Effects of microRNA-320 on learning and memory in mice with vascular cognitive impairment caused via cerebral ischemia

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ABSTRACT

We aimed to explore microRNA (miR)-320's impacts on learning and memory in mice with vascular cognitive impairment induced via cerebral ischemia. After establishment of a cerebral small vessel disease (CSVD) cognitive impairment model, application of corresponding treatment methods was in the model mice to inject miR-320 antagomir/agomir and their negative controls to the lateral ventricles: Test of the learning and memory abilities of mice was conducted; Detection of oxidative stress, inflammation, miR-320, Vascular endothelial growth factor (VEGF) and endostatin (ES) was implemented; Taking mouse hippocampal neuron cells was to detect the cell advancement. MiR-320 was elevated in the CSVD model; MiR-320 was negatively linked with the learning and memory abilities of mice; Repressing miR-320 was available to memorably elevate the learning and memory abilities of CSVD mice; Depressing miR-320 clearly drove CSVD mouse neovascular protein VEGF, but reduced inflammation, oxidative stress response and ES; Restraining miR-320 was available to contribute to mouse neuronal cell advancement. MiR-320 mitigates the learning and memory abilities of cerebral ischemia-induced vascular cognitive dysfunction mice to a certain extent.

Introduction

Cerebral small vessel disease (CSVD) mainly covers a small blood vessel with a diameter of approximately 40-00 mm in the intracranial blood vessels, consisting of skin, capillaries, arterioles, venules and other small veins (1). CSVD is an extremely familiar cerebrovascular disease among middle-aged and elderly people and impacts almost 100% of Chinese elderly over 90 years old. (2). In the light of statistics, the present incidence of CSVD is as high as 3-8%, but its pathogenesis is uncertain (3). In clinical practice, vascular cognitive dysfunction is a familiar phenomenon in CSVD, and cognitive impairment seriously impacts the quality of life and health of patients (2). However, there is currently no reliable treatment method for the cure of vascular cognitive dysfunction induced via cerebral ischemia.

MicroRNA (miRNA) is frequently 18-25 nucleotides in length and is a superfamily composed of small noncoding RNA derived from the genome. It exists in most eukaryotes and is about 22 nucleotides in length (4). In the nervous system, numerous studies have manifested that the clearest pathological change of cerebral ischemia is the death of neurons, which induces the irreversible loss of brain function (5). As we all know, plentiful miRNA genes are involved in the modulation of cell apoptosis and

are closely linked with the occurrence of cerebrovascular diseases. For example, it has been reported that miR-183 functions in the presence, development and prognosis of cerebral ischemia/reperfusion injury in CSVD patients (6). MiR-134-5p, Foxp2, Synapsin 1 participate in the cognitive impairment of early vascular dementia rat models (7). MiR-27a-3p and miR-222-3p are available to be applied as new modulators of phosphodiesterase 3a in brain microvascular endothelial cells, and mitigate endothelial dysfunction induced via cerebral ischemia (8). Hence, it is imperative to further search for brand-new biomarker molecules and determine new targeted therapies, which will help offer new clues for the diagnosis and cure of vascular cognitive dysfunction induced via cerebral ischemia.

MiR-320 is considered to be closely linked with human health and diseases because it controls diversified momentous molecules, especially inflammatory, oxidative stress, and vascular endothelial growth factors, apoptosis genes, etc. (9). Luo reports miR-320 induces axon growth by targeting the cAMP-regulated phosphoprotein 19 (10). Liang *et al.* also testify that miR-320 is available to alleviate cerebrovascular damage by modulating Insulin-like growth factor I during cerebral ischemia/reperfusion in mice, thereby protecting the function of the nervous system (11). However, miR-320's characteristics in cerebral ischemia-induced vascular cognitive dysfunction in mice remain

ambiguous. Therefore, it was speculated that it might take on a crucial role in the treatment of vascular cognitive dysfunction induced via cerebral ischemia.

In this study, it was aimed to identify miRNAs affecting cerebral ischemia-induced vascular cognitive dysfunction and found that miR-320 was regarded as a latent candidate. To verify the hypothesis, separate injection of miR-320 antagomir/agomir was into CSVD cognitive impairment animal models to evaluate their effects on the learning and memory abilities of mice. Moreover, the inflammatory response, oxidative stress response, neovascular protein and endostatin (ES) as well as the protection of neuronal cells were observed, to provide a reliable theoretical basis for miR-320's mitigation of the cognitive dysfunction induced via CSVD.

