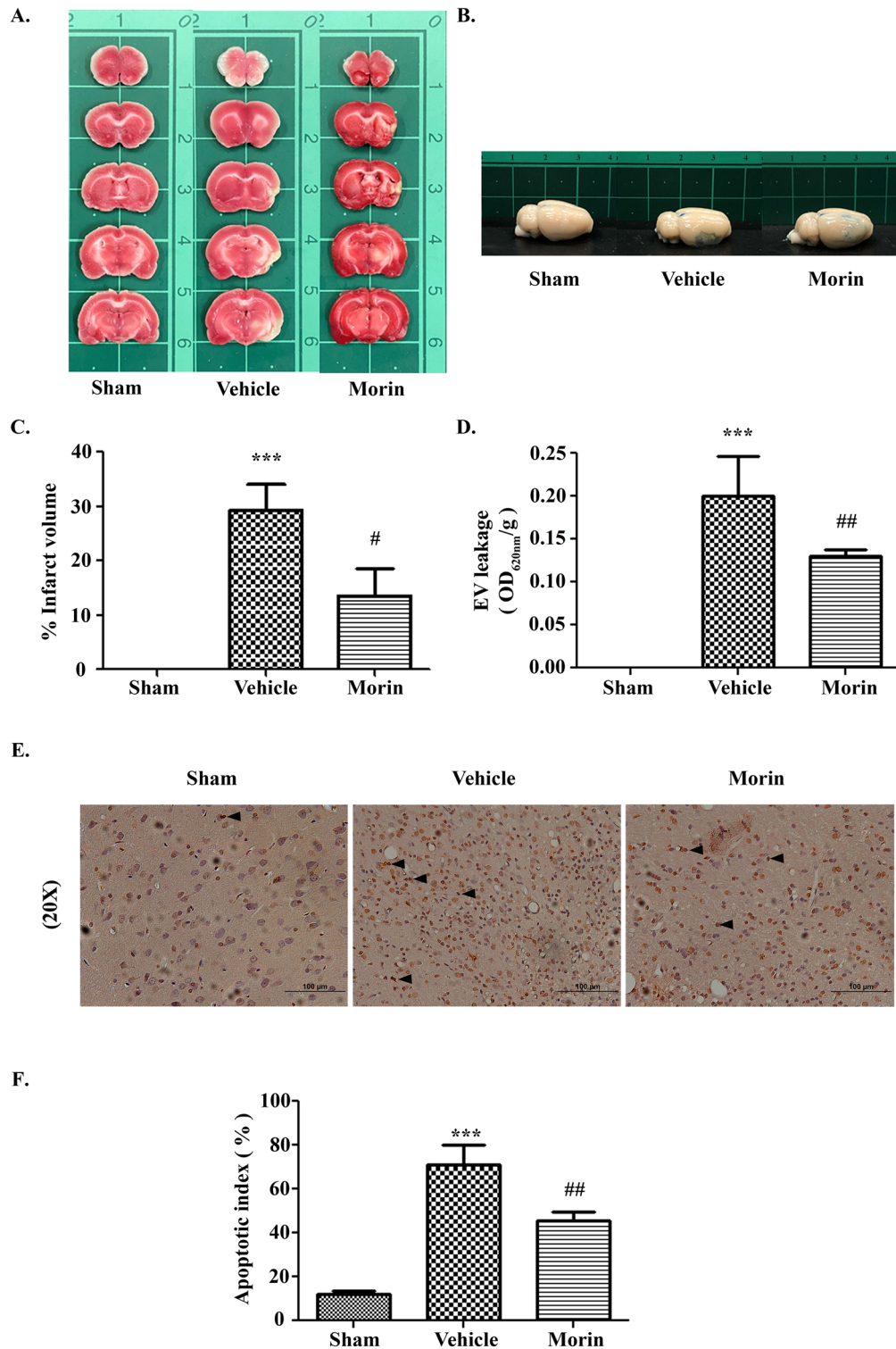
Fig. 2 Effects of morin on cerebral damage at 14 days after cerebral I/R. Representative images of TTC staining and percentage of infarct volume, $n=6$ (**A** and **C**). Representative images of Evans blue leakage in the brain tissues and representation of Evan's blue leakage absorbance, $n=6$ (**B** and **D**). Representative images of TUNEL staining, shown as dark brown particles ($20\times$m agnification). The images were visualized with a light microscope (scale bar $=100$ µm) (**E**). Representation of apoptotic index (AI), $n=6$ (**F**). Representative images of H&E staining (**G**) and Nissl staining (**H**) of cerebral cortex ($20\times$ and $40\times$ magnification). The images were visualized with a light microscope (scale bar $=50$ and 100 µm). The data are presented as the mean $\pm$ standard error of the mean (SEM) from three independent experiments ($^{***}p<0.001$ compared with the sham group; $^{#}p<0.05$, $^{##}p<0.01$ compared with the vehicle group)
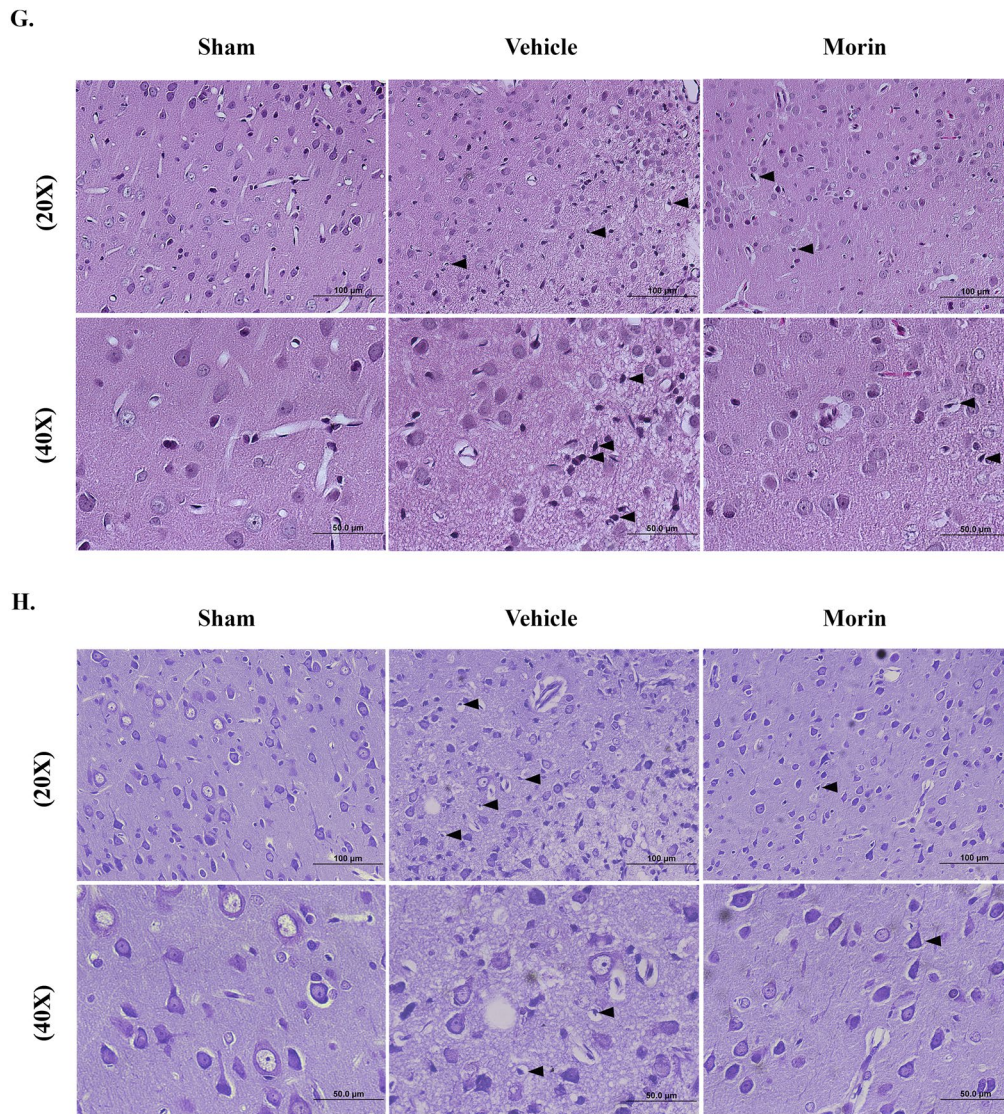
G.

