



The trend of indirect anastomosis formation in a 2-vessel occlusion plus encephalo-myo-synangiosis rat model

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Background: Basic research on the factors influencing indirect anastomosis formation in a 2-vessel occlusion plus encephalo-myo-synangiosis (2VO + EMS) rat model is conducive to improving the efficacy of indirect revascularization surgery in the clinic. However, the time point at which anastomosis between the rat temporal muscle (TM) and brain naturally has the greatest effect after encephalo-myo-synangiosis (EMS) remains unknown. Therefore, we conducted this study to explore the peak time of indirect anastomosis formation in the 2VO + EMS rat model.

Methods: Forty 2VO + EMS rats were randomly divided into five groups (n=8) according to the length of time (by week) after EMS, and 2VO rats were used as the control group (n=8). The expression of vascular endothelial growth factor (VEGF) and CD31 on the EMS side of the brain, perfusion ratio [improvement of cerebral blood perfusion (CBP) on the EMS side] and Morris water maze (MWM) results were compared between groups. Furthermore, the trends of the above variables were explored over weeks.

Results: Overall, the expression of VEGF and CD31, the perfusion ratio and the cognitive improvement in the 2VO + EMS rat model gradually increased over weeks after EMS. The VEGF and CD31 expression (as detected by immunofluorescence), perfusion ratio and number of times crossing the platform area peaked at 4 weeks after EMS. In addition, both the escape latency and the time spent in the target quadrant peaked in the fifth week after EMS.

Conclusions: After establishing the 2VO + EMS rat model, the degree of endothelial cell (EC) proliferation and CBP improvement on the EMS side of the brain peaked at 4 weeks after EMS, whereas the cognitive improvement peaked in the fifth week.

Keywords: Angiogenesis; cognitive function; encephalo-myo-synangiosis (EMS); endothelial cell proliferation; indirect anastomosis formation

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Introduction

Encephalo-myo-synangiosis (EMS), an indirect revascularization surgery, is a conventional method for the treatment of moyamoya disease and some other chronic ischemic cerebrovascular diseases (CICDs) (1). During surgery, chronically ischemic brain tissue is covered by capillary-rich temporal muscle (TM) tissue, and indirect anastomoses (new capillaries) are gradually formed between the TM and the brain (1,2). At 3 to 4 months after EMS, this kind of indirect anastomosis can have the desired effect of improving cerebral blood perfusion (CBP) and preventing stroke (3,4). Unfortunately, more than 50% of adult patients lack sufficient angiogenic potential to form sufficient anastomotic vessels between the TM and brain tissue (5,6). Therefore, the effects of surgery on these patients and their clinical prognoses are poor.

To improve the clinical efficacy of EMS surgery, some researchers have tried to simulate surgery in rats or mice to explore methods and mechanisms of promoting endothelial cell (EC) proliferation and anastomosis formation between the TM and brain (7-10). Although indirect anastomosis formation in these animal models has been revealed, when the anastomosis between the rat TM and brain peaks after EMS remains unclear. Sacrifice of the model rat before the peak anastomosis effect could result in missing potential significant results, whereas sacrifice of the model rat after the peak would undoubtedly prolong the experimental duration and waste experimental funds. Therefore, it is of great significance to determine the peak time of indirect anastomosis in the EMS rat model. Based on this need, we herein aimed to perform EMS on a 2-vessel occlusion (2VO) rat model of chronic cerebral ischemia to comparatively observe the expression of EC markers, the improvement of CBP and the improvement of cognitive function at different time points (different weeks after EMS) to explore the peak time of indirect anastomosis formation. These results will be extremely helpful for most basic research related to angiogenesis and indirect anastomosis formation. To the best of our knowledge, no similar reports have been published. We present the following article in accordance with the ARRIVE reporting checklist (available at <http://dx.doi.org/10.21037/atm-20-2936>).

Methods

Animals and subgrouping

Adult male Wistar rats (280–320 g) were purchased from

the animal center of Sun Yat-sen University, Guangdong, China. All animal experiments were approved by the Institutional Animal Ethics Committee of Sun Yat-sen University (No.: [2019]02-480-12), and treatment conformed to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (publication no. 80-23, revised 1996). Based on the different durations before sacrifice, rats were randomly divided into the (I) 2VO group (control group), (II) 2VO + EMS2 group, (III) 2VO + EMS3 group, (IV) 2VO + EMS4 group, (V) 2VO + EMS5 group, and (VI) 2VO + EMS6 group (*Table 1*), with eight rats in each group. Another 2VO sham group (n=8) was set to detect the baseline level of cerebral blood flow (CBF), CBP and cognitive function. The experimental schedule is presented in *Figure 1*.

Animal model of 2VO

Two-vessel occlusion was performed to establish a chronic cerebral ischemia model (11). After being anesthetized by isoflurane inhalation, the temperature of each rat was maintained at 37 °C on a heating pad. A straight incision in the middle of the neck was made to expose the left common carotid artery (CCA). Once the left CCA was ligated with two 4-0 silk threads, the incision was closed (*Figure 2A,B*). Seven days after left CCA ligation, each rat was fixed on a stereotaxic instrument, and a burr hole was made with an electric drill in the right frontal region (0.8 mm posterior and 3.4 mm lateral to the bregma) after a midline scalp incision was made. In addition, a placement device fixed with cyanoacrylate adhesives for the contact probe was set. Then, the right CCA was exposed by the same procedures. After that, the right CCA was temporarily clamped twice (5 min each time) and finally ligated with two 4-0 silk threads. For the 2VO sham group, the same procedures were performed but without CCA ligation. The CBF before and after the procedure was measured by laser Doppler flowmetry, and the mean CBF values are expressed as a percentage of the baseline value. A decline in CBF to approximately 30% of the baseline before 2VO ligation was required to indicate successful 2VO model establishment (12).

Procedures for EMS surgery in rats

Immediately after ligation of the right CCA, EMS procedures were performed on the right temporal region of 2VO rats. Both the TM tissue and skin were reflected in a U-shape from the skull (*Figure 2C,D*), and a piece of the

Table 1 Animal groups

Groups (n=8)	Procedurals
2VO sham group	Subjected to the same procedure with 2VO but without CCA ligation
2VO group	Ligation of both CCA, and sacrificed 6 weeks later
2VO + EMS group	2VO plus EMS, and sacrificed 2 weeks after EMS
2VO + EMS3 group	2VO plus EMS, and sacrificed 3 weeks after EMS
2VO + EMS4 group	2VO plus EMS, and sacrificed 4 weeks after EMS
2VO + EMS5 group	2VO plus EMS, and sacrificed 5 weeks after EMS
2VO + EMS6 group	2VO plus EMS, and sacrificed 6 weeks after EMS

2VO, 2-vessel occlusion; CCA, common carotid artery; EMS, encephalo-myo-synangiosis.