Materials and Methods

Experimental subjects

The purchase of 50 healthy mice (male to female ratio 1: 1) was from Beijing Wanhe Technology Co., Ltd., at the age of 8-10 weeks, and weighing 320-360 g. The rearing temperature was (22-25)°C, the humidity was 50-65%, and there were 5 animals in each cage. Food and drinking water were not restricted. All animal experiments were conducted in line with the recommendations of the National Institutes of Health's *Guidelines for the Care and Use of Laboratory Animals.* Approval of this study was via the Animal Ethics Committee of Lishui Second People's Hospital.

CSVD mouse model

Modeling of all mice was via carotid artery ligation. After one week of adaptive feeding, anesthetization was via intraperitoneal injection of 10% chloral hydrate (4 mL/kg), and prostration was conducted. A 2 cm incision was made in the center of the neck, and the left common carotid artery was separated. During the operation, paying attention to avoid damage to the rat's muscles, blood vessels and nerve tissues was implemented. After the operation, the wound was sutured and the mice were kept in cages to feed. After 1 week, the right carotid artery was ligated in all patients following the same procedure described above. Test of the burrowing ability was 4 weeks after successful bilateral arterial ligation. Whether the model rat manifested an obviously different burrowing ability from the normal rat was judged to be a successful modeling (3).

Experimental grouping and administration

Assignation of the mice was into five groups in line with the random number table. No statistical difference was presented in basic information such as the age and weight of the mice, and the groups were comparable (*P* > 0.05). Ten mice were initially assigned to the sham operation group (the sham, n = 10). The remaining model mice were administered by lateral ventricle (ICV) injection: CSVD + anta-negative control (NC) (miR-320 negative antagomir), CSVD+miR-320 antagomir (miR-320 antagomir), CSVD+NC agomir (miR-320 negative agomir), CSVD+miR-320 agomir (miR-320 agomir) groups (all n = 10). Putting the back of the rat on a flat plate, placing it on a stereotactic device, and intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg oxygen perazine (0.15 mL/100 g body weight) for anesthesia were conducted. On

the grounds of the mouse brain atlas, careful drilling of two small holes in the skulls on both sides with a surgical drill. The stereotactic coordinate of ICV injection was anterior -0.8 mm, 1.5 mm lateral and -4.5 mm depth. In the CSVD model group, the dissolving of the afore-mentioned drugs was in sterile ddH2O and injection was into both sides of the lateral ventricle with a volume of 5 μl (200 pmol/mouse). When injection of the sham was with the same amount of sterile ddH2O, continuous injection of mice in each group was with ICV for 3 d. The follow-up test was carried out 7 d after the injection (7).

Morris Water Maze (MWM) test

MWM test was conducted to assess spatial memory and learning abilities. A water maze with a black circular pool (180 cm in diameter) was full of opaque water adding black edible paint while maintaining at (25 ± 2)°C. The location of the diving escape platform (20 cm diameter; 2.0 cm underwater depth) was in the center of the first quadrant. Before training, the pupil light reflex test was performed on all mice. The exclusion of mice with impaired pupil light reflection was from the experiment to prevent visual factors from affecting the test. Behavior training covered three trials a day for 6 d. In each behavioral training test, placing of mice was in water facing the transverse wall, and the permission of each rat was to find a platform within 120 s. If the platform was not found within 120 s, guiding them to the platform and allowing them to rest for at least 20 s (in this case, the escape delay was set to 120 s) were conducted. Each rat needed to rest for 15 min before the next test. On the 6th d, a space exploration test was carried out, and the hidden platform was removed. Let the mice swim freely for 90 s to determine their memory retention. After each experiment, the mice were dried. The entire trail was kept clean with water (12).

Brain tissue isolation and cell culture

Anesthetization of mice was with a dose of pentobarbital (50 mg/kg). Collection of hippocampal tissue was in time after euthanasia. Separation of brain tissue in CA1 area was from mouse cortex and hippocampal microvessel fragments. Quick separation of the brain tissue in the CA1 area was from the ice box, freeze was in liquid nitrogen, and store was at −80°C. Separation of the brain tissue of the CA1 area was from the mouse hippocampal neuron cells in each group, and the maintenance medium was replaced with sugar-free Dulbecco's Modified Eagle Medium. Placing the plate was in a sealed tank rinsed with 95% N_2 and 95% N_2 with 5% CO_2 until the oxygen concentration was less than 1%. Seal of the tank and incubation were conducted. The cells are then returned to the normal maintenance medium and incubation was under normal conditions until being harvested. In the sham, incubation of neurons was under normal conditions.

