



Increased Expression of Tight Junction Proteins and Blood-Brain Barrier Integrity in MCAO Rats Following Injection of miR-149-5p

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Cerebral ischemia is a common neurodegenerative disease in which damage to the blood-brain barrier (BBB) is the main consequence. In cerebral ischemia, the level of miR-149-5p and tight junction proteins are decreased, while the level of Calpaine is increased, finally leading to increased BBB permeability. This study investigated the effect of miR-149-5p mimic on the expression of Calpain, Occludin, and ZO-1 and the consequences of cerebral ischemia. Cerebral ischemia model was performed via middle cerebral artery occlusion (MCAO) method on female Wistar rats. Four groups of Wistar rats were studied: Sham, cerebral ischemia without treatment, Scramble miR, and miR-149-5p mimic treatment. Then, neurological defects and BBB permeability (via Evans blue staining), cerebral edema (cerebrospinal fluid percentage), and ZO-1, Occludin, and Calpain expression (by quantitative real time-PCR) were investigated. qRT-PCR results showed miR-149-5p expression decreases after cerebral ischemia induction. In addition, Occludin and ZO-1 expression significantly increased in miR-149-5p group. In contrast, Calpain expression, BBB permeability, brain water content and neurological defects were significantly decreased. It seems that the increased level of miR-149-5p exerts its protective effect on cerebral ischemia due to increasing of tight junction proteins.

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Introduction

Cerebral ischemia is the obstruction of cerebral vessels and the reduction or lack of blood supply to a part of the brain. Blood brain barrier (BBB) defect is a fundamental event following cerebral ischemia and leads to more severe injuries following cerebral ischemia and is influential in the spread of brain damage.

BBB is a barrier between plasma and brain tissue that is created by the endothelial cells of the vessel wall and types of glial cells. BBB has several roles, such as limiting transporting neurotoxic, and cells, and immune and inflammatory cytokines from the blood to the brain, maintaining the balance of ions and water, controlling the secretion of metabolites in the brain, and the level of neurotransmitters and hormones (1).

A wide range of factors plays a role in the function and structure of the BBB, among which vascular endothelial cells are the central element, and tight junction proteins are an essential structure in the connection between these cells. The main of function tight junction proteins limit bypass transfer and cross transfer of substances between brain endothelial cells. In this regard, tight junction proteins such as Occludin and Claudin (tight control over paracellular diffusion within endothelial cells) and ZO1 play an essential role in the formation and integrity of the BBB (2, 4). These tight junctions between endothelial cells are vital for maintaining the integrity of the BBB due to form paracellular permeability limits (3).

The BBB is damaged after ischemia which activates matrix metalloproteinases (MMPs) and Calpain. MMPs, especially MMP-2 and MMP-9, directly lead to increased BBB permeability by destroying tight junction proteins (6-10). Besides, studies showed Calpain could lead to the destruction of Occludin and ZO-1 in tight junctions and thus disturb the integrity of the BBB (12, 13). Calpain is Ca²⁺-activated cytosolic cysteine protease that after cerebral ischemia activated and increasing intracellular calcium leading to the production of free radicals. This process is associated with the loosening of tight junctions and damage to the structure of these junctions (12). BBB permeability lead to getting larger paracellular spaces, allowing blood flow into the brain parenchyma, finally leading to cerebral edema and neuronal death (2). Therefore, factors that prevent damage to tight junctions could protect cerebral ischemia including the increase or decrease of a specific microRNA expression.

Micro RNAs (miRs) are short single-stranded RNAs of about 21 nucleotides that regulate gene expression by destroying or preventing the translation of the target mRNA. Studies showed that some types of miRs are downregulated after cerebral ischemia, which is related to damage to the BBB (14-16). Previous studies have shown that miR-149-5p (miR-149) expression decreases after ischemia, and the increase of miR-149 improved the outcomes of cerebral ischemia (17-19).

In a previous study, we showed that miR-149-5p increases BBB integrity and reduces MMP-2, 9 levels and inflammatory cytokines after cerebral ischemia (17). However, the exact mechanism by which the reduction of MMP-2, 9 leads to increased BBB integrity has not been determined. In line with this, previous studies have shown that proteins associated with tight junctions are among the targets of MMPs and calpain. Hence, we investigated the effect of miR-149-5p mimic on tight junction expression and BBB permeability following cerebral ischemia.