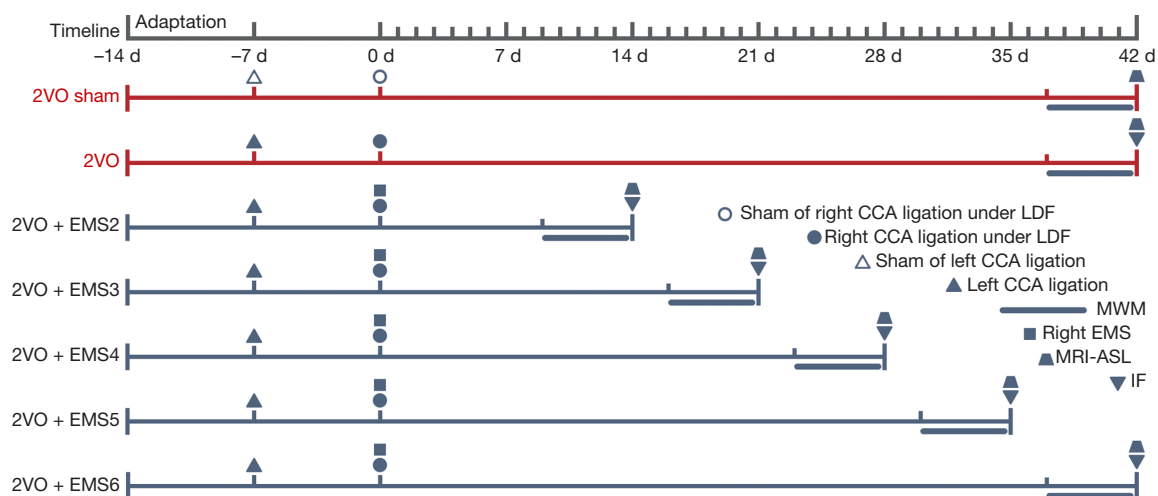


Figure 1 The experimental schedule. CCA, common carotid artery; LDF, laser Doppler flowmetry; 2VO, 2-vessel occlusion; EMS, encephalo-myo-synangiosis; MRI-ASL, magnetic resonance imaging arterial spin labeling; IF, immunofluorescence; MWM, Morris water maze.

skull approximately 4 mm in diameter was removed from the temporoparietal region using an electric drill. After that, the dura mater (DM) was carefully opened with micro-tweezers under the microscope, taking care to avoid damaging the brain surface (Figure 2E). Then, the arachnoid membrane was carefully opened with a 1 ml syringe needle in multiple places. To ensure that the TM tissue can be constantly in close contact with the ischemic brain surface, the edge of the DM and TM tissue were stitched together with a 10-0 prolene suture (Figure 2F). Finally, the EMS area was pressed constantly for 15 s after the skin was sutured. The rat brain and TM tissue were harvested together on the day of sacrifice (Figure 2G,H). A schematic drawing showing the EMS procedure is presented in Figure 3.

Morris water maze (MWM) test

The MWM test was adopted to analyze cognitive improvement after EMS (13). A circular black pool 180 cm in diameter and 60 cm deep was filled with water at a temperature of 22 ± 2 °C to a depth of 30 cm. The pool was divided into 4 equal quadrants. During the training stage, an escape platform was hidden in the center of quadrant III (10 cm × 10 cm, 1 cm below the water surface). The rats were first subjected to a place navigation test. The rats were gently placed in the water and released facing the wall of one of the four quadrants in a random order. They were allowed to find the underwater platform in 60 s, and both the latency to escape onto the platform and the swimming route were recorded. If a rat failed to find the platform, it

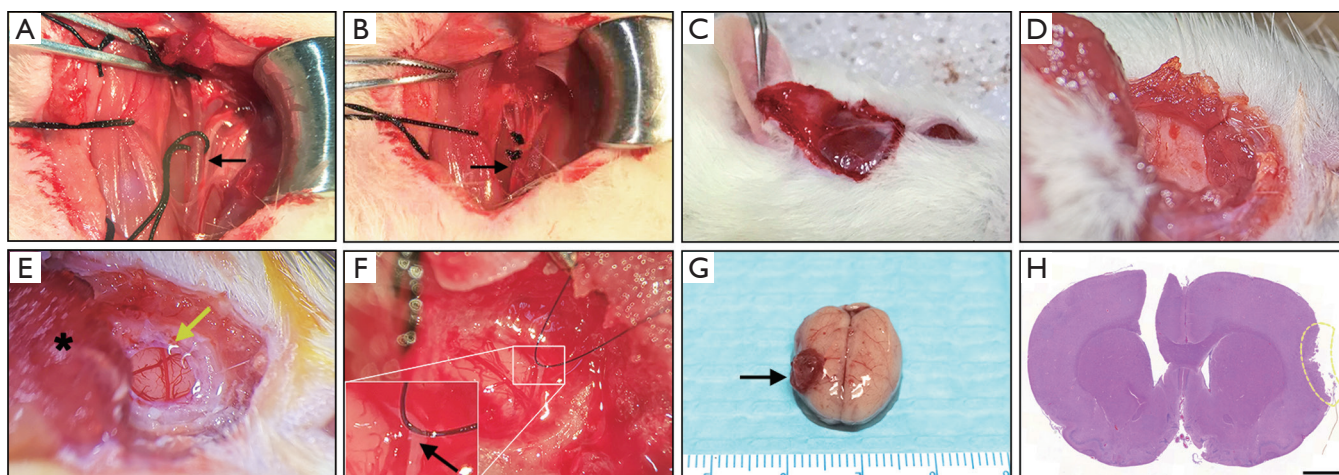


Figure 2 Procedures for modeling the 2VO + EMS rat. (A,B) Procedures for bilateral CCA ligation. The black arrow indicates the left CCA. Representative images showing the EMS procedures, including (C) reflection of the skin, (D) reflection of the TM tissue, (E) opening of the DM (the black asterisk indicates the TM and the yellow arrow indicates the DM margin) and (F) stitching of the TM and DM tissue (the black arrow indicates the DM). (G) Harvested brain samples from 2VO + EMS rats (the black arrow indicates the TM). (H) HE-stained brain slide from a 2VO + EMS rat. The yellow frame indicates the brain cortex involved in EMS. Bar =1 mm. EMS, encephalo-myosynangiosis; 2VO, 2-vessel occlusion; DM, dura mater; TM, temporal muscle; CCA, common carotid artery.

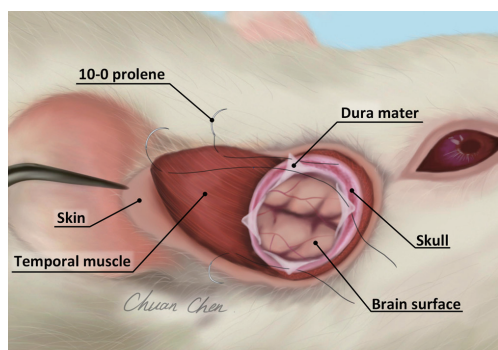


Figure 3 Schematic drawing showing the EMS procedure. EMS, encephalo-myosynangiosis.

was guided onto the platform with a stick, and its escape latency was recorded as 60 s. Each rat was allowed to stay on the escape platform for 10 s regardless of whether they found the platform. The training was conducted twice a day for 5 days, with intervals of approximately 20 min. On the same day that the place navigation test was finished, a 60-s spatial probe test was conducted during which the platform was removed. The time spent in the target quadrant and the cross platform area times were analyzed by a video surveillance system (SMART, Panlab SL, Barcelona, Spain).

In addition, rat motor functions were also assessed by the recorded swimming speed.