Identification of hippocampal neurons

For identifying the isolated primary mouse hippocampal neurons, the neuron morphology was identified by applying an optical microscope (BX53M, Olympus, Tokyo, Japan) on the 3rd, 5th, and 7th d. After incubation of the neurons, wash with phosphate buffer saline (0.01 mol/l, pH7.4), and fixation with 4% paraformaldehyde were conducted. After culture of the cells for 3 d, fixation of the cells was with 2 ml 4% polymethanol. Incubation of neu-

rons was with the anti-MAP-2 antibody (Abcam, ab5392, 1: 500, Cambridge, MA, USA), and the fluorescence-labeled secondary antibody on ice. Resuspension of cells was with 0.5 ml 1% polymethanol, and MAP-2 in hippocampal neurons was identified by flow cytometry (5).

Enzyme-linked immunosorbent assay (ELISA)

ELISA kit (Beyotime, Shanghai, China) was applied to detect inflammatory and oxidative stress factors. In short, anesthetization of mice was with a dose of pentobarbital (50 mg/kg). Collection of hippocampal tissue was immediately after euthanasia, and quick separation of the brain tissue in the CA1 area was from the ice box, the freeze was in liquid nitrogen, and store was at −80°C. Collection of the brain tissues of CA1 area of mice in each group and preparation of tissue homogenates were conducted. In ELISA, strictly following the steps described in the kit, drawing of a standard curve and measurement of the inflammatory factors interleukin (IL)-1β, transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α), oxidative stress factor malondialdehyde (MDA) and superoxide dismutase (SOD) were conducted. The purchase of the above kit was from Wuhan Mskbio Biotechnology Company (13).

Western blot

Western blot analysis was applied to detect ES, Vascular endothelial growth factor (VEGF), Bcl-2 and Bax in each group. To put it simply, the brain tissues of each group of mice were fully ground in the lysis solution and then subjected to ultrasound examination. After centrifugation of lysis buffer, the supernatant was absorbed and aliquoted into Eppendorf tubes. Then measurement of the protein concentration was done by ultraviolet spectrophotometry, with keeping the volume and concentration of all sample proteins equal. Then after loading and storing in a refrigerator at -80°C, extraction of the total protein and conduction of sodium lauryl sulfate-polyacrylamide gel electrophoresis were implemented. Then after electroblot of the protein in the gel onto a polyvinylidene fluoride membrane, incubation was with the primary antibodies anti-Bcl-2 (ab182858), Anti-Bax antibody (ab32503), S6RibosomalProtein (5G10) RabbitmAb (2217) (all 1: 1,000), Anti-VEGF Antibody (C-1) (sc-7269, 1: 200), and the secondary antibody goat anti-rabbit Immunoglobulin G H&L (ab150077, 1: 500, Abcam, Cambridge, MA, USA). Then scanning and quantification of the protein bands were with an Odyssey membrane scanner. The protein to be detected was corrected by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

PCR was employed to detect miR-320, VEGF and ES in the brain tissues of mice in each group. After euthanasia of the mice first, isolation of total mRNA was from the lysed hippocampus of dead mice with the RNA extraction kit. Applying PrimeScript RT kit (TaKaRaBIO, Tokyo, Japan) was for reverse transcription of 1 μg total RNA as the first-strand cDNA to detect mRNA and miRNA. The employment of SYBR Premix ExTaq"II (TaKaRaBIO, Tokyo, Japan) reagent, with GAPDH as the standard, was to evaluate the expression pattern of VEGF and ES mRNA. Determination of the relative miR-320 was by TaqManUniversalMasterMixII (Applied Biosystems, Foster City, CA, USA), with U6 as the standard. The primer sequence list is manifested in Table 1 (3).

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

Detection of cell viability was via MTT method. Simply put, after extracting the neuronal cells of each group of mice, seeding was in a 96-well plate (4×10^3 cells/well) and incubation was conducted. Separate addition of 20 μL MTT solution (5 μmg/mL) at 24, 48, 72, 96 h, seeding, and addition of 200 μL dimethyl sulfoxide to each well were conducted. Finally, the application of a V-1200 spectrophotometer was to measure the optical density (OD) value at 490 nm (14).

