The three-phase enriched environment paradigm promotes neurovascular restorative and prevents learning impairment after ischemic stroke in rats

Yu Zhan, Man-Zhong Li, Le Yang, Xue-Feng Feng, Jian-Feng Lei, Nan Zhang, Yuan-Yuan Zhao, Hui Zhao

School of Traditional Chinese Medicine, Capital Medical University, Beijing, China
Beijing Key Lab of TCM Collateral Disease Theory Research, Beijing, China
Medical Imaging Laboratory of Core Facility Center, Capital Medical University, Beijing, China

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ABSTRACT
Enriched environment (EE) with a complex combination of sensorimotor, cognitive and social stimulations has been shown to enhance brain plasticity and improve recovery of functions in animal models of stroke. The present study extended these findings by assessing whether the three-phase EE intervention paradigm would improve neurovascular remodeling following ischemic stroke.

Male Sprague-Dawley rats were subjected to permanent middle cerebral artery occlusion (MCAO). A three-phase EE intervention paradigm was designed in terms of the different periods of cerebral ischemia by periodically rearranging the EE cage. Morris water maze (MWM) tests were performed to evaluate the learning and memory function. Multimodal MRI was applied to examine alterations to brain structures, intracranial vessels, and cerebral perfusion on the 31st day after MCAO. The changes of capillaries ultrastructure were examined by transmission electron microscope. Double-immunofluorescent staining was used to evaluate neurogenesis and angiogenesis. The expression of angiogenesis-related factors and neurovascular remodeling related signaling pathways including Phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/glycogen synthase kinase-3 (GSK-3)/β-catenin and the axon guidance molecules were detected by Western blot analysis.

MRI measurements revealed that EE treatment significantly increased survival volume of cortex and striatum, improved cerebral blood flow (CBF), amplified anterior azygos cerebral artery (azACA), ipsilateral internal carotid artery (ICA) and anterior communicating artery (AComA) vessel signal compared with standard housed rats (IS). Consistent with these findings, EE reduced ischemic BBB damage of capillary, enhanced endogenous angiogenesis and modified the expression of VEGF, Ang-1 or Ang-2 in ischemic rats. Additionally, this proangiogenic effect was consistent with the increased progenitor cell proliferation and neuronal differentiation in the peri-infarct cortex and striatum after EE intervention. Specifically, EE intervention paradigm markedly increased expression of phosphorylated PI3K, AKT and GSK-3, but reduced phosphorylated β-catenin. Moreover, the axon guidance proteins expression level was significant higher in EE group. In parallel to these findings, EE significantly enhanced recovery of lost spatial learning memory function in MCAO rats without affecting infarct size.

Together, MRI findings along with histological results strongly supported that the three-phase EE paradigm benefited neurovascular reorganization and thereby improved poststroke cognitive function. Moreover, our findings suggest that this type of EE paradigm induced neurogenesis and angiogenesis, at least in part, via regulating PI3K/AKT/GSK-3/β-catenin signaling pathway and activation of the intrinsic axonal guidance molecules in animal models of ischemic stroke.

1. Introduction
Cerebral ischemia is one of the most common causes of adult long-term disability worldwide (Girard et al., 2014). Therapeutic options for ischemic stroke patients remain limited. While the beneficial effect of neuroprotective therapies has been confirmed in animal models of stroke, it is very difficult to translate this pure neuronal approach into clinical applications (Shah et al., 2009; Wu et al., 2019). It is now widely accepted that stroke damages relate to neuronal tissues and cerebrovascular. Thus, the brain should be treated as an entity event...
that requires synergistic interaction of the neural and vascular systems (Liu et al., 2014). In this regard, neurovascular rehabilitation plays a central role in reducing post-stroke impairments and improving functional recovery after stroke (Hatakeyama et al., 2020).

In 1947, Donald Hebb prompted research into enriched environments (EE). EE, the housing environment modified by a larger enclosure and a variety of novel objects, is an intervention designed to increase motor, sensory, cognitive, and social activity by the provision of a stimulating environment. This paradigm represents a basic voluntary use paradigm providing benefits to neuronal function (Rossergen et al., 2019). Due to its multiple salutary actions, EE as a model of rehabilitation has attracted a great deal of attention in stroke (Starkey et al., 2014; Wahl et al., 2019). Evidence accumulated over many years of experimental stroke research indicates EE has great potential to improve physical and cognitive function (Farokhi-Sisakht et al., 2019; Schuch et al., 2016; Wang et al., 2019). In addition to behavioral benefits, EE has been demonstrated to exert neuroprotective effects in animal models of cerebral ischemia (Gonçalves et al., 2018). Likewise, EE is known to enhanced vascular repair, including alleviation of endothelial dysfunction (Shilpa et al., 2017), induction of endogenous angiogenesis (Xie et al., 2019a), and upregulation of endothelial nitric oxide synthase (Koester-Hegmann et al., 2018). These studies indicate that EE might have a more profound effect on post-ischemic neurovascular remodeling.

Despite the long history of investigation of EE in experimental studies, it is not enough to apply this rehabilitation program to stroke patients (Janssen et al., 2012). As the evolution of brain injury after stroke encompasses dynamic, complex, heterogeneous processes which make the environmental setting difficult to tailor to the patient's needs. For example, patients might be restricted with comprehensive EE intervention due to cognitive and/or physical deficits during the period of acute cerebral ischemia. And some studies also suggested that highly intense physical activity very early after injury onset can be risky (Allred et al., 2014). However, in the recovery phase of stroke, high-intensive EE stimulation with a complex combination of physical, cognitive, and social stimulations have been promoted effect better functional outcomes (Janssen et al., 2012). Hence, setting different levels of EE stimulation at different periods following ischemic stroke might have great potential for improving stroke recovery. In this study, we designed a three-phase EE intervention paradigm as rehabilitative strategy to maximize post-stroke recovery. This enriched housing is comprised of many elements that are considered hallmarks of generic EE. In particular, different levels of EE stimulation were designed by periodically rearranging the EE cage following ischemic stroke. Here, we investigated the effects of this EE paradigm on neurovascular reorganization after ischemic stroke.

Improving the collateral circulation and maintaining constant cerebral perfusion are the important strategy for neurovascular repair after ischemic stroke (Stamatovic et al., 2019). Magnetic resonance imaging (MRI) is considered as the most reliable modality to noninvasively reveal the neurovascular aberrations (Ding et al., 2008). Accordingly, we focused on noninvasive MRI methodologies to test the hypothesis that this EE paradigm would provide benefits to the neurovascular remodeling after ischemic stroke.

Previous investigations have demonstrated that EE has beneficial effects on promoting endogenous neurovascular reparative processes involving neurogenesis and angiogenesis after stroke (Kuptsova et al., 2015; Zhan et al., 2019). However, the precise molecular mechanisms underlying above processes have not yet been completely elucidated. Thus, we further investigated whether this EE paradigm could enhance angiogenesis and neurogenesis following ischemic stroke with particular attention given to mechanisms of this coupling. A role for attractive and repulsive axonal guidance cues such as Netrin-1 and Slit-2 in the directional migration of neuroblasts have been reported as well. Notably, axonal guidance cues also influence angiogenesis through their control of endothelial cells migration and new vessel formation (Park et al., 2004). Given that axonal guidance cues play a critical role in the endogenous angiogenesis and neurogenesis, it is imperative to understand the axonal guidance cues involved in EE-mediated neurogenesis and angiogenesis. Moreover, PI3K/AKT axis has been shown to modulate downstream of GSK-3/β-catenin signaling to promote multiple cellular processes such as cell proliferation, growth and apoptosis (Wang et al., 2020; Xu et al., 2020; Zhang et al., 2020). Specially, PI3K/AKT/GSK-3 signal pathway has been recognized to plays an important role in EE mediated neuroprotection in vivo (Jain et al., 2013). On these grounds, we hypothesize that the neurovascular remodeling elicited by EE may be associated with PI3K/AKT/GSK-3-mediated activation of β-catenin. Therefore, the present study noninvasively explored the possible role of the three-phase EE intervention paradigm in neurovascular remodeling through multiple-parameters MRI. In particular, we explored axonal guidance cues and PI3K/AKT/GSK-3/β-catenin pathway to gain an insight into the possible repair mechanism underlying this EE paradigm following an ischemic stroke.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley rats (Vital River Laboratory Animal Technology company, China) weighing 300 to 320 g (aged 8 weeks) at the time of surgery were used. Animals were housed under a 12 h light/dark cycle at 22 ± 1 °C with free access to food and water. All animal procedures were performed in accordance with the guidelines set by National Institute of Health Guide for the Care and Use of Laboratory Animals, and approved by the Capital Medical University Animal Ethics Committee (Permit Number: AEEI-2018-052).