Magnetic resonance imaging (MRI)

We compared the effects of EMS on CBP improvement in the different groups by MRI arterial spin labeling (MRI-ASL) sequence measurements. After being anesthetized by isoflurane inhalation, the rat brains and CBP were assessed by a 7.0-T MRI animal scanner (PharmaScan MRI with ParaVision 7 system, Bruker, Germany). The MRI parameters were set as follows: echo spacing =11 ms, echo time (TE) =33 ms, repetition time (TR) =2,500 ms, TR/TE =2,500/33 ms, matrix size =256×256, field of view (FOV) = 35×35 mm², scan time =2 min 30 s, and section thickness =0.8 mm without gap. The regions of interest (ROIs) were set within the brain cortex in close contact with the TM tissue after EMS, with an area of 10 mm² per ROI. In the 2VO and 2VO sham groups, the ROIs were set in similar locations to those in the other 2VO + EMS groups. The CBP value (mL/100 g·min) of each ROI was assessed and calculated using ParaVision software, and the ratio of the CBP on the EMS side to that on the contralateral side was also calculated and set as the “perfusion ratio”, which was

used to represent the CBP improvement on the EMS side.

Immunofluorescence (IF)

We used IF assays to determine the effects of EMS on EC proliferation. Briefly, brain and TM sections were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 5 min at room temperature, permeabilized, and blocked for 30 min with 1% bovine serum albumin and 0.1% Triton X-100. After that, the fixed sections were washed and incubated for 1 h with primary antibodies against CD31 (1:100; Santa Cruz Biotechnology, Dallas, TX, USA) or vascular endothelial growth factor (VEGF, 1:200; Cell Signaling Technology, Danvers, MA, USA) at 4 °C overnight. Thereafter, the sections were washed three times with PBS and incubated with fluorescence-conjugated species-specific secondary antibodies. Then, to identify the nuclei, the samples were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Beyotime Biotechnology, Shanghai, China), and a fluorescence microscope was used to acquire photographs. The quantities of VEGF- and CD31-positive cells indicated the distribution and proliferation of capillary ECs. The average numbers of positively stained cells in five random visual fields (100 $\mu\text{m} \times 100 \mu\text{m}$) were counted. All experiments were repeated in triplicate.

Statistical analysis

The Statistical Program for Social Science (SPSS) version 22.0 was used to perform statistical analyses. The data are reported as the means \pm standard deviations (SDs). A two-tailed unpaired Student's *t*-test was adopted to analyze the MWM test, MRI-ASL and IF results. Differences were considered to be significant if $P < 0.05$ and very significant if $P < 0.01$.

Results

Mortality rate and effect on CBF changes in the 2VO + EMS model

Of the 53 rats that were subjected to 2VO and 2VO + EMS, 5 (9.4%) died within 7 days after modeling. The remaining rats were randomly assigned to the experimental groups described above. No deaths occurred in the 2VO

sham group. None of the brain surface damage was caused during dura opening, and no symptoms of hemiplegia were observed after modeling. An immediate and sharp decline in CBF was detected by laser Doppler flowmetry after bilateral CCA ligation in each rat from the baseline level of 186.67 ± 13.51 to 42.23 ± 8.95 PU (Figure 4A). The CBF decreased on average to $22.55\% \pm 4.26\%$ of the baseline level before 2VO in this study (excluding the 2VO sham group) (Figure 4B). No significant change in CBF was detected when the EMS procedure was performed. The decrease rate in the 2VO group was significantly lower than that in the 2VO sham group ($22.61\% \pm 2.90\%$ vs. $99.66\% \pm 1.61\%$, $P < 0.001$). No significant difference in the decrease rate was found between the 2VO group and the other 2VO + EMS groups. The operation time was not significantly different between the groups (Figure 4C).

The expression of VEGF and CD31 in the brain cortex of 2VO + EMS rats at different time points

IF was used to detect and quantify the expression of VEGF and CD31 in the EMS-side brain cortex of the 2VO + EMS rats at different time points (Figures 5,6). According to the IF results, the density of VEGF (+) cells was significantly higher in the 2VO + EMS2 group than in the 2VO group (4.25 ± 1.10 vs. 2.61 ± 0.98 , $P = 0.0072$), while the density of CD31 (+) cells in the 2VO + EMS2 group was not significantly different from that in the 2VO group (3.95 ± 0.92 vs. 3.38 ± 0.92 , $P = 0.2330$). The density of VEGF(+) cells in the 2VO + EMS3 group was not significantly different from that in the 2VO + EMS2 group (4.18 ± 1.18 vs. 4.25 ± 1.10 , $P = 0.9872$), whereas the density of CD31(+) cells in the 2VO + EMS3 group was significantly higher than that in the 2VO + EMS2 group (6.39 ± 1.91 vs. 3.95 ± 0.92 , $P = 0.0058$). Furthermore, both VEGF(+) and CD31(+) cells in the 2VO + EMS4 group were significantly denser than those in the 2VO + EMS3 group (VEGF: 6.39 ± 1.70 vs. 4.18 ± 1.18 , $P = 0.0091$; CD31: 8.36 ± 1.66 vs. 6.39 ± 1.91 , $P = 0.0443$). However, there were no significant differences in the densities of VEGF(+) and CD31(+) cells in the 2VO + EMS5 and 2VO + EMS4 groups (VEGF: 6.09 ± 1.40 vs. 6.39 ± 1.70 , $P = 0.7060$; CD31: 8.41 ± 1.68 vs. 8.36 ± 1.66 , $P = 0.9530$) or between the 2VO + EMS6 group and the 2VO + EMS5 group (VEGF: 6.54 ± 1.40 vs. 6.09 ± 1.40 , $P = 0.5306$; CD31: 8.20 ± 1.73 vs. 8.41 ± 1.68 , $P = 0.8064$).