H.

Fig. 2 (continued)

vehicle group. There were no histological changes in the sham group, and dense connective tissue was found in this group. The morin-treated groups showed reduced changes in neuronal cells and slightly loose connective tissue (Fig. 2G, H).

Morin Improves the Functional Outcome at 14 days After Cerebral I/R

We evaluated behavioral tests, including the rotarod test and pole test. In the rotarod test, the results showed that the time spent on the rotarod spindle was significantly decreased in I/R rats at 1, 7, and 14 days after I/R compared to the control rats. The time staying on the rotarod in morin-treated rats was significantly increased compared to the vehicle-treated rats ($p < 0.001$ and $p < 0.01$; Fig. 3A). In the pole test, the

two measurements were taken. Time to turn and the time to down in vehicle-treated rats were significantly increased compared to the control rats and significantly decreased in the morin-treated rats when compared with the vehicle rats ($p < 0.001$, $p < 0.01$, and $p < 0.05$; Fig. 3B, C). These data indicated that morin could ameliorate the behavioral deficits after I/R.

Morin Promotes Angiogenic Factors After Cerebral I/R

We next determined the capacity of morin to induce angiogenesis in I/R rats; we first studied the expression levels of angiogenic factors, including HIF-1α, VEGF, Ang1, Tie-2, and NRP-1, by Western blotting analysis (Fig. 4A). HIF-1α, VEGF, and NRP-1 were significantly decreased in

the vehicle group compared to the sham group. Treatment with morin significantly increased the expression of NRP-1 compared with the vehicle-treated rats ($p < 0.001$; Fig. 4D). However, HIF-1α and VEGF expressions in rats treated with morin tended to increase but with no significant differences when compared to the vehicle-treated group (Fig. 4B, C). In vehicle-treated rats, the expressions of Ang1 and Tie-2 were also significantly reduced compared to the sham group, and the morin-treated group showed significantly increased Ang1 and Tie-2 expressions compared to the I/R group ($p < 0.001$; Fig. 4E, F).

Morin Promotes Vascular Endothelial Cell Proliferation Via Wnt Signaling

To investigate the ability of morin to promote vascular endothelial cell proliferation in I/R rats, we examined the expression levels of Wnt/β-catenin pathway proteins by Western blotting analysis (Fig. 5A). Vehicle-treated rats had significantly reduced Wnt3α and β-catenin levels compared with the control rats. Expression of both proteins was significantly increased in morin-treated rats compared to the vehicle group ($p < 0.001$ and $p < 0.01$; Fig. 5B, C). In addition, β-catenin up-regulates pro-proliferative proteins such as cyclin D1. The results showed cyclin D1 was significantly reduced in the vehicle group compared to the control group, and the morin-treated group showed significantly increased cyclin D1 expression compared to the vehicle group ($p < 0.001$ and $p < 0.05$; Fig. 5D).

Morin Enhances Microvasculature Density After Cerebral I/R

VWF is commonly expressed in endothelial cells and identified as a biomarker of angiogenesis. We investigated the expression of vWF protein by Western blotting analysis. The result showed that vWF expression was significantly decreased in the vehicle group compared with the

control group. The morin-treated group revealed significantly increased vWF expression when compared to the vehicle rats. These results indicated that morin treatment could increase angiogenesis in the penumbra of MCAO rats ($p < 0.001$ and $p < 0.01$; Fig. 6A, B).

Moreover, vWF-positive cells represented the microvasculature density in the cerebral cortex. In the vehicle group, vWF-positive cells were significantly decreased in the brain tissue on day 14 compared with the control group. Treatment with morin significantly increased the vWF-positive cells compared with vehicle group (Fig. 6C, D).