Flow cytometry

Apoptosis analysis was via AnnexinV Fluorescein Isothiocyanate (FITC) and Propidium Iodide (PI) Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA), and detection was by flow cytometry. In simple terms, after collection of the neurons of each group of mice, staining with 5 μL V-FITC and 5 μL PI, and final detection by flow cytometry were put into effect (15).

Statistical analysis

Data analysis was done via adopting Statistic Package for Social Science (SPSS) 20.0 (IBM, Armonk, NY, USA), with data visualization via GraphPadPrism6 (La Jolla, CA, USA). The manifestation of measurement data was as mean ± standard deviation (SD). Two-group comparison of measurement data obeying the normal distribution was

Table 1. Primer sequence.

Genes	Primer sequence (5'-3')
VEGF	Forward: 5′-CATAGCTTGAACGACG-3′
	Reverse: 5′-GCTAGGCTTCGGAGAGACC-3′
ES	Forward: 5′-TCCACCCGCTACGACCACCT-3'
	Reverse: 5′-CCATAGCCACCATCGCACCGTGG-3'
MiR-320	Forward: 5'-ACACTCCAGCTGGGAAAAGCTGGGTTGAGA-3
	Reverse: 5′-ACACTCCAGCTGGGAAAAGCTGGGTTGAGA-3′
U6	Forward: 5′-CTCGCTTCGGCAGCACA-3′
	Reverse: 5′-AACGCTTCACGAATTTGCGT-3′
GAPDH	Forward: 5′-TCCCATCACCATCTTCCA-3′
	Reverse: 5′-CATCACGCCACAGTTTTCC-3′

via adopting an independent sample t-test. One-way analysis of variance was applied in comparison among groups and post-hoc pairwise comparison was via the Least Significant Difference test. Comparison of multiple time points was via repeated measurement analysis of variance. $P <$ 0.05 emphasized obvious statistical meaning.

Results

MiR-320 was elevated in CSVD model, and learning and memory abilities of mice were declined

Detection of miR-320 was in the blood samples of the CSVD and the sham of mice. Figure 1A manifested the concentration of miR-320 in the serum of CSVD mice was up-regulated to about 160% of that in the sham.

Then evaluation of the memory and learning abilities of the two groups of mice was conducted. Among them, the shorter the escape latency and path length in navigation tasks, and the more times to reach the platform in space exploration, the better the memory and learning abilities of mice were. The results of Figure 1B-D clarified vs. the sham mice, the mice in the CSVD had apparently elevated escape latency and path length during navigation tasks, and reduced number of times to reach the platform during space exploration ($P <$ 0.05). The results affirmed mice in the CSVD had poor memory and learning abilities, which proved that the animal model was successful.

All in all, miR-320 was elevated in CSVD model, and learning and memory abilities of mice were declined. Whether the elevation of miR-320 was linked with the decline of learning and memory ability in mice still needs follow-up experiments to verify.

Depressive miR-320 elevates the learning and memory abilities of CSVD mice

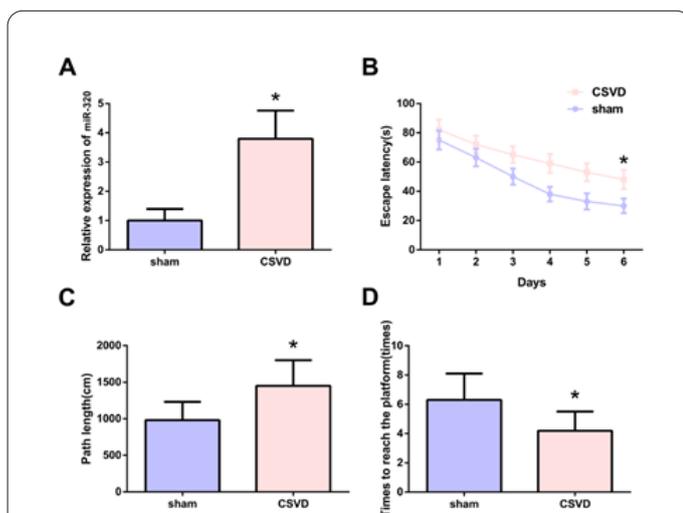For determining the effect of miR-320 on the memory and learning ability of mice. Injection of the mice in the