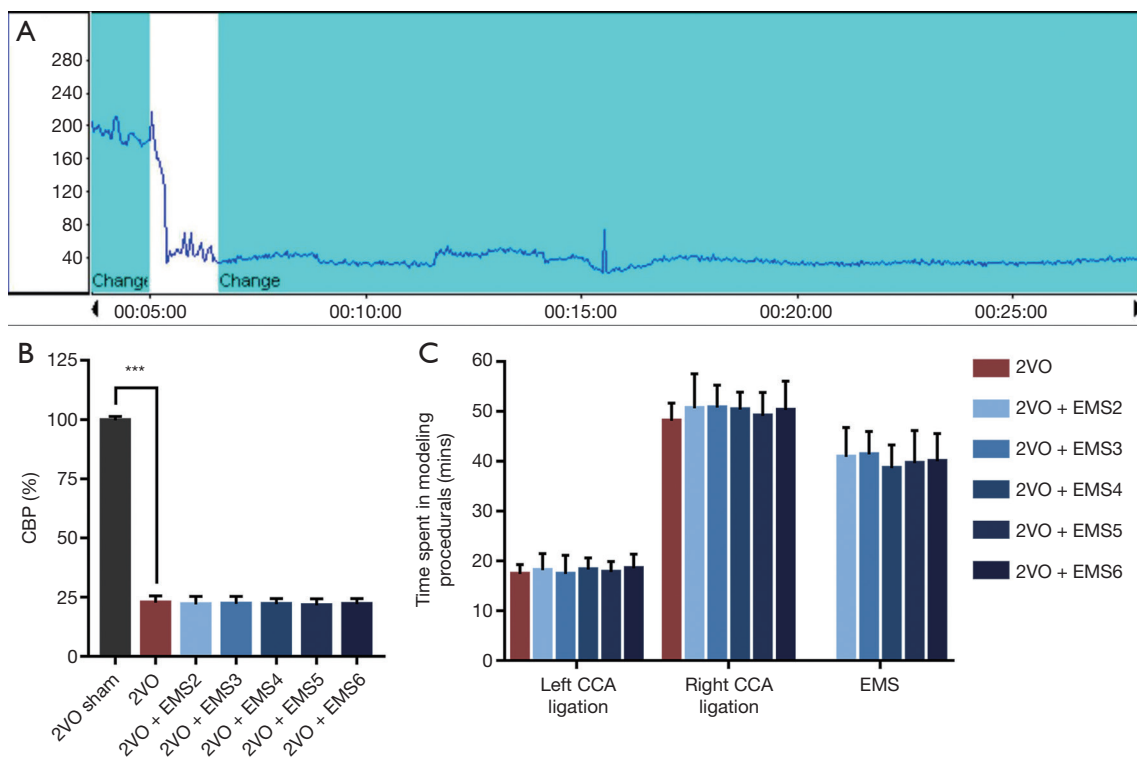


Figure 4 The change in CBF after 2VO. (A) Representative graph showing the CBF changes before and after 2VO; (B) Doppler flowmetry results showing that the CBF values in all the rats dropped to 22.55%±4.26% of the baseline levels, and the decrease rate in the 2VO group was significantly lower than that in the 2VO sham group; (C) the column chart showing that the operation time was not significantly different between groups. *******, $P < 0.001$. 2VO, 2-vessel occlusion; EMS, encephalo-myo-synangiosis; CBF, cerebral blood flow.

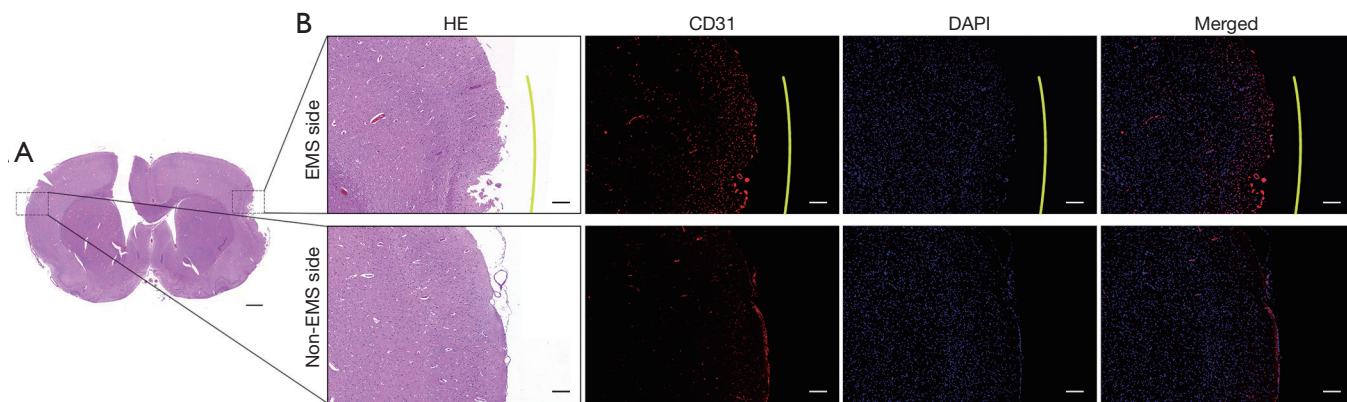


Figure 5 Representative immunofluorescence staining showing CD31 expression on both sides of the 2VO + EMS rat brain. (A) HE-stained brain slides from 2VO + EMS rats. The two black frames indicate the part of the brain in close contact with the TM tissue and the brain on the symmetrical part of the non-EMS side. Bar = 1 mm. (B) HE and immunofluorescence results showing that there were significantly more CD31 (+) cells in the brain on the EMS side than on the non-EMS side. Bar = 200 μ m. The yellow curve indicates the range of the brain surface involved in EMS. HE, hematoxylin and eosin; 2VO, 2-vessel occlusion; EMS, encephalo-myo-synangiosis.