Discussion

In our previous study, Wistar rats were subjected to 2 h of MCAO and then reperfusion for 24 h and 72 h. Our findings revealed morin significantly diminished neurological deficit scores and cerebral infarct volume through attenuating oxidative stress, inflammation, and apoptosis at 24 h after MCAO. Moreover, at 72 h after MCAO, we found morin improved BBB disruption by increasing tight junction proteins and reducing BBB leakage (Khamchai et al. 2020). The present study demonstrates that MCAO rats with morin treatment improve functional outcomes and enhance angiogenesis via promoting the Ang1/Tie-2 axis and Wnt/β-catenin pathway after administration for 14 days.

Angiogenesis is the crucial mechanism for the restoration of cerebral blood flow (CBF) in the penumbra area, which promotes the recovery of neurological function following ischemia (Zhang and Chop 2002; Krupinski et al. 2007; Hayashi et al. 2003). After I/R injury, hypoxic cells trigger the existing vasculature to release angiogenic factors to promote new microvascular formation (Marti et al. 2000). VEGF, a key signaling molecule involved in angiogenesis, is initially released to bind to vascular endothelial growth factor receptor (VEGFR) and NRP-1 on the vascular endothelial cells. NRP-1 also has angiogenic properties,
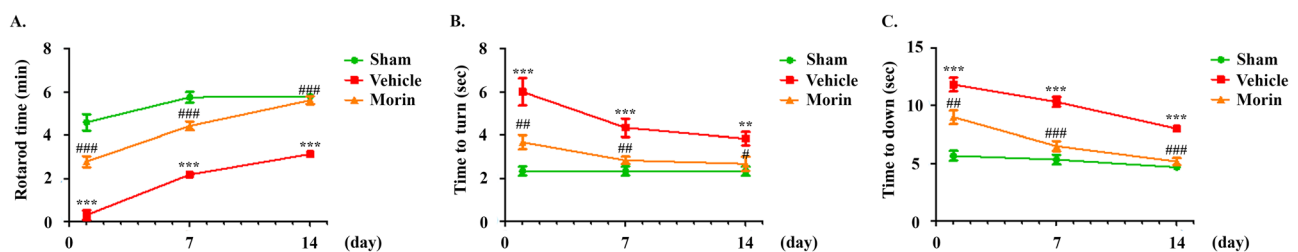