Materials and methods

Animals

Male Wistar rats (240-290 grams) were kept (n=96) under controlled conditions and according to the instructions of the Council for International Organization of Medical Sciences (CIOMS) and approved through Zanzan University of Medical Sciences (Ethical No: IR.ZUMS.REC.1401.004) for working with laboratory animals. They were kept in a temperature range of 22 ± 2 and 12 hours of light and 12 hours of darkness (light from 8 am to 8 pm). Animals were randomly divided into four groups: the Sham group (intact rats) that only received surgical stress without being treated; the MCAO Model group, which was induced ischemia by the MCAO method but did not receive treatment; miR mimics who received miR-149-5p mimic intraventricularly in MCAO rats, and Negative Control group (NC) which Scramble miR (S-miR) was injected intraventricularly. miR mimic and S-miR were injected 30 minutes before the induction of MCAO. Then, each group was divided into three subgroups (n=8). In the first subgroup, neurological defects and BBB permeability were measured. In the second subgroup, the expression level of ZO-1, Calpain, and Occludin genes was measured, and in the third subgroup, cerebral edema (percentage of cere brospinal fluid) was measured (Figure 1).

MCAO modeling and intraventricular injection

The animals were anesthetized by Intraperitoneal (IP) injection of Ketamine (60 mg/Kg), and Xylazine (10 mg/kg), and middle cerebral artery occlusion surgery was performed according to the instructions provided by Lunga *et al.* (20). Briefly, after microscopic surgery, a 0-3 nylon thread was passed through the trunk of the External Carotid Artery (ECA) into the right arterial vein and until it reached the Anterior Cerebral Artery (ACA) along the Internal Carotid Artery (ICA). Contact of the thread and ACA caused the blood flow to be closed from each side to the MCA as far as 20 mm length of the thread from the trunk of the ECA. After 60 minutes of ischemia, the thread was removed and blood flow was restored. Body temperature was measured with a digital thermometer through the rectum and was maintained at around 37 degrees.

Treatment of MCAO

The coordinates (2.5 mm to the sagittal line, 1 mm to the back of the Bregma, and 3.5 mm depth about the surface of the skull) were identified by Atlas Paxinos. Then, the desired coordinates were marked and punched by a dental drill. In NC and miR mimic groups, 5 microliters of mirVana™ miRNA mimic negative control #1 and rno-miR-149-5p mimic (MIMAT0035726, MC12788, Thermo Fisher, USA) by Lipofectamine™ RNAiMax (Thermo Fisher Scientific) were respectively injected intraventricularly. The administration was performed at the desired coordinates in the right lateral ventricle at a rate of one microliter per minute using a Hamilton syringe. The stability of miR-149-5p and S-miR based on its design was 72 hours. The principles of hygiene and working with laboratory animals were considered in all work stages.

Evaluation of neurological defects in rats

Evaluation of motor defects in rats was done 24 hours after ischemia induction based on Baderson *et al.*'s guidelines (21). The score of neurological defects obtained from this study was placed in grades 0-5. Rats with a zero scale did not reveal any neurological complications. Rats with scale 1 showed complete deficiency in the front paws, considered a mild disorder. The rats that turned to the opposite side of the lesion

(left side) got a scale of 2, which is regarded as a moderate defect. The rats falling to the left side were assigned a scale of 3, considered a severe focal deficiency. Rats with a ranking of four had a low level of consciousness and could not walk alone and spontaneously. Rats that died within 24 hours after induction of ischemia, if a large part of the right hemisphere was damaged after staining, were given a scale of 5.

Measurement of BBB permeability by the concentration of EB in brain tissue

BBB integrity was evaluated by the concentration of Evans Blue (EB) in the tissue of the damaged hemisphere (right) and the healthy hemisphere and compared with each other (17). For this purpose, 30 minutes after ischemia induction (obstruction of blood supply to the brain tissue), 4 ml/kg EB 2% solution was injected into the rats through the tail vein. 24 hours after the end of the induction of ischemia (exiting the suture thread from inside the vessel), the animal was under deep anesthesia, opened the chest, and made a small slit in the right atrium. Then 250 ml of saline was injected into the animal's heart through the left ventricle until the colorless liquid perfusion was removed from the right atrium and EB cleared inside the vessels. After this stage, the animal's brain was removed, and the hemispheres were separated. The tissue of the hemispheres was homogenized in 2.5 mL of phosphate buffer, and 2.5 mL of 60% trichloroacetic acid was added to it to precipitate the proteins. The solution was vortexed for 3 minutes and cooled for 30 minutes at 4°C. In the next step, it was centrifuged for 30 minutes at a speed of 1000 rpm. Finally, the optical absorption of the upper part was measured by a spectrophotometer at an absorption of 610 nm, and concentration was calculated according to the standard curve.