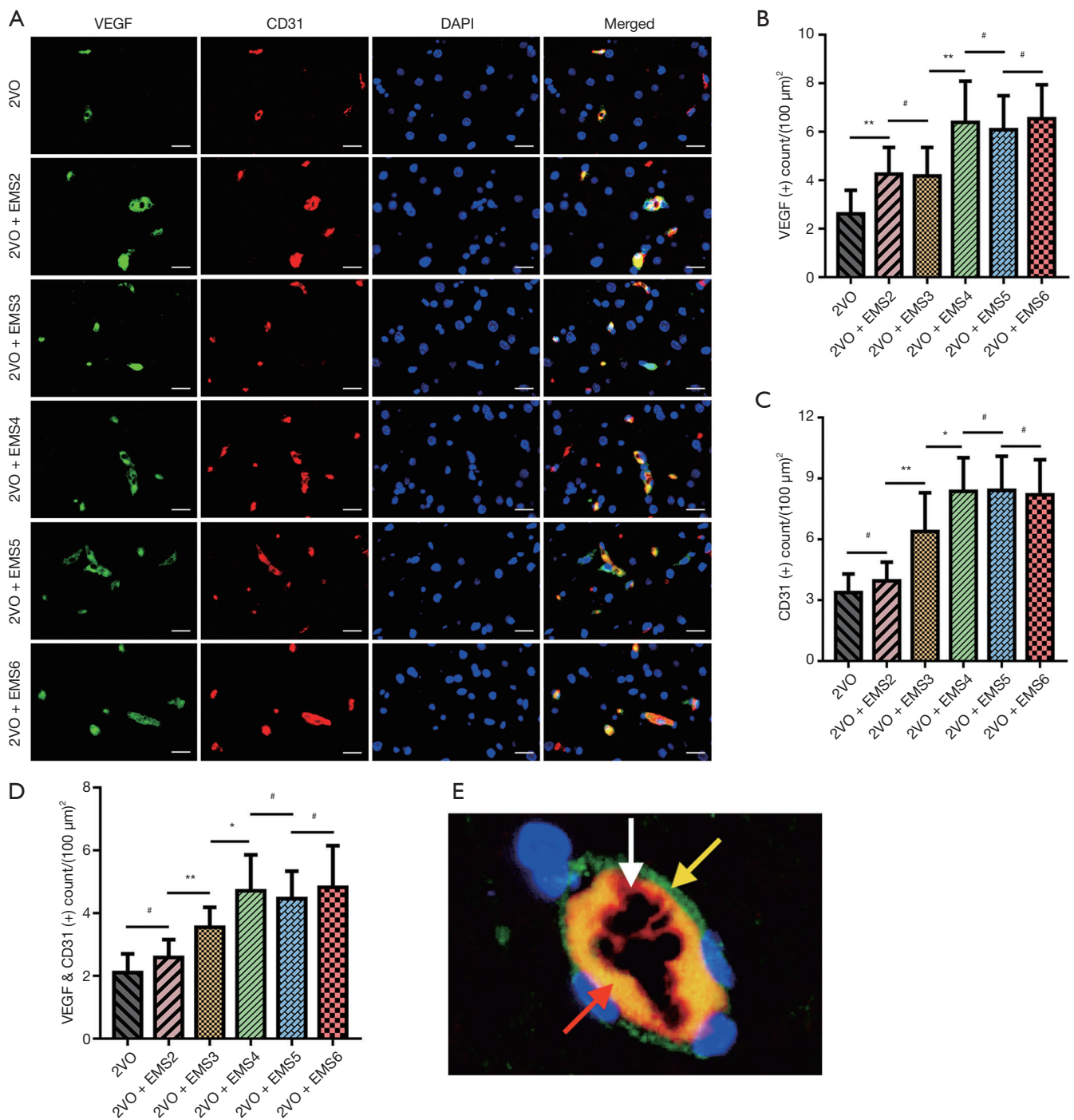


Figure 6 IF results showing both VEGF and CD31 expression in the EMS side brains from each group. (A) Dual IF staining showing VEGF (+) and CD31 (+) cells in the EMS-side brains in each group. Bar =20 μm; (B) counts of VEGF (+) cells in each group; (C) counts of CD31 (+) cells in each group; (D) counts of VEGF & CD31 (+) cells in each group; (E) representative IF results showing both VEGF (+) and CD31 (+) cells. Bar =5 μm. The yellow arrow indicates a VEGF (+) cell, the white arrow indicates a CD31 (+) cell and the red arrow indicates the overlap of VEGF (+) and CD31 (+) cells. The error bars represent the ± SD. *, P<0.05; **, P<0.01; #, P>0.05. IF, immunofluorescence; TM, temporal muscle; VEGF, vascular endothelial growth factor; EMS, encephalo-myo-synangiosis.

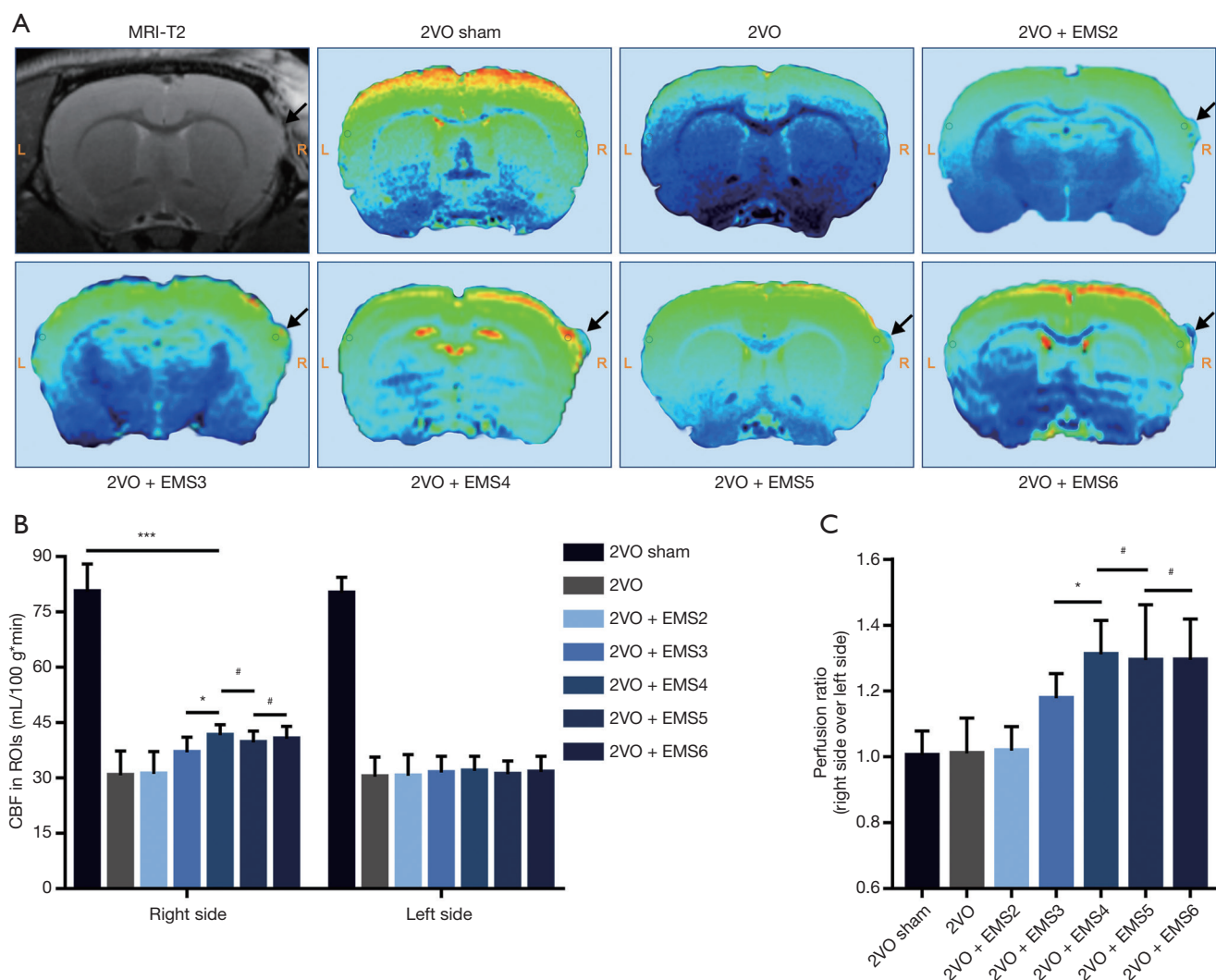


Figure 7 The results of MRI-ASL in each group. (A) Representative MRI-T2 and MRI-ASL films showing the CBP differences between each group. The black arrow indicates the ischemic brain tissue covered by TM. The gray circles indicate the ROIs for CBP detection. (B) A chart showing the CBP values of the ROIs in each group. (C) The results of perfusion ratios in each group. The error bars represent the \pm SD. *, $P < 0.05$; ***, $P < 0.001$; #, $P > 0.05$. CBP, cerebral blood perfusion; ASL, arterial spin labeling; ROI, region of interest; TM, temporal muscle.

CBP improvement in 2VO rats at different time points after EMS

For each group, we used MRI-ASL sequences to determine the CBP values of the ROIs on both the EMS side and non-EMS side and used the perfusion ratio to indicate the degree of increased CBP on the EMS side (Figure 7). For the EMS side, there was no significant difference in the CBP values between the 2VO + EMS2 and 2VO groups (31.11 ± 6.09 vs. 30.71 ± 6.60 mL/100 g-min, $P = 0.9015$). However, the 2VO +

EMS3 group had a higher CBP value on the EMS side than the 2VO + EMS2 group (36.95 ± 4.12 vs. 31.11 ± 6.09 mL/100 g-min, $P = 0.0413$). Furthermore, the CBP value on the EMS side in the 2VO + EMS4 group was significantly higher than that on the EMS side in the 2VO + EMS3 group (41.56 ± 2.87 vs. 36.95 ± 4.12 mL/100 g-min, $P = 0.0210$). Nevertheless, there was no significant difference in the CBP values on the EMS side between the 2VO + EMS5 and 2VO + EMS4 groups (39.66 ± 3.04 vs. 41.56 ± 2.87 mL/100 g-min, $P = 0.2196$) or between the 2VO + EMS6 and 2VO +

EMS5 groups (40.61 ± 3.38 vs. 39.66 ± 3.04 mL/100 g·min, $P=0.5638$). On the other hand, there were no significant differences in the CBP values on the non-EMS side among all six groups. In addition, the rats in the 2VO sham group had a significantly higher CBP value in the right frontal lobe than the rats in the 2VO + EMS4 group (80.50 ± 7.48 vs. 41.56 ± 2.87 mL/100 g·min, $P<0.001$)