2.2. Focal cerebral ischemic model and study protocols

Focal cerebral ischemia was performed by intraluminal occlusion of the right middle cerebral artery (MCAO) as described previously (Longa et al., 1989). The rats were anesthetized with isoflurane (5% for induction and 2% for maintenance) vaporized in a mixture of oxygen/nitrous oxide (30%/70%). An incision in the ventral neck was made. The right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed and isolated from vagus nerves. ECA and ICA were clamped with the microsurgical clips temporarily. A 4–0 monofilament nylon suture (Beijing Sunbio Biotech Co Ltd., China) was inserted through the right ECA and gently advanced into the ICA lumen to occlude the origin of the middle cerebral artery (MCA).

At 1st day after MCAO, neurological dysfunction was evaluated on a five-point scale (Lee et al., 2003; Longa et al., 1989): 0, no neurological deficit; 1, failure to extend left forepaw fully; 2, circling or walking to the left; 3, falling to the left after a slight push; 4, no spontaneous walk. All rats were scored to assess the behavioral deficit in a blinded manner, only rats with a high-grade neurological deficit (2 or greater) were included and further randomized to either EE or standard laboratory cage housing (control). There were no group differences in neurological deficits scores and body weight before treatment. Statistical analyses were performed on data collected by experimenters blinded to the treatment conditions.

Sham-operated animals were undergoing the same surgery without artery occlusion. Rats with successfully induced MCAO were randomly assigned into the ischemic standard-housed group (IS) and ischemic enriched environment group (IE) (n = 27 per group). Sham-operated rats were assigned into sham standard-housed (SS) group and sham enriched environment (SE) group (n = 20 per group).

There were two part of experiment, for neurobehavioral study (study 1), 40 rats (n = 10 per group) were used for learning and memory test in the Morris water maze (MWM). In order to avoided behavioral training and testing influenced subsequent experiments
precision, in the MRI and histological study (study 2), another 40 rats (n = 10 per group) underwent sequential MRI imaging and histological study without undergoing MWM experiments (Fig. 1). The reason was that rats underwent training in the MWM showed structural modifications and rapid changes in MRI indices (Hofstetter and Assaf, 2017; Zhan et al., 2019).

All experiments were performed by observers blinded to the experimental groups. The testing order of the animals was based on a random number list during the experiment (the random was generated by: http://www.99cankao.com/numbers/random-number-generator.php).

2.3. Three-phase EE intervention paradigm

SS and IS groups rats were sorted into standard houses, while SE and IE groups rats were housed in an enriched environment for 12 h every day (8:00 p.m. to 8:00 a.m. the next day), and then housed in the standard cages. The EE intervention paradigm provided different intensity levels of sensory, physical and cognitive stimulation following ischemic stroke (Fig. 2). In the first phase (post-ischemia 2–7 days), twelve rats were housed in a single-layer cage (70 cm long × 50 cm wide × 32 cm high) to encourage complex social interactions. The enriched environment contained wheels to promote voluntary physical activity, and some cabins as a shelter to increase security (Fig. 2A).

In the second phase (post-ischemia 8–14 days), setting a double-layer cage (70 cm long × 50 cm wide × 64 cm high) to expand the scope of activities. Added some objects such as tunnels, swing boards, ladders and balance beams to promote the recovery of limb function after stroke. Multi-point delivered foods to guide animals to explore new environments. The objects were changed every day to ensure continued novelty and complexity (Fig. 2B).

In the third phase (post-ischemia 15–30 days), setting a three-layer cage (70 cm long × 50 cm wide × 96 cm high) to increase the complexity. Added floating cabins and increasing the slope of the balance beams and ladders to promote the recovery of limb function. Placed some palatable foods of rats such as melon seeds and peanuts in the third layer of cage, and reinforced rats to train limbs movement skills and precise exercise. Changed feeding route every day to lure the animals into exploring the environment and improve spatial learning and memory (Fig. 2C).

The standard housing condition (40cm long × 30cm wide × 20cm high) only had food boxes and water bottles. Animals were group housed in sets of 3 per cage.

2.4. Magnetic resonance imaging protocols

MRI studies were using a 7.0 T Bruker animal MRI scanner (Bruker, pharma Scan, Germany) on the 31st day after EE intervention. Rats were anesthetized with isoflurane (5% for induction and 2% for maintenance). During the MRI scan, the animals were put on a feedback-controlled system to ensure a constant body temperature at 37 ± 0.5 °C and the respiration was monitored throughout the experiment.

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Fig. 1. A schematic timeline of the experiments. Two different experimental protocols were used to observe the effects of EE treatment following focal cerebral ischemia in rats. All the rats are conventional raised 3 days. Functional deficits were assessed 1 days after MCAO and rats were randomly assigned to either an enriched environment (EE) or standard conditions starting at 2 days for 30 days. (A) In study1, rats were undergoing 5 sequential days learning and memory test in the MWM. (B) In study 2, rats were undergoing sequential MRI imaging at 31 days post-treatment and follow by histological study.

Fig. 2. Photograph of the enrichment cage in different rehabilitation phases. (A) In the first phase (post-ischemia 2–7 days), setting a single-layer cage. (B) In the second phase (post-ischemia 8–14 days), setting a double-layer cage to expand the scope of activities. (C) In the third phase (post-ischemia 15–30 days), setting a three-layer cage to increase the complexity and lure the animals into exploring the environment and improve spatial learning and memory.
2.4.1. Evaluation of ischemic brain lesion by T2-weighted images and T2 relaxometry mapping

T2-weighted imaging (T2WI) was performed to evaluate the tissue injury by using a fast spin-echo pulse sequence. Ischemic injury size was determined from the hyper-intensity region in the T2WI. Pixels in the lesion area with image intensities higher than the mean plus twice the standard deviation (mean ± 2SD) of the intensity in the mirrored contralateral normal tissue were defined as the infarct area (Chan et al., 2009). The infarct volume was obtained by adding all infarct areas measured on individual slices multiplied by slice thickness (0.7 mm) using NIH Imagej software (Liu et al., 2011).

The survival tissue was identified by subtracting the infarct tissue and the lateral ventricle from the ipsilateral hemisphere. The survival volume was determined by the sum of the survival tissue areas on individual slices and multiplying each area by slice thickness (Li et al., 2018). The cortex and striatum were measured on T2WI at a fixed structural location. The volume of the cortical and striatal survival tissue was obtained in the ipsilateral cortex and striatum respectively by subtracting the corresponding infarct tissue using the same criteria as described above and determined by the sum of the survival areas across all slices, multiplied by slice thickness (Li et al., 2018).

T2 relaxometry mapping was performed to evaluate the degrees of tissue injury using a multi-slice multi-echo sequence. Regions of interest (ROIs) were manually drawn in the bilateral cortex and striatum on coronal T2 relaxometry maps (bregma 0.8 mm, coronal direction) according to the Paxinos and Watson atlas (Schober, 1986). T2 values were obtained from each ROI and data were expressed as a percentage of the ipsilateral T2 values compared to the contralateral T2 values.