Figure 1. MiR-320 is elevated in CSVD model, and learning and memory abilities of mice are declined. (A) RT-qPCR method detection of miR-320 in the two groups of blood samples; (B) MWM test detection of the comparison of escape latency between the two groups; (C) MWM test detection of the comparison of path lengths between the two groups; (D) MWM experiment detection of the comparison of the number of arrivals to the platform between the two groups; The data in the figures were all measurement data, in the form of mean ± SD; * vs. the sham, n = 10, $P <$ 0.05.
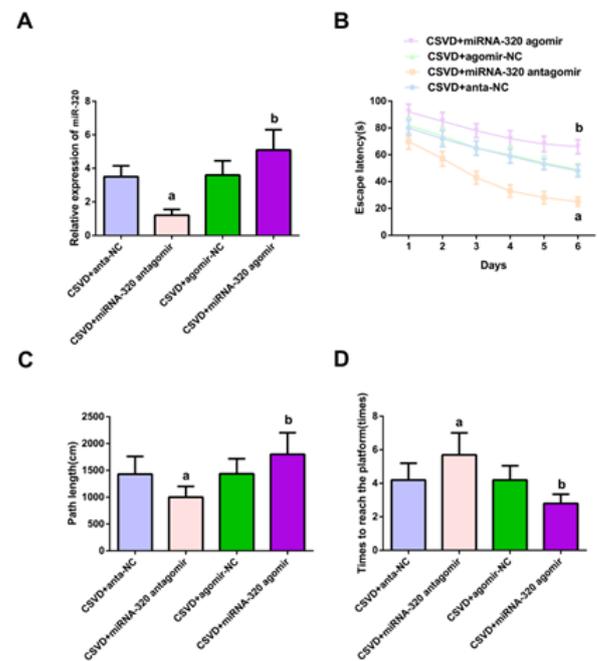

Figure 2. Depressive miR-320 clearly elevates the learning and memory abilities of CSVD mice. (A) RT-qPCR detection of miR-320 in the brain tissue of CSVD mice in each group; (B) MWM test of comparison of the escape latency of each group; (C) MWM test to compare the path length between the two groups; (D) MWM experiment detection of the comparison of the number of arrivals between the two groups; The data in the figures were all measurement data, in the form of mean ± SD; a vs. the CSVD + anta-NC, n =10, $P <$ 0.05; b vs. the CSVD+NC agomir, n =10, $P <$ 0.05.

CSVD was with miR-320 agomir/antagomir and agomir/anta-NC. Detection of miR-320 was in CSVD mice of each group. It came out (Figure 2A), vs. the NC, miR-320 was enhanced after injection of miR-320 agomir in CSVD model mice, and reduced after injection of miR-320 antagomir ($P <$ 0.05).

Then evaluation of the memory and learning abilities of CSVD mice groups was conducted. As manifested in Figure 2B-D, mice injected with miR-320 antagomir had memorably shorter escape latency and path length during navigation tasks, and clearly elevated number of times to reach the platform during space exploration, which was opposite to the results of mice treated with miR-320 agomir.

In summary, miR-320 was negatively linked with the learning and memory abilities of mice. Repressing miR-320 was available to clearly contribute to the learning and memory abilities of CSVD mice.

Down-regulated miR-320 mitigates the vascular damage caused via cerebral ischemia in CSVD mice

For exploring whether repressive miR-320 could mitigate the inflammation and oxidative stress response in mice, detection of the concentration of inflammatory response indicators (IL-1β, TGF-β1) and oxidative stress response indicators (MDA, SOD) in mouse brain tissue was conducted. As clarified in Figure 3A-D, injection of miR-320 antagomir in CSVD model mice was available to memorably reduce IL-1β, TGF-β1, and MDA, while apparently elevating SOD. Injection of miR-320 agomir in

model mice found the opposite trend. It revealed the injection of miR-320 antagomir reduced the inflammation and oxidative stress in CSVD mice after cerebral ischemia.

VEGF is highly specific, and can elevate vascular permeability, extracellular matrix degeneration, vascular endothelial cell migration, proliferation and angiogenesis (16). ES is an endogenous angiogenesis antagomir, which can restrain the growth of blood vessels (17). Detection of VEGF and ES was in the brain tissues of mice in each group. As clarified in Figure 3E-H, after miR-320 was refrained, VEGF was clearly elevated, but ES was distinctly decreased (all $P < 0.05$); after miR-320 was up-regulated, VEGF was memorably reduced, but ES was obviously augmented (all $P < 0.05$). The above experimental results all manifested down-regulation of miR-320 could contribute to neovascular protein but refrain ES in CSVD mice.