Accordingly, the perfusion ratio in the 2VO + EMS2 group was not significantly different from that in the 2VO group (1.02 ± 0.07 vs. 1.01 ± 0.11 , $P=0.8524$). In addition, the 2VO + EMS3 group had a higher perfusion ratio than the 2VO + EMS2 group (1.18 ± 0.08 vs. 1.02 ± 0.07 , $P=0.0008$). Furthermore, the perfusion ratio in the 2VO + EMS4 group was significantly higher than that in the 2VO + EMS3 group (1.31 ± 0.10 vs. 1.18 ± 0.08 , $P=0.0105$). However, there was no significant difference in the perfusion ratio between the 2VO + EMS5 and 2VO + EMS4 groups (1.29 ± 0.17 vs. 1.31 ± 0.10 , $P=0.8056$) or between the 2VO + EMS6 and 2VO + EMS5 groups (1.30 ± 0.12 vs. 1.29 ± 0.17 , $P=0.9867$).

Pattern of cognitive function improvement in 2VO + EMS rats

MWM tests were performed to evaluate the cognitive function improvement in the 2VO rats after EMS (Figure 8). The escape latency 2 weeks after EMS surgery (2VO + EMS2 group) was not significantly different from that of the control group (29.38 ± 5.37 vs. 31.50 ± 5.83 s, $P=0.4609$). However, three weeks after EMS, the escape latency of 2VO + EMS rats began to shorten significantly (2VO + EMS3 vs. 2VO + EMS2: 24.38 ± 3.62 vs. 29.38 ± 5.37 s, $P=0.0465$). The escape latency in the fourth week (2VO + EMS4 group) was significantly shorter than that in the third week (2VO + EMS3 group) (19.86 ± 4.26 vs. 24.38 ± 3.62 s, $P=0.0390$). By the fifth week after EMS surgery, the escape latency was still significantly shortened (2VO + EMS5 vs. 2VO + EMS4: 15.63 ± 2.88 vs. 19.86 ± 4.26 s, $P=0.0346$). However, there was no significant difference in the escape latencies between the sixth and fifth weeks after EMS (2VO + EMS6 vs. 2VO + EMS5: 16.25 ± 3.28 vs. 15.63 ± 2.88 s, $P=0.6916$). In addition, the escape latency of the 2VO sham rats was significantly shorter than that of the 2VO + EMS5 group (9.13 ± 1.81 vs. 15.63 ± 2.88 s, $P<0.001$).

For the time spent in the target quadrant, the result in the 2VO + EMS2 group was not significantly different from that in the 2VO group (17.88 ± 3.00 vs. 17.50 ± 4.75 s, $P=0.8529$). Three weeks after EMS, the time spent in the target quadrant began to increase significantly (2VO

+ EMS3 vs. 2VO + EMS2: 22.00 ± 2.83 vs. 17.88 ± 3.00 s, $P=0.0484$). Furthermore, the rats in the 2VO + EMS4 group spent more time in the target quadrant than the rats in the 2VO + EMS3 group (26.00 ± 4.90 vs. 22.00 ± 2.83 s, $P=0.0419$), and the time spent in the target quadrant by the 2VO + EMS5 group rats was significantly longer than that spent by the 2VO + EMS4 group rats (32.02 ± 4.96 vs. 26.00 ± 4.90 s, $P=0.0289$). However, there was no significant difference in the time spent in the target quadrant between the 2VO + EMS6 and 2VO + EMS5 groups (29.50 ± 6.23 vs. 32.02 ± 4.96 s, $P=0.3896$). In addition, the time spent in the target quadrant of the 2VO sham rats was significantly longer than that of the 2VO + EMS5 group (37.88 ± 5.38 vs. 32.02 ± 4.96 s, $P=0.0395$). For the number of times crossing the platform area, there was no significant difference between the 2VO + EMS2 and 2VO groups (1.50 ± 0.93 vs. 1.38 ± 0.52 , $P=0.7438$). However, the number of platform area crossings began to increase significantly from the third week after EMS (2VO + EMS3 vs. 2VO + EMS2: 1.75 ± 1.16 vs. 1.50 ± 0.93 , $P=0.0465$). Furthermore, the number of times crossing the platform area was continuously more frequent at 4 weeks after EMS than at 3 weeks after surgery (2VO + EMS4 vs. 2VO + EMS3: 3.00 ± 1.07 vs. 1.75 ± 1.16 , $P=0.0431$). Nevertheless, there was no significant difference in the number of times crossing the platform area between the 2VO + EMS5 and 2VO + EMS4 groups (2.63 ± 1.19 vs. 3.00 ± 1.07 , $P=0.5176$) or between the 2VO + EMS6 and 2VO + EMS5 groups (2.88 ± 1.13 vs. 2.63 ± 1.19 , $P=0.6723$). Furthermore, the number of times crossing the platform area of the 2VO sham rats was significantly greater than that of the 2VO + EMS4 group (4.38 ± 1.30 vs. 3.00 ± 1.07 , $P=0.0368$).

In addition, there was no significant difference among groups in swimming speed during the experiments, indicating that the 2VO or 2VO + EMS procedures did not cause motor dysfunction in this study.

Discussion

In the present study, we revealed, for the first time, the trend in EC proliferation and indirect anastomosis formation induced and promoted by EMS surgery in the chronically ischemic brains of 2VO rats. We found that the degree of EC proliferation and CBP improvement on the EMS side of 2VO + EMS rat brains peaked in the fourth week after EMS, whereas cognitive improvement peaked in the fifth week after EMS. Undoubtedly, this finding is extremely helpful and illuminative for most basic studies