2.4.2. Magnetic resonance angiography

Three-dimensional time-of-flight (3D-TOF) magnetic resonance angiography (MRA) was applied to determine the status of the circle of Willis by using a fast low angle shot sequence (Holskenberg et al., 2003). The coronal multi planar reformation (MPR) and maximal intensity projection (MIP) of MRA were acquired by Paravision version 5.1 software (Bruker, Pharmascan, Germany) (Naganawa, 2015). The vascular signal intensities in the anterior cerebral artery (ACA), anterior communicating artery (AComA), anterior azygos cerebral artery (azACA), MCA, ICA, posterior cerebral artery (PCA) and basilar artery (BA) were obtained (Kara et al., 2012).

2.4.3. Arterial spin labeling

Regional cerebral blood flow (CBF) was monitored by Arterial spin labeling (ASL) with an echo-planar imaging fluid-attenuated inversion recovery (EPI-FLAIR) sequence. ROIs were first placed in the bilateral cortex and striatum on the T1 image and then copied to CBF map (bregma -0.2 mm) to acquire corresponding CBF. The relative CBF was further calculated as the ipsilateral CBF divided by the contralateral CBF (Yang et al., 2015).

2.5. Tissue processing

At the end of MRI scanning, six rats from each group were anesthetized with isoflurane and perfused transcranial with 0.9% saline followed by 4% paraformaldehyde in 0.1 M/L phosphate-buffered solution (PBS, pH 7.4). The brains were removed and post-fixed in the same fixative at 4 °C overnight. Random four brain of each group blocks (bregma -0.4 to 0.4 mm) were cut according to a rat brain atlas (Büttnér-Ennever, 1996), processed and embedded in paraffin. A series of 5 μm thickness coronal brain sections were sliced from the paraffin block for immunostaining.

The rest of two perfused brain sections were used for transmission electron microscope observation. Peri-infarct tissues (1mm 3, Bregma −0.8 to −0.2 mm) were cut and post fixed in 2.5% electron microscopy-specialized glutaraldehyde, then they were embedded in Epon812 epoxy resin. Serial ultrathin sections (5 μm) were stained with uranyl acetate and lead citrate and examined by transmission electron microscope (H7700TEM, Hitachi, Tokyo, Japan) according to a previously described method (Nahirney et al., 2016).

All the remaining rats (4 rats per group) were sacrificed for western blot analysis. The brains were quickly dissected and the peri-ischemic tissues were separated for analysis according to a previously described method (de Boer et al., 2019).

2.6. Immunofluorescence staining

Double-label immunofluorescence staining were carried out as previously described (Zarruk et al., 2018). Free-floating slices at bregma level − 0.2 mm was rinsed three times in phosphate buffer saline buffer and incubated in blocking solution for 1 h at room temperature (RT). Angiogenesis was visualized using double immunofluorescence labeled with Ki67 (a marker for proliferating cell) and CD31 (a marker for endothelial cell) or CD31 and NG2 (a marker for pericyte) (Teichert et al., 2017). Neurogenesis were visualized with Ki67 and Map-2 (a marker for neuron) double-label immunofluorescence staining (Gu et al., 2000). Briefly, brain sections were stained with primary antibody at 4 °C for 48 h, and then incubated for 2 h at 37 °C with secondary antibodies conjugated with species- and isotype-appropriate Alexa 488 (diluted 1:400; Abcam, cat. No. ab150077) and 594 (diluted 1:200; Abcam, cat. No. ab150116). The primary antibodies used were: mouse anti- Ki67 (diluted 1:200; Servier, cat. No. GB14102), rabbit anti- CD31 (diluted 1:100; Abcam, cat. No. ab222783), mouse anti- NG2 (diluted 1:100; Abcam, cat. No. ab50009), mouse anti- Map-2 (diluted 1:3500; Abcam, cat. No. ab32454) and rabbit anti- Ki67 (diluted 1:800; Abcam, No. ab16667). Negative control slices were processed in the same manner without primary antibodies during the staining procedure to check the specificity of the staining. All coverslips were mounted with Vectashield Mounting Medium (Vector Laboratories, Burlingame, cat. No. Vector H-1200) containing the correct dilution of DAPI (SouthernBiotech, cat. No. 0100–20) to counterstain DNA in the nuclei.

Immunofluorescence images were captured using a fluorescence microscopy (Nikon 80i, Nikon, Tokyo, Japan) equipped with a 3-CD colour video camera (Sony DSC-970MD interfaced with a Micro Computer Imaging Device (MCID) image analysis system (Imaging Research, St Catharines, Canada). Immuno-positive stained cells were determined under high magnification (20 ×, 40 × or 63 × objective). For microscopic analysis, four non-overlapping regions were randomly sampled in the peri-infarct cortex and striatum separately for counting Ki67 +, Ki67+/CD31 +, CD31+/NG2 + and Ki67+/Map-2 + cells. Data were presented as the number of positive cells/mm 2 for quantitative analysis of cell-type-specific markers (Haldorsen et al., 2014). The immunostaining analysis was performed by an investigator blinded to the experimental groups.

2.7. Transmission electron microscopy

Electron micrographs of capillaries were analyzed using Image pro plus software from sham-operated (SS = 2 rats; SE = 2 rats) or stroke-affected rats (IS = 2 rats; IE = 2 rats). Five randomly selected brain areas in the ipsilateral peri-infarct of each section were chosen and magnified from 8000× to 32,000× for the visualization of capillaries detail. Inclusion criteria for microvessel analysis were as follows: the diameter was < 8 mm, the lumen was not occluded by blood cells or plasma, and the vessel was located in cortical layers 2-5 and within 300 mm of the infarct border.

For each parameter and region, measurements from 20 vessels were averaged to give a single biological repeat. Lumen circularity was calculated using the Image pro plus circularity function (Circularity = \(4\pi\frac{Area}{Perimeter^2}\)), with a value of 1 indicating a perfect circle (Haley and Lawrence, 2017). Vesicles of the endothelium was
quantified by normalizing the number of vesicles to the circumference of the vascular endothelium. Pericyte coverage of endothelium cells were calculated based on the total length of inner pericyte processes around each vessel relative to the perimeter of the endothelium (Nahirney et al., 2016). The basement membrane (BM) thickness was the mean value measured in straight line segments at four cardinal points in the microvessel (Nahirney et al., 2016). The astrocytic swelling scores were measured on a scale of zero to five according to the astrocyte mitochondrion damage degree as previously described (Haley and Lawrence, 2017). The severity of the astrocyte pathology was graded as follows: Grade 0, normal; Grade 1, astrocytic swelling and astrocytic mitochondrion with prominent crista; Grade 2, astrocytic swelling and astrocyte mitochondrion cloudy swelling; Grade 3, astrocytic swelling and astrocyte mitochondrion formed vacuoles; Grade 4, astrocytic swelling and astrocyte mitochondrion cristae dissolved; Grade 5, astrocytic visibly compressing the vessel lumen and astrocyte mitochondrion disintegrated.