Fig. 3 Effects of morin on functional outcome after cerebral I/R. Representative rotarod time (min) at 1 day, 7 days, and 14 days after I/R (**A**). Representative pole test including time to turn (sec) (**B**) and time to down (sec) (**C**). The data are presented as the mean ± standard error of the mean (SEM) from three independent experiments (**$p < 0.01$, ***$p < 0.001$ compared with the sham group; #$p < 0.05$, ##$p < 0.01$ and ###$p < 0.001$ compared with the vehicle group; $n = 6$)
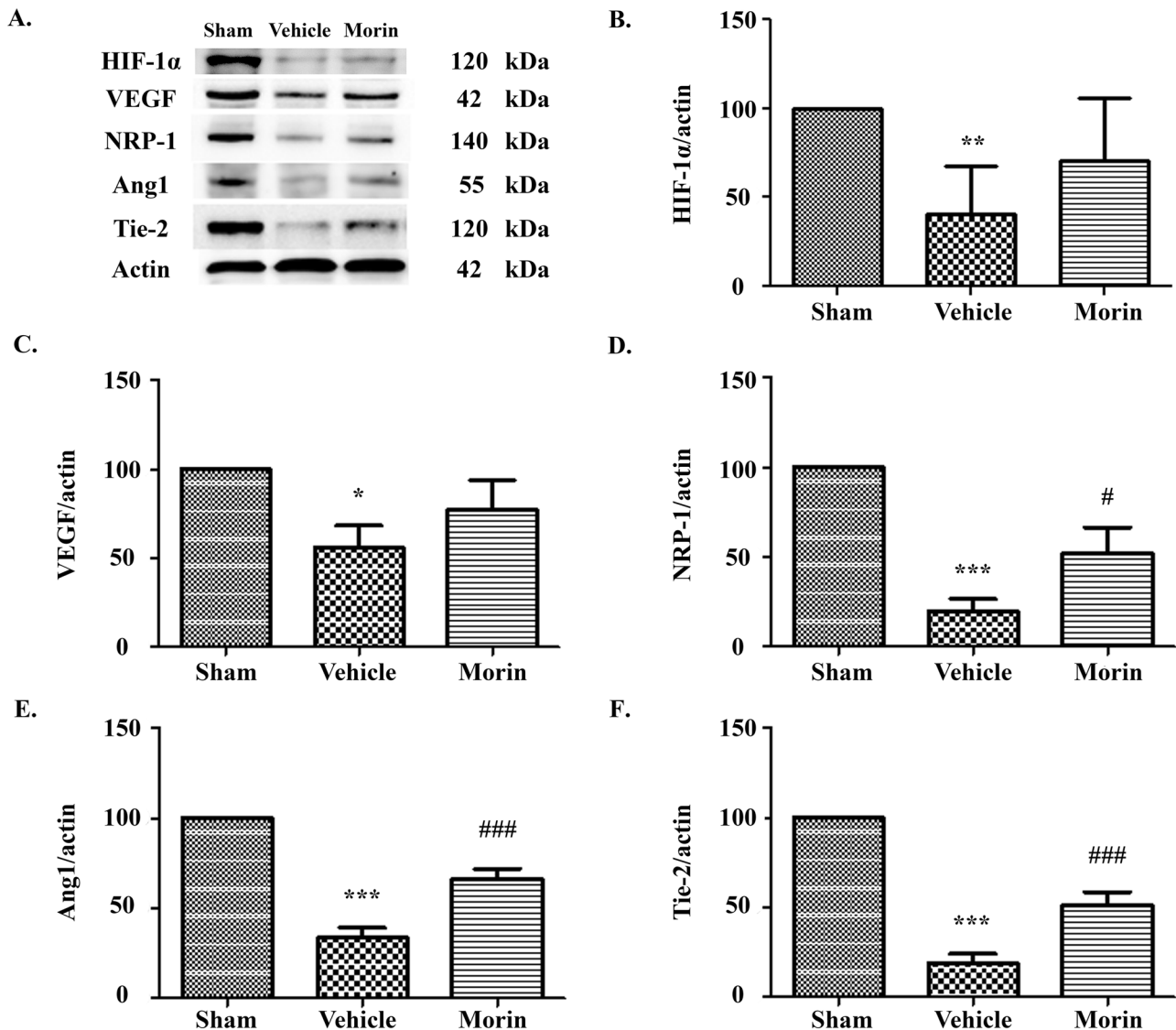
Fig. 4 Effects of morin on the expression of angiogenic factors at 14 days after cerebral I/R. Western blot analysis of HIF-1α, VEGF, Ang1, Tie-2, and NRP-1 (**A**). Quantitative analysis of the expression of the proteins normalized using β-actin (**B**–**E**). The data are presented as the mean ± standard error of the mean (SEM) from three independent experiments ($^*p < 0.05$ and $^{***}p < 0.001$ compared with the sham group; $^\#p < 0.05$ and $^{\#\#\#}p < 0.001$ compared with the vehicle group; $n = 6$)

such as being a co-receptor for VEGF or forming complexes with VEGFR. The expression of NRP-1 also corresponds with the expression of VEGF (Mey et al. 2013). The previous studies demonstrated that VEGF peaks at 24–72 h after cerebral ischemia (Croll and Wiegand 2001). However, the expression of VEGF is reduced at 14 days after onset (Janyou et al. 2020). Our data showed that I/R could suppress the expression of HIF-1α, VEGF, and NRP-1 in the vehicle group at 14 days after I/R. However, morin administration increased the expression of HIF-1α, VEGF, and NRP-1 to promote the formation of new microvessels in MCAO rats at 14 days after I/R (Fig. 4B, C).