Measurement of brain water content

After killing the animals with a high dose of anesthetic, the animals' heads and brains were removed. A surgical blade separated the cerebellum, pons, and olfactory bulbs and the two hemispheres, and the wet weight of the brain hemispheres (WW) was measured. Then the dry weight (DW) was measured after being placed in the oven at 120 °C for 24 hours. Finally, brain water content was calculated based on the formula $(WW-DW)/WW \times 100$.

qRT-PCR

Table1. Primer sequences for qRT-PCR.

Genes	Primer Sequences
miR-149-5p	Forward: 5'-TCTGGCTCCGTGTCTTCACTCCC-3' common reverse primer in BON microRNA QPCR Master mix kit
ZO-1	Forwar: 5'-AGGACACCAAAGCATGTGAG-3' Reverse: 5' - GGCATTCTGCTGGTTACA-3'
Occludin	Forward 5'- CCTTCTGCTTCATCGCTTCCTTA-3' Reverse 5'-CGTCGGGTTCACTCCCATTAT-3'
Calpain	Forward: 5'-CAAG-ATGCCCTGTCAACTCCA-3' Reverse:5'-CGAACCAAACACCGAA-CAAA-3'
β-actin	Forward: 5'-GCTCTGGCTCCTAGCACCAT -3' Reverse: 5'-GCCACCGATCCACACAGAGT-3'

First, total RNA was extracted using by RNX-Plus (Sinaclon, Iran). Then, the purity of the extracted RNA was determined using a spectrophotometer (nanodrop 2000, Wilmington, USA), and the reverse transcription process was performed using the instructions of the mRNA/miRNA cDNA Synthesis kit (Stem

Cell Technology Research Center (BON209002). RT-qPCR was performed using primers (Table 1) for the desired genes, and the data were analyzed using the Ct comparison method. The gene expression level was normalized by β -actin and the level of miR-149-5p by Snord (Table 1).

Statistical analyses

Data were analyzed using SPSS Statistics 24. The data obtained from qRT-PCR and EB concentration were evaluated using ANOVA and Tukey's post hoc test. The Kruskal-Wallis test also evaluated the data obtained from examining the neurological defects of rats. In all tests, $p < 0.05$ was considered significant.

Results

miR levels decreased following ischemia

In this study, it was found that 24 hours after the induction of ischemia, the level of miR-149-5p in the damaged hemisphere (right) decreased significantly, so there is a significant difference between the level of miR-149-5p MCAO Model and sham groups were observed ($P < 0.001$) (Figure 1). The use of S-miR did not considerably affect the level of miR-149-5p in the damaged hemisphere, and no significant difference was observed between the NC and MCAO groups. In contrast, the use of miR-149-5p mimic led to a significant increase ($P < 0.001$) in the miR-149-5p level. RT-qPCR was shown, miR-149-5p in MCAO treated by miR-149-5p mimic was higher than MCAO in the group (Figure 2).

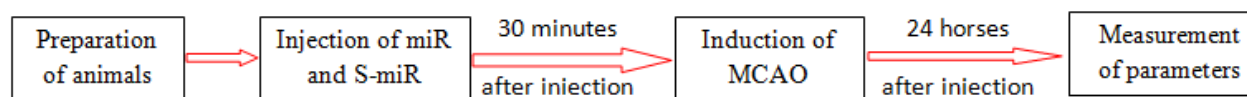


Fig.1. Study timeline

miR mimic injection reduced the neurological defects.

This study used a scale of 0 to 5 to evaluate the severity of neurological damage after MCAO Model. Induction of cerebral ischemia caused movement disorders in rats ($P = 0.003$), and miR-mimic group was reduced of neurological defects compared to the MCAO Model group ($P = 0.004$). The use of S-miR in the NC group did not reduce the movement disorders of the rats, and no significant difference was observed between the NC group and MCAO model (Figure 3).

miR mimic injection increased BBB integrity.

The higher concentration of EB in the brain tissue indicated the disruption in the BBB and its permeability. EB assay showed that EB's concentration in the rats' damaged hemisphere was increased significantly after cerebral ischemia. (Figure 4A). In addition, a significant difference was observed between the right and left hemispheres of the rats in the MCAO group ($P < 0.001$). Intraventricular injection of miR-149-5p mimic led to a considerable decrease in EB concentration in the rats' damaged hemisphere compared to the MCAO ($P = 0.002$) and NC ($P = 0.001$) group. S-miR injection could not cause a significant change in EB concentration in the brain tissue of the NC and MCAO groups. Also, there was no significant difference between the right hemispheres of the studied groups ($P < 0.001$) (Figure 4B).