2.8. Western blot analysis

Western blots were performed to determine protein levels in perivascular tissues (Deeguchi et al., 2004). Proteins were extracted in RIPA buffer (Applygen, China, cat. No. C1053) containing 1% phe- nylmethanesulfonfyl fluoride (Applygen, China, cat. No. A1100) and 1% protease inhibitor (Applygen, China, cat. No. P1260). Protein concentrations were estimated by BCA protein assay kit (Applygen, China, cat. No. P1511). 7 µL total proteins were separated on 10% SDS-poly- acrylamide gels, and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA, cat. No. IPVH0010). The membranes were blocked with 5% nonfat milk or BSA for 1 h at RT and then incubated overnight at 4 °C with the primary antibodies: vascular en- dothelial growth (VEGF) (diluted 1:2000; Abcam, cat. No. ab61654), Ang-1 (diluted 1:2000; Millipore, cat. No. AB10516), Ang-2 (diluted 1:1000; Abcam, cat. No. ab125692), p-PJIK p85α Y607 (diluted 1:1000; Abcam, cat. No. ab182651), PI3K p85α (diluted 1:1000; Abcam, cat. No. ab125692), p-PI3K p85α Y607 (diluted 1:1000; Abcam, cat. No. ab125692), Akt (diluted 1:10000; Cell signaling, cat. No. 9601S), GSK 3α/β (diluted 1:10000; Cell signaling, cat. No. 9272S), GSK 3β (diluted 1:100000; Abcam, cat. No. ab40870), GSK 3β (diluted 1:10000; Gene Tex, cat. No. GTX111192), p-AKT (diluted 1:5000; Cell signaling, cat. No. S473), AKT (diluted 1:10000; Cell signaling, cat. No. 9601S), β-catenin (diluted 1:50000; Abcam, cat. No. ab6302), Nefrin-1 (diluted 1:40000; Abcam, cat. No. ab126729), DCC (diluted 1:50000; Abcam, cat. No. ab125280), Slit-2 (diluted 1:60000; Abcam, cat. No. ab134166), Robo-1 (diluted 1:40000; Abcam, cat. No. ab85312), GAPDH (diluted 1:40000; CWBIO, cat. No. NBL01c). After washing, the membranes were incubated with secondary horseradish peroxidase-labeled anti-rabbit (diluted 1:20000; CWBIO, cat. No. CW0103S) or anti-mouse (diluted 1:20000; CWBIO, cat. No. CW0102S) IgG at RT for an hour. Immunoreactive bands were visualized using the super ECL plus kit (Applygen, China, cat. No. P15050) and visualized with a DRAFT-Fluor Chem Q apparatus (Alpha Innotech Corporation, San Leandro, CA, USA). Quantitation of target protein intensities was performed with the use of ImageJ software.

2.9. Behavioral tests

The MWM test was performed from 31st to 35th day after EE intervention which consisted of hidden platform tests in 4 consecutive days for the evaluation spatial learning and one day of probe trial for the evaluation spatial memory (Bengoetxea et al., 2018). The MWM consisted of a circular water tank filled with water (18–20 °C) and divided into four quadrants of equal surface area (quadrants I, II, III, and IV). In the hidden platform test, the platform was placed 1.5 cm below the water surface in the middle of the quadrant I and kept in the same position throughout the entire experiment. Two sessions were finished per day which including 1 h intervals, and each session contained two trials. For each trial, the rat was allowed to swim for at most 60 s before locating the submerged platform. If the rat failed to find the platform, the researcher gently guided it to the platform and allowed it to remain there for 10 s. A video camera connected to an image analyzer (JLBev-MWMG, Jiliang Software Technology Co. Ltd., Shanghai) recorded latency and swim path length to find the platform. The probe trial was conducted 24 h after the last hidden platform test to assess spatial memory capacity. The platform was removed from the maze and rats were allowed to swim for 30 s in the pool. The percentage of time spent by rats in the target quadrant (quadrant I) was calculated. The data analysis was done by an experimenter blinded to the experimental conditions of the animals.

2.10. Statistical analysis

Data were presented as mean ± standard error of the mean (SEM). All data were analyzed using the SPSS 21.0 (SPSS Inc., USA) software. Data from the hidden platform test in the MWM were analyzed by two-way repeated measures ANOVA (between subject factor - treatment; within subject factor - time) with Bonferroni’s post-hoc test. Infarct volume, MCA signal intensity, Ki67/CD31 +, CD31/NG2 +, K67 + and K67/Map-2 + positive cells numbers were analyzed by Student’s t-test. The others were performed by two-way ANOVA (with MCAO and EE as main factors) followed by Bonferroni correction. Pearson linear regression analysis was applied to examine the correlation between relative CBF and survival volume in the cortex and striatum. Significance was defined as P < 0.05.

3. Results

3.1. EE ameliorated cortical and striatal lesion without affecting infarct volume in MCAO rats

T2WI obtained on 31st day after EE intervention revealed a hyper-intense signal in the MCA territory representing infarction (Fig. 3A). Two-way ANOVA revealed a significant interaction between the MCAO and EE in survival volume of striatum (F_int (1,36) = 13.453, P = 0.001). EE made main effect in cortex survival volume (F EE (1, 36) = 11.35, P = 0.002). Post-hoc comparisons revealed that the cortical and striatal survival volume were significantly decreased in IS rats compared with the SS rats (P < 0.001). EE treatment significantly increased the survival volume of the cortex (P < 0.01) and striatum (P < 0.001) in ischemic rats (Fig. 3C and D). While EE showed a trend to alleviate infarct volume, but there was no significantly difference compared to IS group rats (Fig. 3B).

3.2. EE improved cerebral blood flow that correlates with cortical and striatal surviving volume in MCAO rats

CBF perfusion was measured by ASL. (Fig. 4A). Two-way ANOVA showed there was significant interaction between MCAO and EE for the relative CBF of the cortex (F_int (1,36) = 11.752, P < 0.01) and striatum (F_int (1,36) = 8.739, P < 0.01). EE made main effect in the cortex relative CBF (F EE (1, 36) = 8.184, P = 0.007). Post-hoc comparisons revealed that MCAO markedly decreased relative CBF in the ipsilateral cortex and striatum compared to the SS group (P < 0.001). Notably, EE intervention increased cortical and striatal relative CBF in comparison with the IS rats (P < 0.001). (Fig. 4B-C).

Then Pearson linear regression analysis was performed to assess the correlation between the CBF and survival volume. The CBF showed significantly and strongly positive correlation with the survival volume in the cortex (R = 0.8339, P < 0.001) and striatum (R = 0.8813, P < 0.001), suggesting the preservation of CBF may causatively contribute to the increased survival volume after stroke (Fig. 4D-E).
3.3. **EE improved cerebral vascular signal in MCAO rats**

MRA was used to examine the status of intracranial vessels (Fig. 5A-E). Two-way ANOVA analysis showed that there were significant MCAO and EE interactions (BA: $F_{\text{int}} (1,36) = 6.223$, $P < 0.05$; contralateral MCA: $F_{\text{int}} (1,36) = 23.872$, $P < 0.001$; contralateral ICA: $F_{\text{int}} (1,36) = 8.373$, $P < 0.01$; contralateral PCA: $F_{\text{int}} (1,36) = 8.145$, $P < 0.01$; ipsilateral PCA: $F_{\text{int}} (1,36) = 5.494$, $P < 0.05$). EE made main effect in signal intensity of the MCA (ipsilateral: $F_{\text{EE}} (1, 36) = 9.035$, $P = 0.005$), AComA (ipsilateral: $F_{\text{EE}} (1, 36) = 8.025$, $P = 0.007$), azACA ($F_{\text{EE}} (1, 36) = 6.2753$, $P = 0.013$) and BA ($F_{\text{EE}} (1, 36) = 6.223$, $P = 0.017$) signal intensity. Post-hoc comparisons revealed that MCAO enhanced BA, azACA, contralateral ACA, AComA, ICA, MCA, and bilateral PCA signal intensity, while decreased ipsilateral ICA signal intensity compared with SS rats ($P < 0.05–0.001$). EE treatment increased azACA, ipsilateral AComA and ICA signal intensity, but reduced contralateral ICA, MCA, bilateral PCA and BA signal intensity ($P < 0.05–0.01$) (Fig. 5F-L). Notably, there was no significant difference between IE and SE in the BA, ipsilateral AComA, contralateral MCA, ICA and bilateral PCA signal intensity.