In the last step of angiogenesis, Ang1 is up-regulated, which binds to the receptor Tie-2 for stabilization and remodeling of newborn blood vessels. Previous studies indicated that Ang1/Tie-2 is expressed 2–21 days after I/R and is also involved in vessel stability (Chen et al. 2009; Beck et al. 2000). Our results revealed that the expressions of Ang1 and Tie-2 were suppressed in the vehicle group. In the morin-treated group, the level of both proteins was significantly increased at 14 days after MCAO compared to the vehicle group (Fig. 4D, E). Moreover, the Wnt/β-catenin pathway also plays a key role in angiogenesis. β-catenin activates the pro-proliferative protein cyclin
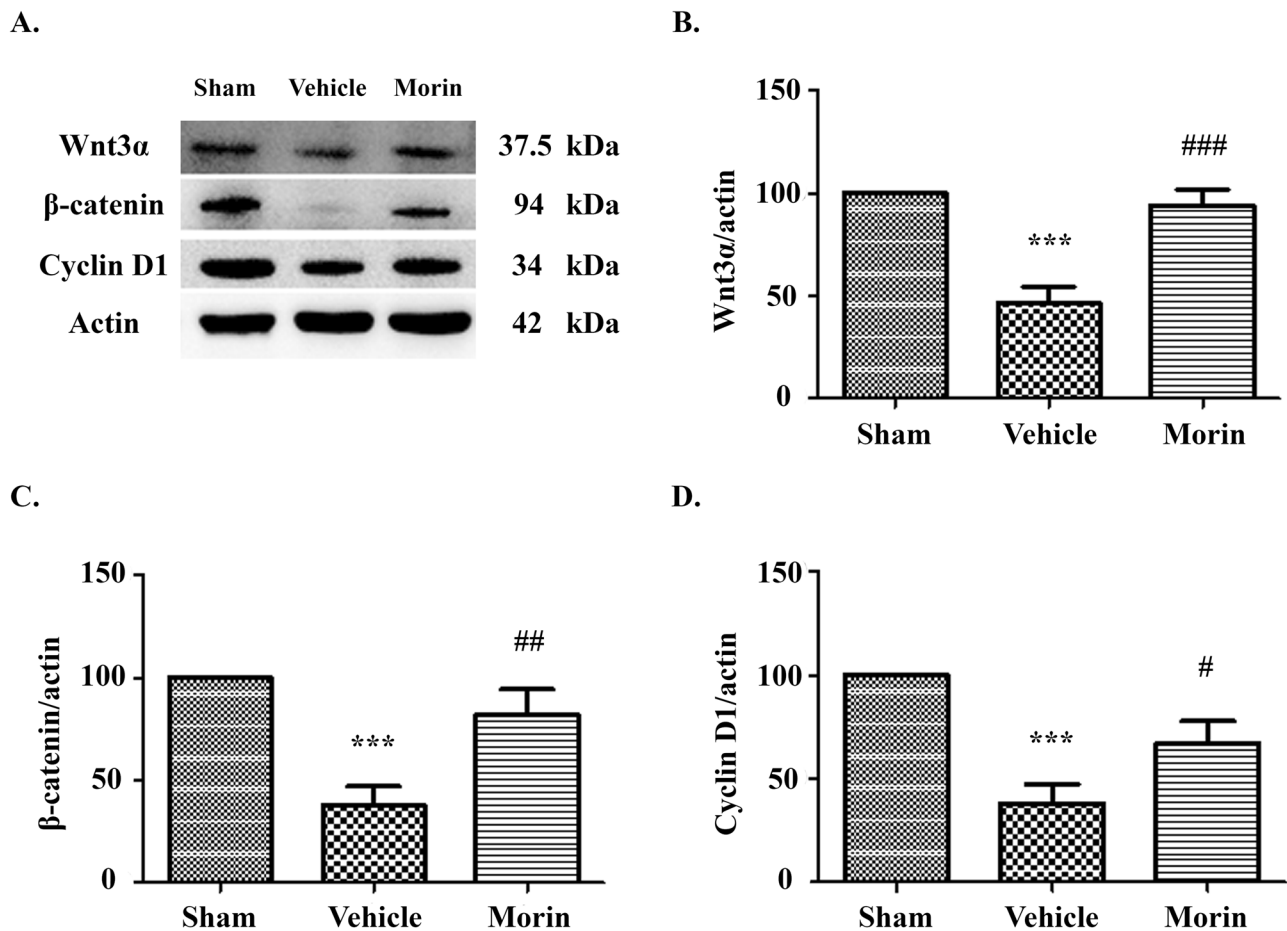
A.