Up regulating of miR mimic decreased brain edema.

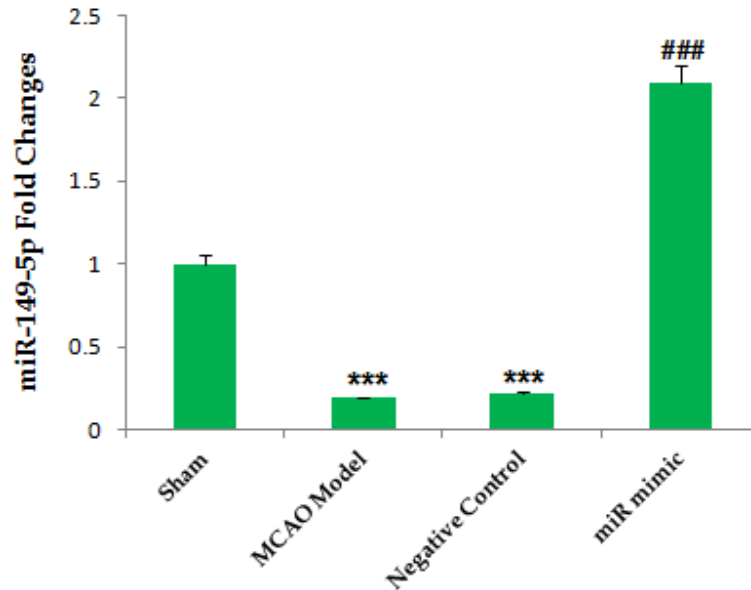


Fig.2. Changes of miR-149-5p following cerebral ischemia. Induction of cerebral ischemia led to a decrease in the level of miR-149-5p in the damaged hemisphere. The level of this miR in the model group showed a significant difference compared to the sham group ($P<0.001$). The injection of miR mimic led to a considerable increase in the level of miR-149-5p compared to the model group ($P<0.001$). S-miR injection did not affect increasing miR levels. The data are expressed as the mean \pm SD. *** $P<0.001$ vs. Sham group, ### $P<0.001$ vs. MCAO Model and NC Groups. (n=8).

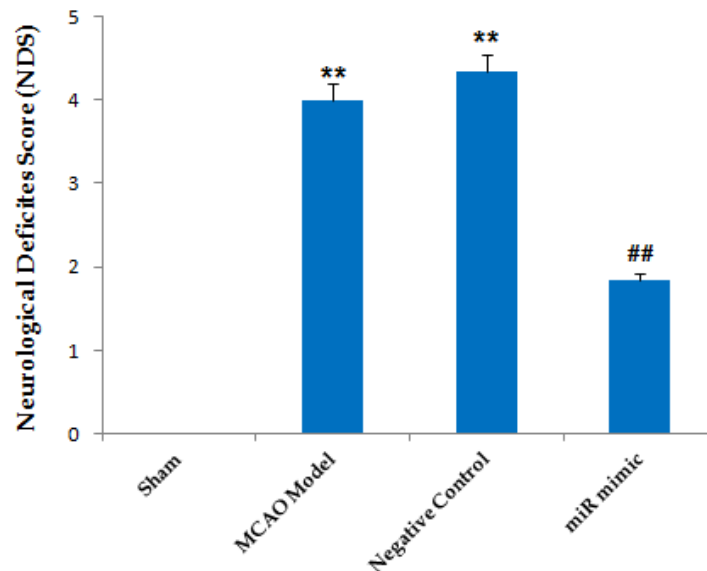


Fig.3. Comparison of neurological defects in the studied groups. Cerebral ischemia led to movement disorders in rats, and miR-149-5p mimic group significantly reduced neurological defects in miR mimic group rats compared to the model group ($P<0.001$). However, S-miR injection did not significantly change the score of neurological defects. The data are expressed as the mean \pm SD. ** $P<0.01$ vs. Sham group, ## $P<0.01$ vs. MCAO Model and NC Groups. (n=8).