In summary, repressive miR-320 could reduce the inflammatory damage and oxidative stress process of cerebrovascular tissue, repair damaged blood vessels and accelerate vascular regeneration, to cure cerebrovascular diseases.

Neuronal cell advancement in CSVD mice in each group

For further studying the effects of miR-320 on CSVD neuronal cells, the primary mouse hippocampal neurons were first isolated. After the successful culture of the neurons, the neurons were morphologically identified, and markers of neuronal differentiation (MAP-2) were identified. The results of morphological identification clarified on the 3rd d, neuronal synapses were augmented, and the neural network gradually formed; on the 5th d, the connections of the neural network were formed in the synapses; on the 7th d, the neurons were full and matured (Figure 4A). Microtubule-associated protein 2 (MAP-2) is a sign of neuronal differentiation, and its function is controlled by phosphorylation after neuronal development and synaptic activity differentiation to maintain neuronal health (18).
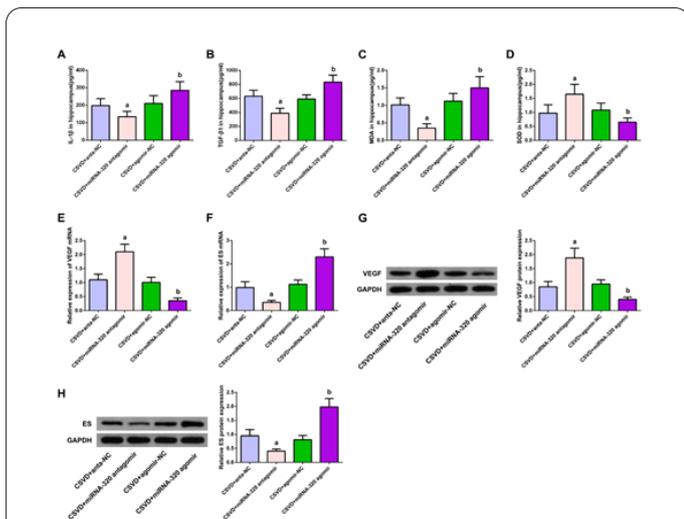

Figure 3. Down-regulated miR-320 mitigates the vascular damage caused via cerebral ischemia in CSVD mice. (A-D) Comparison of IL-1β, TGF-β1, MDA and SOD in each group; (E-H) Comparison of VEGF and ES in hippocampus of mice in each group; The data in the figures were all measurement data, in the form of mean ± SD; a vs. the CSVD + anta-NC, n =10, $P < 0.05$; b vs. the CSVD+NC agomir, n =10, $P < 0.05$.
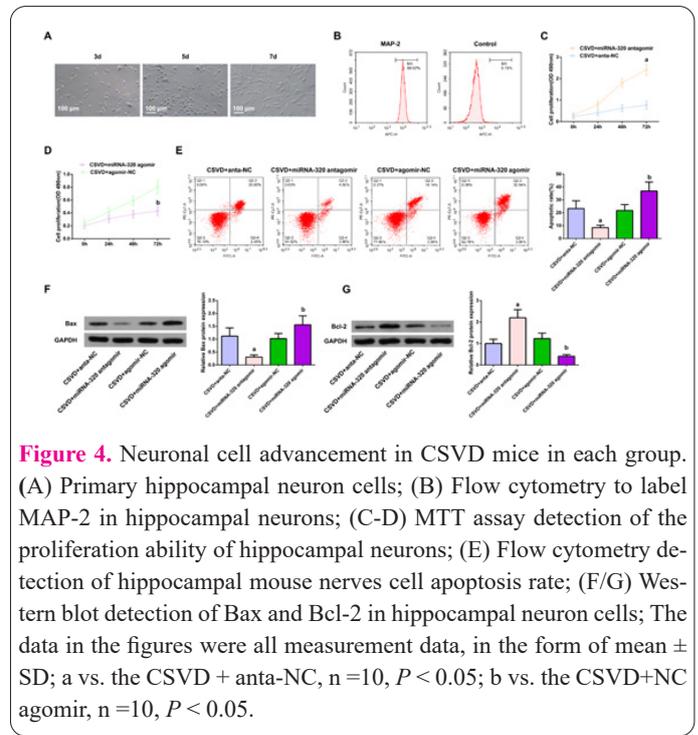

Figure 4. Neuronal cell advancement in CSVD mice in each group. (A) Primary hippocampal neuron cells; (B) Flow cytometry to label MAP-2 in hippocampal neurons; (C-D) MTT assay detection of the proliferation ability of hippocampal neurons; (E) Flow cytometry detection of hippocampal mouse nerves cell apoptosis rate; (F/G) Western blot detection of Bax and Bcl-2 in hippocampal neuron cells; The data in the figures were all measurement data, in the form of mean ± SD; a vs. the CSVD + anta-NC, n =10, $P < 0.05$; b vs. the CSVD+NC agomir, n =10, $P < 0.05$.