B.

C.

D.

Fig. 5 Effects of morin on the expression of levels of Wnt/β-catenin pathway proteins at 14 days after cerebral I/R. Western blot analysis of Wnt3α, β-catenin, and cyclin D1 (**A**). Quantitative analysis of protein expression normalized using β-actin (**B–D**). The data are presented as the mean ± standard error of the mean (SEM) from three independent experiments ($^{***}p < 0.001$ compared with the sham group; $^{#}p < 0.05$, $^{##}p < 0.01$, and $^{###}p < 0.001$ compared with the vehicle group; $n = 6$)

D1, helping to enhance vasculature maturation (Martowicz et al. 2019; Xu et al. 2016). Recent evidence revealed that Wnt signaling is crucial in vascular endothelial cells and functions involved in angiogenesis. Our data demonstrated that morin treatment significantly increased the expression of Wnt/β-catenin and cyclin D1 in the morin-treated rat at 14 days after I/R (Fig. 5). The new microvessels formation is started from 14 to 21 days after cerebral ischemia (Chen et al. 2017; Gandin et al. 2016). However, at 14 days after I/R, our study showed that MCAO rats had a decrease in the microvascular density, but we found an increase of microvessels in the morin-treated rats, corresponding with an increase in the expression of vWF protein (Fig. 6). These results suggest that morin induced angiogenesis and promoted vasculature stability.

In rodents, as in humans, MCAO rats have the predominant characteristic and functional deficit, such as hemiparesis. Accordingly, the rotarod and pole tests, widely used to assess the long-term functional outcomes in

MCAO rats, were performed in the present study (Janyou et al. 2020). Our data demonstrated MCAO rats had a decrease in the duration on the rotarod and an increase in the time to turn and time to down in the pole test. Nevertheless, daily administration of 30 mg/kg morin for 1, 7, and 14 days significantly improved the functional deficit in MCAO rats (Fig. 3). Morin administration also reduced cerebral damage at 14 days after I/R by decreasing cerebral infarction and BBB leakage. Additionally, we observed fewer TUNEL-positive cells in the ischemic brain of the morin-treated group, which presented a decrease in apoptotic cells. Our results also showed that morin treatment reduced brain morphological changes in the cerebral cortex. We found that the neuronal cells showed pyknotic nuclei and overall shrinkage in the vehicle group (Fig. 2). These results suggest morin can improve brain damage in the recovery phase of cerebral I/R injury by promoting angiogenesis via Wnt/β-catenin signaling pathway.
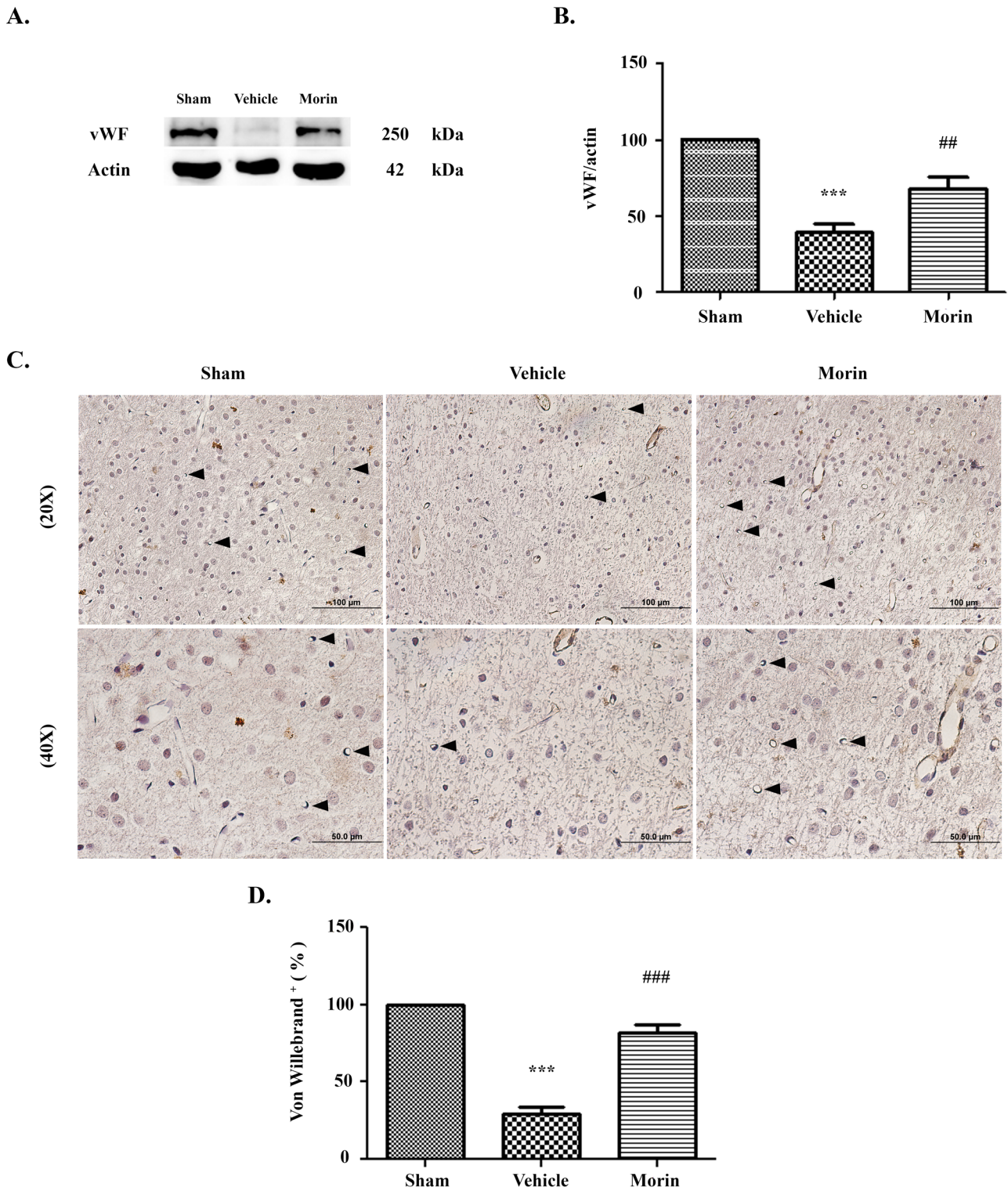
A.