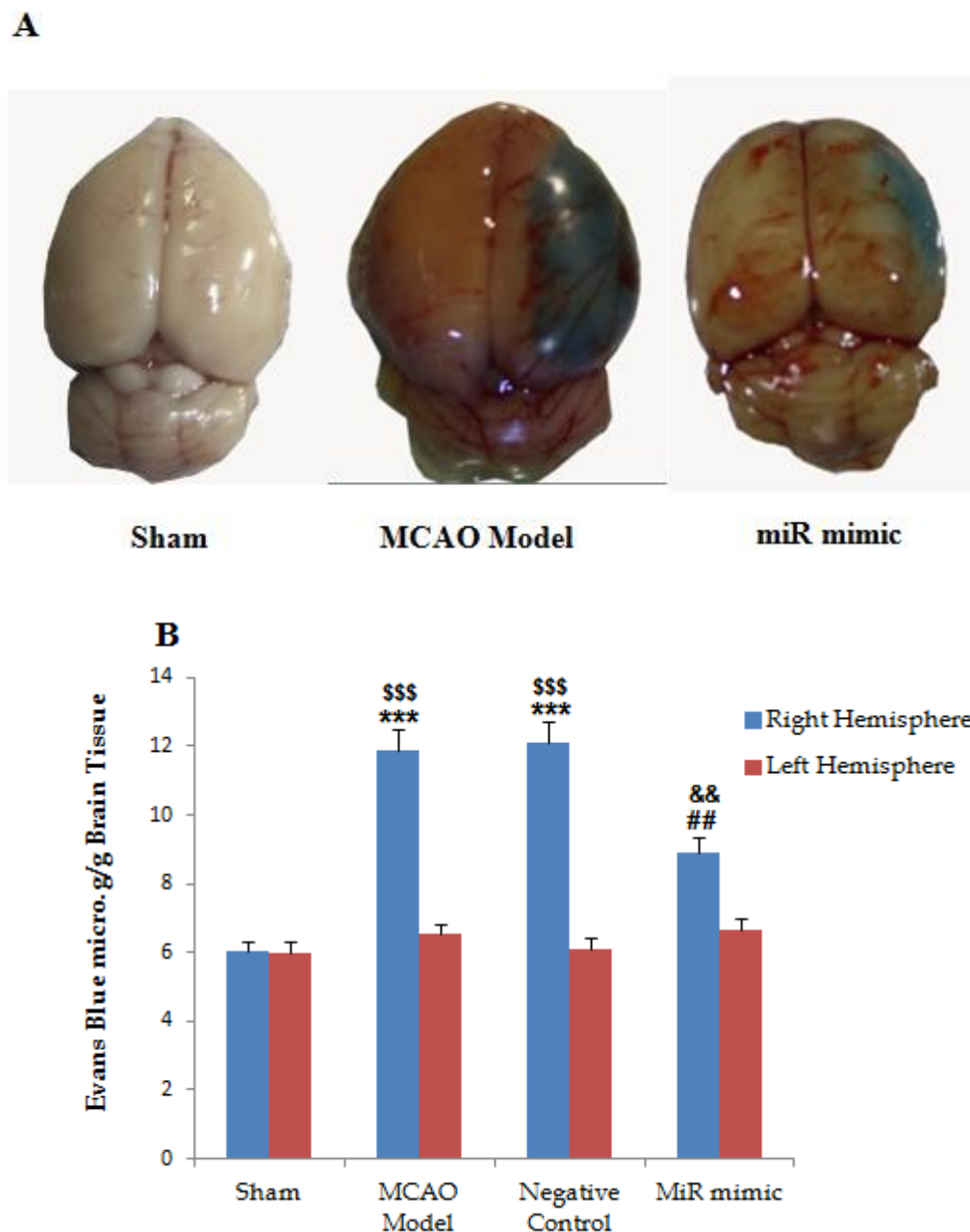


Fig.4. Brain samples containing EB in the affected hemisphere. (A) Comparison chart of EB concentration in different groups (B). and Comparing the absorption of the samples obtained from the brain hemispheres of rats with the standard graph showed that following the induction of cerebral ischemia, the concentration of EB in the damaged hemisphere increased, and there was a significant difference in the concentration of EB in the healthy and damaged hemispheres ($P=0.000$). The injection of miR mimic caused a considerable decrease in EB concentration in the damaged hemisphere compared to the damaged hemisphere of the model group ($P<0.001$). In contrast, the injection of S-miR did not have a significant effect. There was no significant difference in the concentration of EB in the healthy hemispheres of different groups ($P=0.000$). The data are expressed as the mean \pm SD. $***P<0.001$ vs. Sham group, $##P<0.01$ vs. NC Group, $&&P<0.01$ vs. MCAO Model, $$$$P<0.001$ vs. Opposite hemisphere in the same group. ($n=8$).