3.4. **EE promoted perilesional angiogenesis in MCAO rats**

To determine the effects of EE on angiogenesis, the numbers of Ki67/CD31$^+$ and NG2/CD31$^+$ cells were measured (Fig. 6A-C). EE significantly increased the number of Ki67/CD31 and NG2/CD31 double-labeled cells in the peri-infarct cortex and striatum when compared with the of IS group rats ($P < 0.001$; Fig. 6D-E). These data suggested that EE enhances angiogenesis following stroke.

Besides, angiogenesis-related factors including VEGF/Ang-1/Ang-2 were investigated by western blot. Two-way ANOVA revealed significant interactions between the MCAO and EE for the protein level of VEGF ($F_{\text{int}} (1,12) = 8.161$, $P = 0.008$). EE made main effect in the Ang-1 protein level ($F_{\text{EE}} (1,12) = 14.95$, $P = 0.001$). Post-hoc comparisons revealed that IS rats had lower level of VEGF and higher level of Ang-2 than SS rats ($P < 0.01$). Post stroke EE intervention significantly increased the protein levels of VEGF and Ang-1 and decreased the level of Ang-2 compared with the IS group ($P < 0.05–0.01$). Remarkably, there was no significant difference between IE and SE rats in protein level of VEGF, Ang-1 and Ang-2 (Fig. 6F-I).

3.5. **EE ameliorated ultrastructural injuries of the cerebral microvessels in MCAO rats**

Transmission electron microscopy showed normal appearance of capillaries and surrounding astrocytes in the SS and SE group rats. MCAO elicited ultrastructural abnormalities in the microvasculature. Vessel lumen showed stenosis and capillary endothelia displayed...
numerous vacuoles in its cytoplasm. The BM was thickened, while pericytes showed swelling with increased coverage enclosing endothelial cells. And perivascular astrocyte end-feet showed severe swelling (Fig. 7A). After EE treatment, the size of endothelium vesicles was significantly reduced, the thickness of BM was reduced and the swelling of pericytes were effectively alleviated. Furthermore, very mild edema was found at the astrocyte foot processes after EE intervention. (Fig. 7A-D).

Two-way ANOVA analysis showed significant interactions between MCAO and EE for the numbers of ECs vesicles ($F_{\text{Int}} (1,4) = 74.834, P < 0.001$), BM thickness ($F_{\text{Int}} (1,4) = 32.386, P < 0.001$) and astrocyte swelling score ($F_{\text{Int}} (1,4) = 11.688, P = 0.001$). There were significant main effects for EE in lumen circularity ($F_{\text{EE}} (1,4) = 16.480, P < 0.001$), number of ECs vesicle ($F_{\text{EE}} (1,4) = 38.130, P < 0.001$), BM thickness ($F_{\text{EE}} (1,4) = 26.366, P < 0.001$), pericyte coverage ($F_{\text{EE}} (1,4) = 5.394, P = 0.028$) and astrocyte swelling score ($F_{\text{EE}} (1,4) = 13.238, P = 0.001$).

Post-hoc comparisons revealed there were more endothelial vesicles in IS group rats than SS rats ($P < 0.001$). On the other hand, the ECs vesicle in the IE group was scarcely observed ($P < 0.001$) (Fig. 7B). Notably, MCAO without interference elicited remarkably narrowed lumen, increased BM thickness, as well as swelling perivascular astrocyte end-feet when compared with SS rats ($P < 0.01–0.001$). In contrast, post-ischemic intervention with EE significantly prevented vascular narrowing ($P < 0.01$), alleviated BM thickness ($P < 0.001$), ameliorated pericyte coverages ($P < 0.05$), and reduced astrocyte swelling score ($P < 0.001$) compared with IS group (Fig. 7E-I). Note that the lumen circularity and pericyte coverages of the IE rats return to the normal levels seen in the SE rats. Taken together, these findings demonstrated that EE attenuated the alterations in cerebral microvasculature and perivascular edema after ischemic stroke.

3.6. EE boosted the endogenous neurogenesis in MCAO rats

To examine the effect of EE on neurogenesis in the ischemic brain, the numbers of Ki67$^+$ and Ki67/MAP-2$^+$ cells were measured in the peri-infarct cortex and striatum (Fig. 8A-B). The results revealed that EE significantly increased the number of Ki67$^+$ and Ki67/MAP-2$^+$ cells when compared with the IS rats, suggesting that EE promoted cell proliferation and enhanced the neuronal differentiation in the peri-infarct cortex and striatum. ($P < 0.05$) (Fig. 8C-D).

T2 relaxometry mapping was conducted to determine the ischemic lesion of the cortex and striatum (Fig. 8E). Notably, two-way ANOVA revealed EE main effect and EE × MCAO interaction on relative T2 value in the ipsilateral striatum ($F_{\text{EE}} (1,36) = 20.157, P < 0.001$; $F_{\text{Int}} (1,36) = 20.009, P < 0.001$) and cortex ($F_{\text{EE}} (1,36) = 18.633, P < 0.001$; $F_{\text{Int}} (1,36) = 18.488, P < 0.001$). Post-hoc comparisons revealed that higher relative T2 values were detected in ipsilateral cortex and striatum of IS rats compared with SS rats ($P < 0.001$). After EE intervention, markedly reduced relative T2 values were identified in the corresponding areas when compared with IS group rats ($P < 0.001$) (Fig. 8G). Taken together, these data indicated that EE might play an important role in the protection and repair of the ipsilateral cortex and striatum induced by focal cerebral ischemia.