The result of MAP-2 labeling clarified MAP-2 was positively manifested in hippocampal neurons (Figure 4B).

Subsequently, detection of hippocampal neurons' advancement was in each group. The results of Figure 4C-E manifested after down-regulating miR-320, cell advancement was motivated; while enhancive miR-320 was inverse.

As we all know, Bcl-2 and Bax are anti-apoptotic and pro-apoptotic proteins, respectively. They participate in apoptosis by modulating the permeability of the mitochondrial outer model and are crucial modulators of apoptosis (19). Detection of Bcl-2 and Bax proteins in each group was conducted. The results of Figure 4F-G revealed elevated Bcl-2 but reduced Bax in neuronal cells after miR-320 was down-regulated.

In summary, down-regulation of miR-320 could mitigate neuronal cell advancement.

Discussion

In recent years, with the changes in the country's population structure and the rapid augment in the elderly population, the incidence of CSVD has been on the rise (2). CSVD is the main reason for functional loss, cognitive ability decline and disability in the elderly (20). Cognitive impairment seriously impacts the quality of life and health of patients. However, the current clinical lack of standardized cure and high-quality research on this vascular cognitive dysfunction caused by cerebral ischemia. Hence, discovering brand-new targeted therapies will help offer new clues for the diagnosis and cure of vascular cognitive dysfunction induced via cerebral ischemia.

MiRNA is a tiny non-coding RNA derived from the genome. Its basic physiological function is to control gene expression through RNA silencing or post-transcriptional modulation (21). Recently, elevated studies have manifested the crucial influence of miRNA in cerebrovascular diseases. This is due to their specific localization and enrichment, some of which are highly manifested in the brain, covering miR-183(6), miR-134-5p (22), miR-27a-3p and

miR-222-3p (8) etc. In this study, it was discovered a new miRNA (miR-320) impacting the cognitive dysfunction of the CSVD mouse model. The research results manifested that down-regulating miR-320 had anti-inflammatory, anti-oxidative stress response, repairing vascular damage, reducing hippocampal neuronal apoptosis, and mitigating the cognitive function of CSVD mice.

Initially, the manifestation of miR-320 in CSVD model mice and its effect on cognitive function were discussed. Detection of miR-320 in the blood samples and hippocampus of each group of mice found the elevation in CSVD model mice. Subsequently, the CSVD model mice and normal mice treated with miR-320 for loss or gain of function were tested for their learning and memory abilities. The experiments affirmed down-regulation of endogenous miR-320 could clearly shorten the escape latency of mice in navigation tasks and the path length during space exploration, and clearly elevate the number of times to reach the platform. MWM is a commonly applied method to test learning and memory ability, which can intuitively reflect the formation of learning and memory in mice (3). For example, it is previously reported that in the MWM test it is observed that miR-375-3p aggravates the cognitive impairment caused by HIBD in mice, and affects the mood and circadian rhythm of mice treated with HIBD (23). Wei *et al.* also announce that miR-150 knockdown observed in the MWM can alleviate the cognitive dysfunction of VD model mice and enhance the learning and memory ability (12). The results illustrated depressive miR-320 clearly elevated the learning and memory abilities of cognitively impaired mice.