Cerebral ischemia led to an increase in the brain water content in the damaged hemisphere, so the percentage of brain water in the right hemisphere of the MCAO group showed a significant increase compared to the left hemisphere of the same group ($P < 0.001$). In the miR mimic group, the water content of the right hemisphere decreased significantly compared to the MCAO group ($P < 0.001$). The injection of S-miR in the negative control group did not affect the water content of the brain hemispheres, and no significant difference was observed in the percentage of brain water in the right hemisphere of the MCAO. Also, different groups observed no significant difference in brain water percentage between the healthy hemispheres (Figure 5).

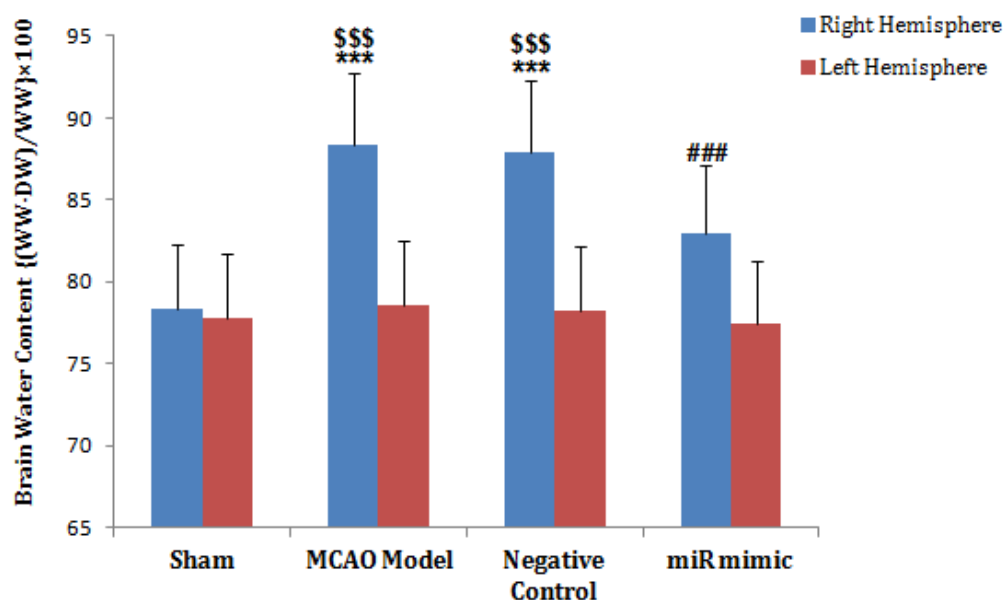


Fig.5. Comparison of brain water content in the studied groups. An increase in cerebrospinal fluid content occurred after cerebral ischemia. The injection of miR-149-5p mimic showed a significant decrease in the percentage of cerebrospinal fluid ($P < 0.001$). There was no significant difference in brain water content between the right hemispheres of the studied groups. The data are expressed as the mean \pm SD. ^{***} $P < 0.001$ vs. Sham group, ^{###} $P < 0.001$ vs. MCAO Model and NC Groups, ^{\$\$\$} $P < 0.001$ vs. Opposite hemisphere in the same group. (n=8).

The upregulation of miR increased the level of ZO-1 and Occludin.

The present study showed that the level of ZO-1 and Occludin was downregulated following ischemia induction. Also, ZO-1 and Occludin expression in the MCAO group significantly differed from the sham group ($P = 0.001$) (Figure 5). On the other hand, the level of these two genes in the group receiving S-miR did not significantly change compared to the MCAO group, which shows that S-miR does not affect the expression of these two genes. In contrast, the miR-mimic group increases significant levels of ZO-1 and Occludin in the damaged hemisphere of rats related to the MCAO group ($P < 0.001$) (Figure 6).

The upregulation of miR decreased the level of Calpain.

RT-qPCR analysis showed that the level of Calpain increases significantly after cerebral ischemia. So, it revealed a significant difference in the level of Calpain between the sham group and the MCAO group ($P < 0.001$). Calpain expression after miR-149-5p mimic injection was decreased compared to the MCAO group ($P < 0.001$), but S-miR injection was not a significant change compared to the MCAO group (Figure 7).