3.7. EE activated the PI3K/AKT/GSK3/β-catenin signaling pathway and axon guidance molecules in MCAO rats

Given that axonal guidance cues play a critical role in the endogenous angiogenesis and neurogenesis, this study investigated whether axonal guidance cues involved in the neurovascular remodeling elicited by EE. Two-way ANOVA revealed remarkable main effect for EE in the attractive axon guidance molecules (Netrin-1, $F_{\text{EE}} (1,12) = 5.290, P = 0.029$; DCC, $F_{\text{EE}} (1,12) = 8.826, P = 0.006$) and repulsive axon guidance molecules (Slit-2, $F_{\text{EE}} (1,12) = 6.780, P = 0.015$; Robo-1, $F_{\text{EE}} (1,12) = 4.186, P = 0.050$). There were also interactive effects of EE and MCAO on the Netrin-1 ($F_{\text{Int}} (1,12) = 6.873, P = 0.015$) and Slit-2 ($F_{\text{Int}} (1,12) = 4.634, P = 0.040$). Post-hoc comparisons revealed significantly decreased Netrin-1/DCC ($P < 0.01–0.001$) and Slit-2/Robo-1...
Fig. 5. Effect of EE on cerebral vascular signal in MCAO rats. Representative (A) axial, (B) coronal and (C) sagittal MIP maps of MRA were obtained from various groups. (D) The amplified azACA (1, 2) and AComA (3, 4) signal pictures. (E) The slices of 3D data images indicating the regions used for the signal intensity evaluation. Quantitative analysis of (F) MCA, (G) azACA, (H) AComA, (I) ACA, (J) ICA, (K) PCA and (L) BA vascular signal intensity. (ACA, anterior cerebral artery; AComA, anterior communicating artery; MCA, middle cerebral artery; ICA, internal carotid artery; PCA, posterior cerebral artery; BA, basilar artery; azACA, anterior azygos cerebral artery; Contra, contralateral; Ipsi, ipsilateral). Data were presented as mean ± SEM, n = 10, ⁎⁺⁺P < 0.01, ⁎⁎⁎P < 0.001 vs. SS; ^^^P < 0.001 vs. SE.
Fig. 6. Effect of EE on the angiogenesis in MCAO rats. (A) Representative double immunofluorescent staining for Ki67/CD31+ in the peri-infarct cortex and striatum of different groups (arrow heads indicated co-located cells; white arrows indicated CD31+; empty arrows indicated Ki67+; empty arrow heads indicated DAPI). (B) Typical confocal photographs of double immunofluorescent staining for CD31/NG2+ (arrow heads indicated co-located cells; white arrows indicated NG2+; empty arrows indicated CD31+; empty arrow heads indicated DAPI). (C) A representative T2WI image showed the peri-infarct areas (yellow square, cortex; green square, striatum). Quantitative data in the number of Ki67/CD31+ and CD31/NG2+ cells per mm² in the (D) peri-infarct cortex and (E) striatum. (F) The representative stripes of western-blot analysis for VEGF/Ang-1/Ang-2. (G-I) The protein expression of VEGF/Ang-1/Ang-2 were determined by western blot analysis. The protein level of was quantified using GAPDH as the loading controls. Data are expressed as means ± SEM, \( n = 4 \) for each group. \(*P < 0.05, **P < 0.01\) vs. IS; \( \#P < 0.01\), vs. SS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 7. Effects of EE on capillaries ultrastructure in MCAO rats. (A) A single layer of endothelial cells (En) was surrounded by a single layer of basement membrane (BM) and enclosed by astrocytes end-feet, forming an intact BBB. Endothelial cells created nearly rounded lumen in SS, SE and IE rats, but vessel lumen showed stenosis in IS rats (black dotted line). Astrocyte processes surrounding capillaries showed a normal morphology in SS and SE rats. After MCAO, astrocytes end-feet were severely swelling (shaded in blue) and contained swollen mitochondria with disorganized or absent cristae (III') in IS rats. IE astrocytes end-feet were slightly swollen (shaded in blue) with relatively normal appearing mitochondria (IV'). Insets (I, II and IV) right each image showed a relatively thin and intact BM, meanwhile IS rats (III) showed a thickness BM with fracture (black arrow heads). (B) Endothelial cytoplasm packed with scattered small vesicles (white arrow heads) in SS, SE and IE rats. However, IS rats contained dense caveolae-like vesicles (long white arrow) in endothelial cytoplasm. (C) The representative normal endothelium nucleus in SS and SE rats. After MCAO, the endothelium nucleus membrane being sunken (white open arrow heads) in IS rats, while slightly bended in IE rats. (D) The pericytes were relatively thin with regular nucleus in SS and SE capillaries. After MCAO, pericyte swelling increased along with its coverage of the endothelium in IS rats. However, in IE rats, pericytes appeared swollen but reduced coverage of the endothelium than IS rats. IS rats also contained sunken nucleus membrane in the pericyte (white open arrow heads). Quantitative analysis of (E) lumen circularity, (F) number of endothelial vesicles, (G) BM thickness, (H) pericyte coverage and (I) astrocyte swelling score. Data were presented as mean ± SEM, \( n = 2 \), \(^*P < 0.05, **P < 0.01\) vs. IS; \(^*P < 0.001\) vs. SS; \(^*P < 0.05\) vs. SE. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
protein level in the peri-ischemic tissues of IS group rats compared with the SS rats. Furthermore, Netrin-1/DCC (P < 0.01) and Slit-2/Robo-1 (P < 0.05) protein level were significantly increased by EE treatment after stroke compared to IS group, and returned to the normal level seen in the SE rats (Fig. 9A-B).

Specially, PI3K/AKT/GSK-3/β-catenin signal pathway, which exhibits important role in cell proliferation and growth, has been recognized to plays an important role in EE mediated neuroprotection. This study further investigated the expression of PI3K/AKT axis and its downstream targets GSK-3 and β-catenin to gain an insight into the possible repair mechanism underlying this EE paradigm following an ischemic stroke. Two-way ANOVA revealed that MCAO and EE treatment made significant interactions on proteins expression of p-AKT (F Int (1, 12) = 4.634, P = 0.040), p-GSK-3β Ser9 (F Int (1, 12) = 6.966, P = 0.013) and p-β-Catenin (F Int (1, 12) = 4.624, P = 0.040). EE made main effect in protein level of p-PI3K (F EE (1, 12) = 5.282, P = 0.040) and p-β-catenin (F EE (1, 12) = 6.648, P = 0.016). Post-hoc comparisons revealed that IE have significant higher p-PI3K and p-AKT ser473 protein level than IS group (P < 0.05) (Fig. 9C-D). IS rats showed sharply decreased p-GSK-3β Ser9 compared with the SS group rats (P < 0.05). Notably, EE treatment markedly increased p-GSK-3β Ser9 and p-GSK-3α Ser21 (P < 0.05–0.01), but decreased the p-β-catenin protein level (P < 0.05) compared with the IS group (Fig. 9E-G). There was no significant difference between IE and SE rats in the protein level of p-PI3K, p-AKT, p-GSK-3β Ser9, p-GSK-3α Ser21 and p-β-catenin. These results demonstrated the beneficial effect of EE on neurovascular reorganization, was at least in part, attributed to regulating PI3K/AKT/GSK-3/β-catenin signaling pathway and intrinsic axonal guidance molecules in ischemic rats.

3.8. EE improved the spatial learning and memory in MCAO rats

MWM test was performed to evaluate spatial learning and memory capacity of all rats. In the hidden platform test, the repeated measures of ANOVA revealed significant main effects of time and treatment on the path length (time: F(7, 266) = 94.442, P < 0.001; treatment: F(3, 114) = 18.221, P < 0.001) and escape latency (time: F(7, 266) = 88.414, P < 0.001; treatment: F(3, 114) = 154.950, P < 0.001). These results suggested all group rats gained spatial learning over testing days and treated rats performed better than that of rats without intervention. Post hoc analysis showed that IS rats took longer path and time to find the hidden platform compared with SS rats from the 2nd up to first session of 4th day (P < 0.05–0.001). IS rats also displayed longer time to find the hidden platform in the second session on the 1st day compared with SS rats (P < 0.05). However, IE rats showed reduced escape latency and path length in locating the hidden platform as compared with the IS rats on the 2nd and 3rd day (P < 0.05–0.001), and returned to normal level seen in the SE rats (Fig. 10A-B). There was no significantly difference in the swimming speed among the four group, indicating that no motor impairments influenced the acquisition of spatial learning (Fig. 10E).

The probe test was used to assess memory retention (Fig. 10C). Two-
way ANOVA revealed a significant main effect for EE ($F_{EE}(1, 39) = 5.190, P = 0.029$) in the time spent by rats in the target quadrant. Post hoc analysis showed that IS rats displayed fewer time spent in the target quadrant compared with SS group ($P < 0.001$). EE treatment displayed good spatial memory as evidenced by a remarkably increased time spending in the target quadrant compared with IS group ($P < 0.001$). Notably, there was no significantly difference between the IE rats and SE rats (Fig. 10D).

4. Discussion

Here, our findings demonstrated that the three-phase EE intervention paradigm ameliorated cortical and striatal lesion, benefited neurovascular remodeling, and improved spatial learning and memory in a permanent MCAO rat model. Meanwhile, PI3K/AKT-mediated activation of GSK-3/β-catenin and axonal guidance signaling Netrin-1/DCC and Slit-2/Robo-1 would be participated the process of EE promoted angiogenesis and neurogenesis in the ischemic brain.

As a noninvasive imaging modality, T2WI accurately identifies the ischemic lesion size of the brain (Castaneda et al., 2017). Currently, serial T2 images showed a large scale of infarction in the MCA-supply areas of the ipsilateral hemisphere after MCAO. EE treatment did not significantly reduce the ischemic lesion size consistent with previous studies (Xie et al., 2013), but did enhance the surviving volume of ipsilateral cortex and striatum compared with rats housed in standard conditions on the 31st day after MCAO. This indicates that post-ischemic EE might provide additive benefits for the reduction in brain tissue loss after ischemic stroke.