B.

C.

D.

Fig. 6 Effects of morin on the microvasculature density at 14 days after cerebral I/R. Western blot analysis of vWF, $n=6$ (**A**). Quantitative analysis of vWF normalized using β-actin (**B**). Representative images of vWF positive cells in the penumbra, shown as ring-like or tubular structures (20× and 40× magnification).The images were visualized with a light microscope (scale bar = 50 and 100 μm) (**C**). Representative vWF positive cells as a percent of control, $n=6$ (**D**). The data are presented as the mean ± standard error of the mean (SEM) from three independent experiments ([**]$p<0.01$, [***]$p<0.001$ compared with the sham group; [##]$p<0.01$ compared with the vehicle group)

In conclusion, our study provided evidence that morin has a neuroprotective effect on cerebral ischemia/reperfusion at 14 days after I/R injury. It enhanced angiogenic factors through the Ang1/Tie-2 pathway and Wnt/β-catenin signaling and also improved functional outcomes. We found that morin promoted angiogenesis in the recovery phase after I/R injury.

Author Contribution Conceptualization: Satchakorn Khamchai, Jiraporn Tocharus; Methodology: Satchakorn Khamchai, Wijitra Chumboatong, Janejira Hata, Chainarong Tocharus, Apichart Suksamrarn; Formal analysis and investigation: Satchakorn Khamchai, Jiraporn Tocharus; Writing-original draft preparation: Satchakorn Khamchai; Writing-review and editing: Jiraporn Tocharus, Apichart Suksamrarn; Funding acquisition: Jiraporn Tocharus, Apichart Suksamrarn; Supervision: Jiraporn Tocharus.

Availability of Data and Material Data available on request from the corresponding author.

Declarations

Competing Interests The authors declare no competing interests.

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