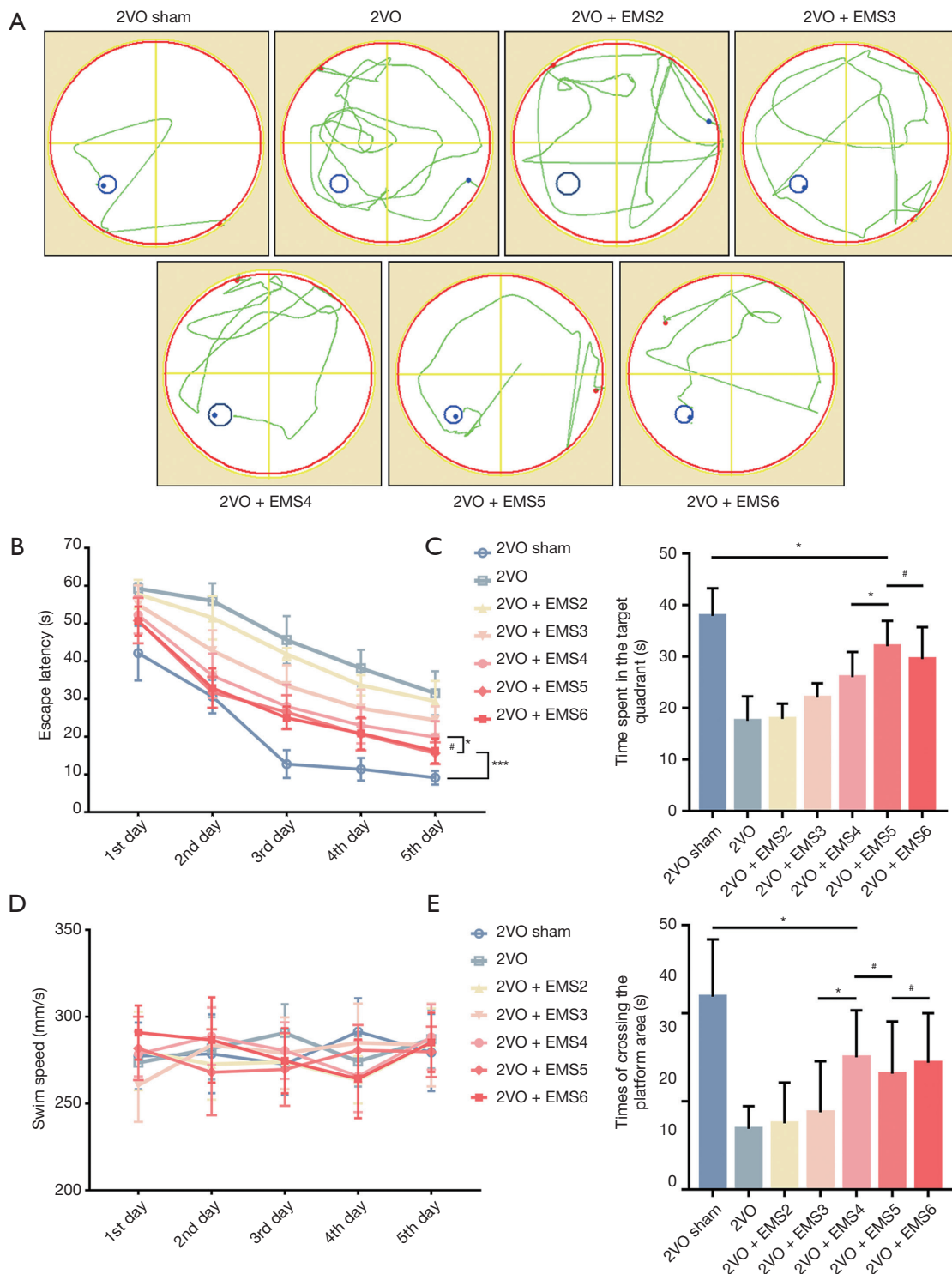


Figure 8 MWM test results showing the cognitive improvement in each group. (A) Representative swimming path of each group. (B) Average escape latency of each group. #, $P > 0.05$, 2VO + EMS6 vs. 2VO + EMS5; *, $P < 0.05$, 2VO + EMS5 vs. 2VO + EMS4; ***, $P < 0.001$, 2VO sham vs. 2VO + EMS5. (C) Time spent in the target quadrant of each group. *, $P < 0.05$; #, $P > 0.05$. (D) Average swimming speed of each group. (E) Times of crossing the platform area of each group. *, $P < 0.05$; #, $P > 0.05$. The error bars represent the \pm SD. MWM, Morris water maze; 2VO, 2-vessel occlusion; EMS, encephalo-myelo-synangiosis.

related to angiogenesis and indirect anastomosis formation. Moreover, the novel method used to establish the 2VO + EMS rat model in this study had low mortality and was highly effective; thus, this method should be promoted. A discussion is presented in detail as follows.

The trends and significance of EC marker expression and CBP and cognitive improvement in the 2VO + EMS rat model

Unquestionably, EC proliferation is the basis of angiogenesis (14,15). Therefore, this study inferred the degree of angiogenesis by quantifying the expression of VEGF and CD31 (an EC marker). VEGF is a key angiogenesis-promoting cytokine that has an effect on increasing EC proliferation, migration and permeability (16-18). CD31 (platelet EC adhesion molecule-1, PECAM-1), which concentrates at the sites of interendothelial cell contact, is commonly used to detect the existence of ECs (19,20). In this study, the IF results showed that the expression of both VEGF and CD31 peaked at the fourth week after the EMS procedure. Thus, for basic studies performed using 2VO + EMS rat models, cytokine detection should optimally be performed at 4 weeks after EMS. On the one hand, this time point will optimally ensure that significant experimental results are obtained, and the difference between the experimental and control groups should be maximized. On the other hand, this time point could shorten the experimental period and save money in some cases. However, we also found that the expression of VEGF in 2VO + EMS rats was significantly higher at 2 weeks after EMS compared to that in the 2VO group, whereas the expression of CD31 was not significantly higher until the third week after EMS. This result probably indicates that VEGF is involved in the processes of EC proliferation and angiogenesis before CD31, and this finding could be explained by the different biological functions of these two cytokines (mentioned above).

In addition, to objectively and intuitively reflect the effect of EMS on promoting EC proliferation and angiogenesis, we used MRI-ASL sequences to quantify the perfusion ratio of CBP in each group. The perfusion ratio of 2VO + EMS rats increased slightly 2 weeks after EMS compared to that of 2VO rats, but the difference was not significant. However, the perfusion ratio increased significantly in the third week after EMS, peaked in the fourth week, and then entered the platform stage. The trend of CBP improvement on the EMS side was basically consistent with that of EC

proliferation (especially the increased pattern of CD31 expression), which also confirmed the feasibility of using EC markers to represent the degree of angiogenesis.

Finally, we also assessed whether CBP improvement could improve the cognitive functions of 2VO + EMS rats by using the MWM test to evaluate cognitive function improvement over weeks after EMS. The results suggested that the cognitive function of 2VO + EMS rats was not significantly improved in the second week after EMS and began to show significant improvement beginning in the third week, continued to improve in the fourth week, and peaked in the fifth week after EMS (the escape latency and time spent in the target quadrant peaked in the fifth week, but the number of times crossing the platform area peaked in the fourth week). The improvement in cognitive function lagged slightly behind that of EC proliferation and CBP (which peaked in the fourth week) (*Figure 9*), but this lag is explainable. Taking patients with symptomatic CICD as an example, their recovery of affected neurological functions requires additional processes even if they undergo revascularization surgery (such as extracranial-intracranial bypass and middle cerebral artery recanalization) as soon as possible. In other words, after the formation of new capillaries, the repair of neurons needs to initiate another complex mechanism (21-23).

Advantages of and surgical techniques for the novel method for establishment of the 2VO + EMS rat model in this study

The traditional 2VO + EMS rat modeling method involves the simultaneous ligation of the bilateral CCAs and performance of EMS 1 week later (or at any given interval) (10,24). However, in this study, we first ligated the left CCA and then ligated the right CCA 1 week later and performed EMS simultaneously. Similar to the traditional method, our novel method also requires two anesthesia sessions and 1 week of experimental duration, but it has the following two advantages. First, the method of two separate unilateral CCA ligations allows the rat to adapt to the hypoperfusion state after unilateral CCA ligation (based on our prior experimental results, a decrease in CBF was not obvious when only one side of the CCA was ligated and soon returned to the baseline level). With this strategy, the sudden and sharp decrease in CBP caused by the simultaneous ligation of both CCAs can be avoided. In addition, clamping the right CCA repeatedly before ligating allows the rat to adapt to the hypoperfusion state after bilateral CCA