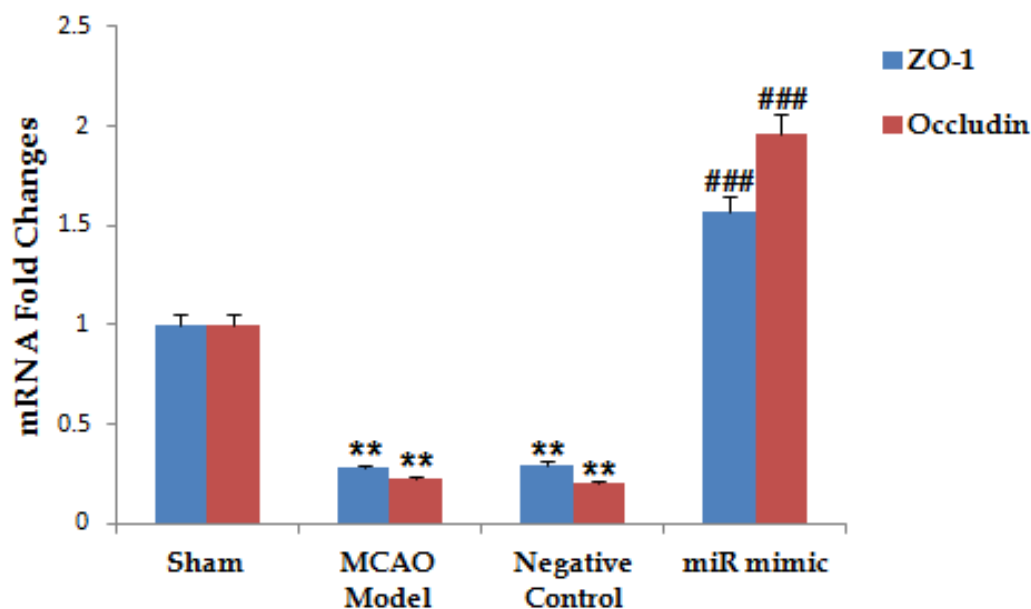


Fig.6. Comparison of ZO-1 and Occludin levels in the studied groups. Following the induction of ischemia, ZO-1 and Occludin showed a significant decrease in the damaged hemisphere of rats, and a significant difference between the levels of these two genes was revealed in the model and sham groups ($P=0.003$). The injection of miR mimic caused a considerable increase in the level of ZO-1 and Occludin compared to the model group ($P=0.004$). In contrast, the injection of S-miR did not affect the level of these two genes compared to the model group. The data are expressed as the mean \pm SD. ** $P<0.01$ vs. Sham group, ### $P<0.001$ vs. MCAO Model and NC Groups. ($n=8$).

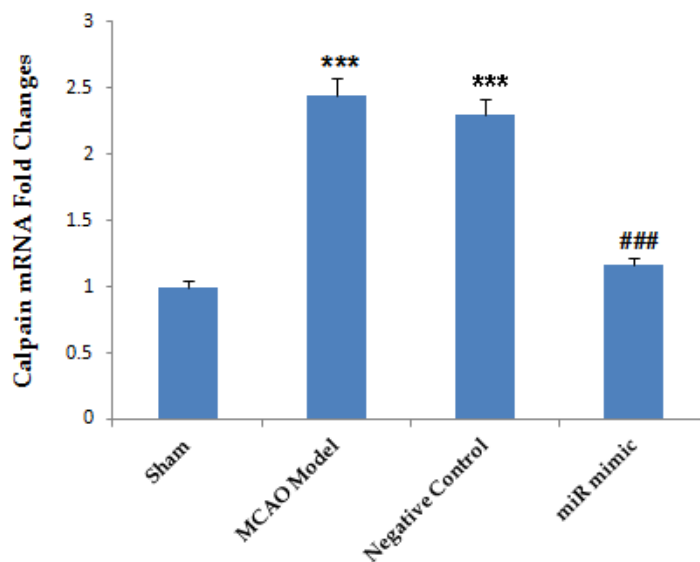


Fig.7. Calpain level changes in the studied groups. Cerebral ischemia led to a significant increase in the Calpain level in the model group compared to the sham group ($P=0.000$), and the treatment of rats with miR-149-5p mimic led to a rise in its level in the miR mimic group compared to the model group ($P<0.001$). Treatment with S-miR did not affect Calpain downregulation. The data are expressed as the mean \pm SD. *** $P<0.001$ vs. Sham group, ### $P<0.001$ vs. MCAO Model and NC Groups. ($n=8$).

Discussion

The current study showed that following the induction of cerebral ischemia, the level of miR-149-5p, Occludin, and ZO-1 decreased and the level of Calpain increased significantly compared to the sham group. The injection of miRmimic led to an increase in miR-149-5p, followed by a decrease in Calpain expression and a significant increase in Occludin and ZO-1. Also, intraventricular injection of miR-mimic decreased the water content and increased the strength of the BBB, followed by a decrease in cerebrospinal fluid content and neurological defects.