Improving of collateral circulation and maintaining constant cerebral perfusion are the important strategy for neurovascular remodeling after ischemic stroke (Stamatovic et al., 2019). It has been well documented that EE stimulated angiogenic growth factors (Yu et al., 2020), promoted angiogenesis (Xie et al., 2019b) and increased peri-infarct vascular density (Xie et al., 2019b; Zan et al., 2019). These changes caused by EE may play an essential role in maintaining perfusion following ischemic stroke.

In the present study, ASL perfusion imaging showed that unilateral MCA occlusion led to significant hypoperfusion within the MCA territory of the ischemic side, while EE significantly increased relative CBF in the ischemic cortex and striatum after stroke. Especially we analyzed the correlation between CBF and survival tissue volume and noticed higher CBF value corresponded to increased survival volume in the ischemic striatum and cortex following ischemic stroke. The data herein indicated that improved global CBF is a critical step for the repair of ischemic brain following post-stroke EE intervention.

The circle of Willis constitutes the main network of collateral
circulation, which plays a critical role in maintaining regional CBF in case of acute large artery occlusion caused brain damage (Romero et al., 2009). In the present study, MRA images showed that EE led to augmented signal intensity of the ipsilateral ICA, AComA and azACA in rats and correspondingly, increased CBF value in the ischemic region following ischemic stroke. The AComA bridges the bilateral ACAs supporting the anterior portion of the circle of Willis. The azACA is the extension of ACA that supplies both medial territories of the anterior cerebral hemispheres (Saleh et al., 2018). Based on the structural and functional properties of the cerebral neurovascular system detected by MRI, the present study strongly supported the beneficial effects of EE on improving cerebral collateral circulation and maintaining perfusion after ischemic stroke.

Growing evidence suggests that angiogenesis, is required for restoration of oxygen/nutrients and maintaining regional CBF during the subacute stage of cerebral ischemia (Chen and Chopp, 2006). Angiogenesis, defined as formation of new microvessels from pre-existing vessels, is a multistep process controlled by an intricate interplay between migrating pericytes and endothelial cells. The ECs proliferation initiates the angiogenic process, and pericytes contribute to the early growth of microvessels by recruiting and guiding endothelial cells to organize the microvessel wall and form an integral new vessel (Virgintino et al., 2007). In this study, ECs were identified by the endothelial marker CD31 and the pericytes in newly formed blood vessels (Virgintino et al., 2007). Our results revealed that treatment of EE were identified through the specific marker NG2 proteoglycan and the pericytes in newly formed blood vessels (Virgintino et al., 2007). In this study, ECs were identified by the endothelial marker CD31 and the pericytes in newly formed blood vessels (Virgintino et al., 2007). In this study, ECs were identified by the endothelial marker CD31 and the pericytes in newly formed blood vessels (Virgintino et al., 2007). In this study, ECs were identified by the endothelial marker CD31 and the pericytes in newly formed blood vessels (Virgintino et al., 2007).

Angiogenesis in the brain is regulated by a variety of proangiogenic factors (Fallah et al., 2019). VEGF, the most important angiogenic growth factor, exhibits important role in ECs proliferation, promotion of migration and capillary formation (Lee et al., 2009). Ang-1 and Ang-2 depend on VEGF to modulate angiogenesis. Ang-1 as pericyte-derived paracrine signal promotes blood vessel maturation and stabilization during late stages of vascular development. And Ang-2, an endogenous antagonist of Ang-1, is expressed mainly in ECs leading to vessel destabilization during angiogenesis (Armulik et al., 2005; Liu et al., 2014). In the present study, we found that EE robustly increased the protein expression of VEGF and Ang-1 but decreased expression of Ang-2. The increase of Ang-1 expression and endogenous stimulation of VEGF can act in combination to enhance angiogenesis and vascular integrity (Zacharek et al., 2007). While Ang-2 and VEGF worked in concert to enhance angiogenesis after stroke, but can ultimately lead to BBB disruption unlike Ang-1 (Zhu et al., 2005). Our findings shed light on the dynamic interplay among VEGF, Ang-1, and Ang-2 in EE-mediated angiogenesis following ischemic stroke. These results, along with the information obtained from MRI, likely reflected that enhanced angiogenesis after EE treatment is likely a major contributor to the elevation of CBF in the ischemic region which was essential for maintaining circulatory homeostasis following ischemic stroke.

It’s worth noting that enhanced angiogenesis does not always result in increased CBF in the brain. Cerebrovascular system stability relies on blood-brain barrier (BBB) integrity (Bowman et al., 2007; Thal et al., 2012; Winkler et al., 2018). BBB is a dynamic structure which comprises multiple cell types, including ECs, pericytes, BM and vascular astrocytes (Nirwane et al., 2019). ECs form the inner lining of blood vessels and limit diffusion of blood-borne solutes to keep vascular stability. Pericytes constitute an umbrella-like cover wrapping ECs and play an important role in regulating focal CBF (Duz et al., 2007). In addition, BM builds a physical contact bridge between ECs and pericytes (Caporarello et al., 2019). Furthermore, astrocyte end-feet almost completely cover blood vessels influencing the function and integrity of the BBB (Caporarello et al., 2019). As described above, multiple cell types of BBB collectively regulate blood flow and maintain the homeostasis of the brain microenvironment (Caporarello et al., 2019). In the present study, we further investigated the pathological response in the BBB ultrastructure of capillary by electron microscopy and found that MCAO elicited increased endothelial vesicles (Haley and Lawrence, 2017). The increased number of capillary endothelial vesicles often correlates with BBB permeability after cerebral ischemic (Haley and Lawrence, 2017). Combined with the change of ECs, pericytes were swelled with elongated coverage that sheathed the vascular endothelium, which might potentially lead to ‘no-reflow’ phenomenon in which blood flow could not return to capillaries after recanalization of larger vessels in the brain after ischemic stroke (Haley and Lawrence, 2017). Specifically, the thickness of the BM increased and astrocyte

**Fig. 10.** Effects of EE on MWM performance deficits in MCAO rats. (A) Path length and (B) escape latency of rats in the hidden platform test. (C) Typical training traces of rats in hidden platform test (top panel) on second session of 4th day and typical traces in probe test (bottom panel). (D) Percentage time spent by rats in the target quadrant in the probe trial. (E) Swim speed of rats in the hidden platform test. Data were presented as mean ± SEM, n = 10, *P < 0.05, **P < 0.01, ***P < 0.001 vs. IS; NS: P > 0.05, **NS: P < 0.01, ***NS: P < 0.001 vs. SS.
end-feet were significant edematous, resulting in compress vessel lumen and then limited blood reperfusion after stroke. These findings strongly suggested that ischemic injury led to abnormal structural modifications of the microvascular system. Conversely, EE intervention significantly decreased the number of endothelial vesicles and reduced pericyte coverage. Furthermore, the cumulating thickness of the BM and edema in astrocyte end-feet were alleviated by EE treatment. Based on these results, we confirm that EE could reduce ischemic damage of BBB in the capillary. Overall, MRI findings along with histological results strongly supported that EE benefited vascular remodeling including improving collateral flow, increasing angiogenesis and protecting BBB integrity.

A better collateral circulation and amplified angiogenesis have been shown to be coupled with neurogenesis during brain tissue remodeling after ischemic stroke. Neurogenesis is the process of producing new functional neurons from endogenous neural stem/progenitor cells (NSCs) which is essential for repair of the injured cerebral tissue (Hayashi et al., 2018). Several recent studies have shown that treatment of stroke with EE increased neurogenesis (Wu et al., 2018). Our results were in accordance with previous studies reporting that EE not only induced angiogenesis, but also promoted the proliferation and differentiation of progenitor cells as evidenced by increased number of Ki67 and Ki67/MAP-2 positive cells in peri-infarct striatum and cortex (Wu et al., 2018). The present study further supported the role of EE in promoting endogenous neurovascular remodeling involving neurogenesis and angiogenesis at a late stage after cerebral ischemia.