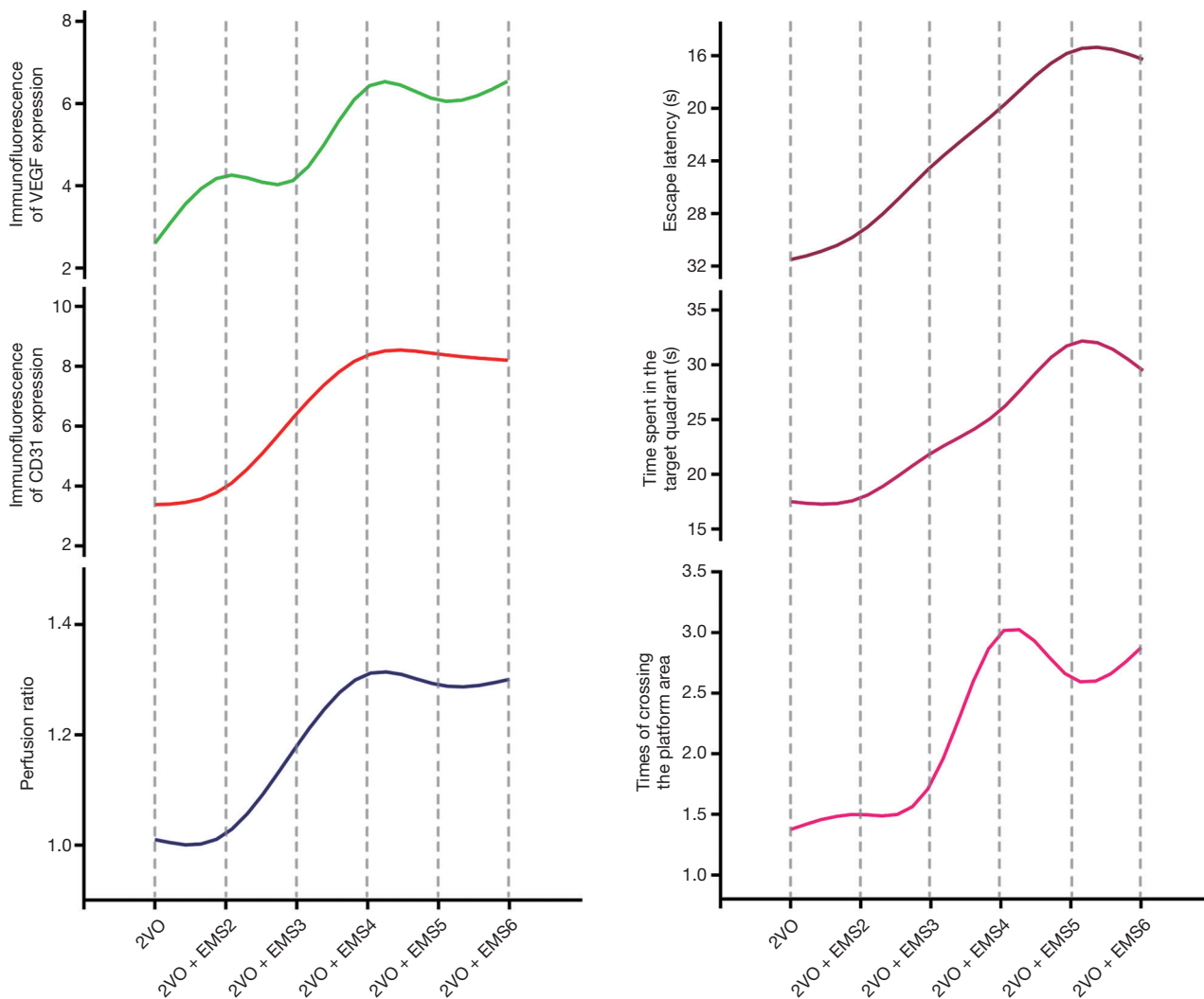


Figure 9 Diagrams of curves showing the trends of VEGF expression, CD31 expression, perfusion ratio improvement and cognitive improvement over weeks.

ligation. Therefore, the novel modeling method described herein theoretically has a lower 7-day mortality rate than the traditional method. The 7-day mortality rate was 9.43% (5/53) in this study (the EMS procedure caused almost no brain damage, and its lethal risk was negligible), whereas the previously reported mortality rates of the traditional method ranged from 16% to 20% (12,25-28). Therefore, our novel method may have a lower mortality rate and be safer than the traditional method. Second, the immediate performance of indirect revascularization in a moyamoya patient before the natural establishment of adequate blood flow compensation generally leads to an ideal degree of anastomosis formation (for example, the effect of indirect

revascularization in children with moyamoya disease is significantly better than that in adult patients) (29,30). However, the traditional method involves the performance of EMS 1 week after the establishment of 2VO. During this week, the affected anterior circulation blood flow of 2VO rats may be compensated to a certain degree (e.g., open posterior communicating artery), which has a negative effect on anastomosis establishment. In our modeling method, EMS was completed immediately after 2VO was established (when the ischemia was most severe). Therefore, compared with the traditional method, our new method can theoretically lead to better anastomosis formation, is more effective and is more conducive to experimental

observation.

In addition, some useful surgical techniques can be utilized to ensure the safety and effectiveness of the 2VO + EMS model to the greatest extent. First, the rat DM is extremely soft and thin, and direct cutting with scissors or a scalpel will very likely damage the underlying brain tissues, resulting in dysfunction or death. We recommend gently scratching the dura with micro-tweezers to form a wrinkle and then tearing the wrinkled dura directly with two micro-tweezers, which can avoid damaging brain tissue. Second, the arachnoid membrane is thought to act as a barrier between the brain tissue and the TM, which can be a negative factor affecting the efficacy of indirect revascularization (31,32). Therefore, when modeling, a 1 mL syringe needle should be used to poke the arachnoid membrane in multiple places to promote anastomosis formation. Third, similar to the clinical EMS procedure (33), this method involves the suturing of the TM together with the edge of the DM (with 10-0 prolene) to ensure that the brain is covered tightly by TM. Fourth, after the skin is sutured, we recommend gently pressing the EMS area for 15 s to stop TM bleeding and to ensure that the brain is fully covered by TM. With all the above surgical techniques, anastomosis was observed in every 2VO + EMS rat in this study, and the mortality rate was very low; moreover, no cases of hemiplegia were observed (the lack of significant differences in the swimming speed between the groups also supports our point).

Limitations

The present study has two potential limitations. First, to reduce the number of rats needed, we did not establish more control groups to evaluate EC proliferation in the brain tissues of 2VO rats at the 2nd, 3rd, 4th, or 5th weeks. Second, our research results cannot clarify whether a part of EC proliferation was caused by physical exercise during the MWM test. Third, when we studied the effect of EMS surgery on CBP improvement, we used the CBF improvement in the ROIs (the regions with the best CBF improvement) to represent the CBF improvement in the whole cerebral hemisphere. Although this measure is representative of the overall CBF changes, it is not sufficiently rigorous. Therefore, in future work, we will further determine better methods to quantify the CBF improvement in the whole EMS-side hemisphere.

Conclusions

After establishing the 2VO + EMS rat model, the degree of EC proliferation and CBP improvement on the EMS side of the brain peaked at 4 weeks after EMS, whereas the cognitive improvement peaked in the fifth week after EMS. For basic experiments related to angiogenesis and indirect anastomosis formation, this finding will optimally ensure that the differences between groups are maximized and that significant experimental results are obtainable.

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Footnote

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