Numerous studies have manifested cerebral ischemia can lead to free radical production, hemodynamic disorders, inflammatory response and blood-brain barrier damage, leading to secondary damage to brain tissue (5). It has previously been reported that the miR-124-5p/NOX2 axis controls the generation of ROS and the inflammatory microenvironment during brain I/R injury, and ultimately protects brain I/R injury (24). Cai also reports that miR-195-5p has anti-inflammatory, anti-apoptotic and neuroprotective effects, and can be applied to reduce vasospasm, apoptosis and secondary brain damage caused by SAH (25). Later, it was speculated that miR-320 had the effects of anti-inflammatory, anti-oxidative stress response, protection of the nervous system, etc., and could repair blood vessels, elevate vascular regeneration, and ease cognitive function. Detection of the concentration of inflammatory response indexes (IL-1β, TGF-β1) and oxidative stress response indexes (MDA, SOD) in mouse hippocampus was put into effect, and it was found that down-regulation of miR-320 could reduce IL-1β, TGF-β1 and MDA, and at the same time elevate SOD in mouse hippocampus. Inflammatory factors are involved in diversified diseases of the human body. Reducing inflammation is conducive to anti-infection and immune modulation, and can indirectly lead to changes in cytokine levels. Oxidative stress refers to the imbalance between the body's oxidation and antioxidant effects. It is a negative impact produced by free radicals and is a momentous factor in aging and disease (3). It could be seen that down-regulation of miR-320 had an active therapeutic impact on the hippocampus of CSVD mice against inflammation and oxidative stress. Detection of neovascular protein (VEGF) and ES found that down-regulating miR-320 could elevate neovascular

protein, but repress ES in the hippocampus of CSVD mice. VEGF is highly specific, can elevate vascular permeability, extracellular matrix degeneration, vascular endothelial cell migration, proliferation and angiogenesis (16). ES is an endogenous angiogenesis antagomir, which can restrain the growth of blood vessels (17). It could be seen that down-regulation of miR-320 had the effect of repairing damaged blood vessels and promoting vascular regeneration. A former study reported that miR-320 mitigates Müller cell inflammation and oxidative damage by modulating nuclear factor kappa B (NF-κB) and Nuclear factor erythroid 2-related factor 2 pathways in retinopathy (26). Therefore, it was speculated that miR-320 might mitigate hippocampal neuronal inflammation and oxidative stress, which might be linked with these two signaling pathways, which need to be further explored in subsequent studies.

Apoptosis is a momentous way of neuron death after cerebral ischemia. For further exploring whether miR-320 could protect the central nervous system of CSVD mice, taking mouse hippocampal neurons was to detect cell advancement. It was discovered that down-regulated miR-320 could contribute to neuronal cell advancement. It was previously reported that miR-146a alleviates the apoptosis of hippocampal neurons induced by microglia activation by targeting TNF receptor-associated factor 6 (27). Jia *et al.* report elevated miR-146a refrains hippocampal neuronal advancement in mice with cerebral hemorrhage by modulating autophagy (28). Zeng *et al.* also announce that miR-129-5p controls the advancement of AD hippocampal neuronal cells by targeting SIX6, thereby alleviating the nerve damage and inflammation in Alzheimer's disease mice (29). These studies further support that miR-320 can impact the apoptosis of hippocampal neurons after cerebral ischemia, thereby elevating the recovery of neurons and cognitive dysfunction. Moreover, it was also verified the effect of miR-320 on apoptosis-linked proteins in the hippocampus of mice, the detection of Bax and Bcl-2 were conducted. This study found down-regulation of miR-320 clearly refrained the CSVD-induced Bcl-2 up-regulation and Bax down-regulation. As we all know, Bcl-2 and Bax are anti-apoptotic and pro-apoptotic proteins, respectively. They participate in apoptosis by modulating the permeability of the mitochondrial outer model and are crucial modulators of apoptosis (19). Recent studies have testified that BCL-2 and Bax can be targeted by a variety of miRNAs, covering miR-195(30), miR451(31), miR-181d (32), etc. A previous study reports the occurrence of CSVD-induced cognitive impairment controls the pro-apoptotic protein Bax and anti-apoptotic protein BCL-2 by modulating the TLR4 and NF-κB pathways (33). Hence, it was speculated that miR-320 was supposed to modulate neuronal apoptosis in CSVD via targeting the two pathways, which requires further exploration in later studies.

Of course, this study still has many limitations. Although it was found that down-regulation of miR-320 could mitigate the learning and memory ability of mice with vascular cognitive dysfunction caused by cerebral ischemia, the specific therapeutic mechanism of miR-320 for CSVD-induced cognitive dysfunction remains to be explored. Moreover, clinical trials have not been conducted yet and the effect of treatment on patients cannot be evaluated. Therefore, a more complete experimental analysis is needed to be conducted in the future to solve all the above limitations.

All in all, miR-320 is elevated in the CSVD mouse model, and a repressive one is available to mitigate the learning and memory abilities of cerebral ischemia-induced vascular cognitive dysfunction mice.

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Conflict of Interests

The authors declared no conflict of interest.

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