The underlying mechanisms of how EE-induced neurogenesis and angiogenesis are linked together are yet to be elucidated (Ruan et al., 2015). Attractive and repulsive axonal guidance cues such as Netrin-1 and Slit-2 play a crucial role in angiogenesis and neurogenesis. Netrin-1 mainly participates in guiding axon projection and neuroblasts migration. Additional evidence suggested that Netrin-1 also induces a proangiogenic response with increased sprouting, proliferation, migration, and tube formation in the ECs after ischemic injury (Guo et al., 2019; Rust et al., 2019). Similarly, the Slit-2/Robo-1 signaling is initially characterized as a repulsive guidance cue mediating migration of neural progenitor cells crossing the midline in the brain (Borrell et al., 2012), and also involved in regulating angiogenesis (Blockus and Chédotal, 2016). In this study, we detected the changes of axonal guidance cues and found that EE-treated rats exhibited a significant upregulation of Netrin-1/DCC and Robo-1/Slit-2 expression in the peri-infarct tissue after stroke. The finding suggested that axonal guidance signaling might be involved in EE induced angiogenesis and neurogenesis following cerebral ischemic injury.

Recently, PI3K/AKT/GSK-3 signal pathway has been recognized to play a major role in EE mediated neuroprotection against hypoxia-induced neurodegeneration in rats (Jain et al., 2013). Specifically, several lines of evidence support the critical role of GSK-3β and the transcriptional activator β-catenin in angiogenesis and neurogenesis (Wang et al., 2020; Xu et al., 2020; Zhang et al., 2020). In the present study, significantly up-regulated p-PI3K protein was detected in EE group rats after stroke. In accordance with the change of PI3K, significantly increased phosphorylation of AKT at Ser473 was measured in EE-treated MCAO rats. Evidences have demonstrated that the activation of AKT by phosphorylating Ser473 might further regulate downstream effector proteins to promote ECs migration, capillary formation and neuroblasts adhesion, migration (Shi and Walsh, 2002). One of the most important downstream targets of Akt is GSK-3. GSK-3 is inhibited by serine phosphorylation in either of the two isoforms of GSK-3, Ser9 in GSK-3β or Ser21 in GSK-3α. In the present study, we specially analyzed the expression of phosphorylation of GSK-3β Ser9 and GSK-3α Ser21, and found significantly hypo-phosphorylated GSK-3β at Ser9 in the IS rats. In contrast, treatment of EE showed a significant upregulation of GSK-3β at Ser9 and an increase of phosphorylation level of GSK-3α at Ser21 in the ischemic brain suggesting EE triggered phosphorylation of GSK-3 and hence inactivates GSK-3. Our findings might bring some new clues for understanding the mechanism of EE induced- neurovascular remodeling following ischemic stroke. However, there is a gap in the understanding of how GSK-3α inhibition mediating the neurovascular effects of EE, which also deserve future investigation.

It is now evident that GSK-3β influences cell adhesion and migration through its control of transcription factor β-catenin. GSK-3β can destroy β-catenin accumulation in cytosol or translation into nucleus by phosphorylating β-catenin. In the absence of GSK-3β signaling, stabilized β-catenin will lead to neural progenitor cells proliferation and ECs migration, differentiation into network structures (Skurk et al., 2005). We hypothesized that up-regulation phosphorylated GSK-3β at Ser9 and subsequent activation of its transcription factor β-catenin might play a critical role in mediating the neurovascular effects of EE. In this study, consistent with increased phosphorylated GSK-3β at Ser9, down-regulation of phosphorylated β-catenin was measured in EE rats in the later phases of stroke recovery. This implies that GSK-3β phosphorylation mediated by EE might subsequently allow β-catenin to escape the inhibitory phosphorylation after ischemic stroke. Since β-catenin has been linked to angiogenesis and neurogenesis, β-catenin over-expression may contribute to EE-induced promotion of neurovascular remodeling after ischemic stroke. Collectively, the present results showed that post-stroke EE increased phosphorylated PI3K-Akt followed by the phosphorylation of GSK-3α/β at Ser21/9, resulting in inactivation of GSK-3 and attenuated phosphorylation of β-catenin. The simultaneous changes of these proteins suggested that EE-induced neurogenesis and angiogenesis, at least in part, via activating PI3K/AKT/GSK-3β-catenin signaling pathway after ischemic stroke.

In addition to physical deficits, cerebrovascular dysfunction impairs cognitive function and ultimately causes irreparable cognitive impairment. Thus, improving neurovascular remodeling after stroke may offer long-term functional restoration. Currently, it is of particular interest to investigate the effect of EE on the recovery of cognitive function. Our MWM results showed that EE improved spatial learning and memory in ischemic rats as evident from decreased latency and path length in the hidden platform test, and increased time spent in target quadrant during the probe test. Our findings were consistent with previous reports indicating that EE significantly improved spatial learning and memory following ischemic stroke (Gonçalves et al., 2018; Tang et al., 2019; Wang et al., 2019). It should be noted that EE-induced improvement in the spatial memory was not accompanied by reduced infarct volume which was in agreement with previous studies (Söderström et al., 2009; Xie et al., 2013). Although far from being completely understood, accumulating evidences suggested that EE had multi-effects on neuralplasticity, including stimulating neurogenesis and angiogenesis (Xie et al., 2019a) increasing synaptic plasticity and dendritic complexity (Greifzu et al., 2018; Hannan, 2014), and upregulation the expression of neurotrophic factors and growth-associated proteins (Chen et al., 2017; Xie et al., 2013; Farokhi-Siasakht et al., 2019; Zhao et al., 2001). Furthermore, results from present study proposed that the beneficial effects of the EE on neurovascular restorative, all of that were essential for brain tissue remodeling and long-term functional recovery after ischemic stroke.

There are several limitations in our study that should be mentioned. First, we only imaged rats at one time point, 31 days after surgery. MRI data obtained from more time points would be useful for better understanding of the spatiotemporal profile of neurovascular alterations that occur during different stages of ischemic stroke. Future investigations designed to perform longitudinal MRI study of EE-induced neurovascular restoration are very necessary. Moreover, we set the three-phase EE intervention paradigm after ischemic stroke and explored the possible role of this type of EE paradigm on spatial learning and memory comparing with the standard environment. However, little is known about the differences between the current three-phase EE and the traditional EE. Future investigations that designed to compare the differences between the current three-phase EE and the traditional EE are very necessary, which would more precisely elucidate the rehabilitation effects of the three-phase EE paradigm on neurovascular remodeling.
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restorative and functional recovery following ischemic stroke. Although further experimental studies are needed, the three-phase EE intervention paradigm appears an effective interventional strategy for neurovascular restorative by improving CBF, ameliorating BBB integrity, promoting endogenous neurogenesis and angiogenesis coupled with regulating the PI3K/AKT/GSK-3β-catenin signal and axon guidance molecules, all of which may contribute to the improvement of learning and memory function after ischemic stroke. These data provide further evidence that set different levels of EE stimulation at different periods following ischemic stroke might have great potential for improving stroke recovery.

Author contributions

YZ performed the research, analyzed the data, and wrote the manuscript. MI performed the MRI experiments, and YZ and FL analyzed MRI data. LY reared animals and NZ performed the behavioral tests. XF helped capture the TEM photos. QZ provided professional suggestions. HZ designed the study, supervised the study and critically evaluated the manuscript.

Credit author statement

The authors claim that none of the material in the paper has been published or is under consideration for publication elsewhere. And the Authors declare no conflict of interest.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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