Cerebral ischemia causes a change in the level of various miRs, which is related to BBB damage. Some studies show that the level of miR-149-5p is also down-regulated in neurodegenerative disorders, including cerebral ischemia (17-19), Parkinson's (22), and Alzheimer's (23). The up-regulation of this miR expression by miR mimics improves the complications caused by neurological disorders. Several mechanisms have been reported for the neuroprotective effects of miR-149-5p, such as reduced migration of pericytes (18) and inflammatory factors, and the expression of MMPs and P53 (17, 19). Also, the present study showed injection of miR-149-5p mimic preventing the destruction of tight junction proteins.

Occludin is a common protein in TJ and consists of two extracellular rings and four transmembrane segments. Its C-terminal domain directly binds to ZO-1, an intracellular protein that facilitates the connection between Occludin and the intracellular skeleton and enables signal transduction in endothelial cells. The Occludin damage causes a disturbance in the structure and TJ function and thus increases the possibility of BBB permeability (24).

Recent studies have found that the Occludin and ZO-1 expression in blood and brain tissue directly correlates with the severity of BBB damage. Studies revealed that in cerebral ischemia the Occludin and ZO-1 expression in the brain decreased and in the peripheral blood increased indicating these factors are released from the brain into the bloodstream. At the same time, changes in Occludin and ZO-1 in the brain and blood are associated with the concentration of EB in the brain tissue, indicating relation with BBB integrity (25, 26). Occludin and ZO-1 decrease in the brain from the first moments after MCAO and then increase in a time-dependent manner (25, 26).

It has been found that MMPs and caspases can break down Occludin into 55 and 31 kDa fragments (27). Following 3-4 hours from the induction of cerebral ischemia, 55 and 31 kDa fragments of Occludin in the blood increase and remain high during the next 24 hours, associated with an up-regulation of MMPs and Occludin down-regulation in the brain (25, 26). The present study also showed that the induction of cerebral ischemia caused the down-regulation of Occludin and ZO-1. In this regard, miR-149-5p mimic treatment increased the expression of Occludin and ZO-1 and the BBB integrity. In our previous study showed the expression of MMP-2, 9 increases in the brain after cerebral ischemia. Regarding the current study result, we suggested that following the rise of miR-149-5p, the expression of these two factors decreases, finally increasing BBB integrity. One of the mechanisms by which miR-149 increases BBB integrity and reduces brain edema is preventing the destruction of Occludin and ZO-1 by decreasing the expression of MMPs. In line with this, several studies showed that MMPs, especially MMP-2, 9 directly affects TJ proteins and leads to their destruction (6-10).

Calpain is a calcium-dependent proteolytic enzyme activated following an increase in cytoplasmic calcium. It is involved in physiological processes such as cell cycle regulation, differentiation, signal transduction, and long-term potentiation. Calpain is expressed in various brain cells, including Purkinje cells, cortical neurons, and glial cells (28). The dysfunction of the sodium-potassium pump and the calcium pump disrupts the ion gradient decreasing the energy level following brain ischemia. As a result, this process leads to sodium and calcium accumulation inside the cell (29) which causes Calpain to be activated (12). Some studies show that Calpain activation is associated with damage to tight junction proteins, in a manner the use of Calpain sealer prevents the destruction of ZO-1 and thus increases the BBB integrity. ZO-1 plays a vital role in maintaining the integrity of the BBB by transmembrane binding proteins of tight junctions to actin of the cytoskeleton (30). On the other hand, calpain activation following an increase in intracellular calcium has been shown to cleaving the Occludin (12). Therefore, modulating intracellular calcium or preventing Calpain activation seems to effectively reduce the consequences of cerebral ischemia. It is known that miRs modulate the activity of Calpain by changing the concentration of intracellular calcium or the level of expression of genes (31). For example, miRs 223 and 145 are negative regulators of Calpain (32). In the present study, it was also found that the increase in the expression of miR-149-5p decreases the expression of Calpain after cerebral ischemia, thus preventing damage to tight junctions, reducing BBB permeability, and preventing the increase in cerebral edema. The precise identification of the mechanism of the effect of this miR on Calpain requires more extensive studies to determine whether miR-149 directly affects Calpain mRNA or affects the expression of other genes that led modulates intracellular calcium.

It seems that miR-149-5p can exert its protective effect on the consequences of cerebral ischemia by increasing calpain expression, reducing the damage to tight junctions, and increasing the strength of the BBB, thereby preventing the accumulation of water in the brain tissue and finally preventing neurological defects. However, more studies are needed to accurately identify the mechanism of miR-149-5p effects on Calpain and tight junction proteins.

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