European Review for Medical and Pharmacological Sciences 2020; 24: 6338-6345

MiR-199a modulates autophagy and inflammation in rats with cerebral infarction *via* regulating mTOR expression

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Abstract. – OBJECTIVE: The aim of this study was to investigate the roles of micro ribonucleic acid (miR)-199a in rats with cerebral infarction by regulating mammalian target of rapamycin (mTOR).

MATERIALS AND METHODS: A total of 36 Sprague-Dawley rats were randomly assigned into three groups, including: sham group (n=12), model group (n=12) and miR-199a mimics group (n=12). In sham group internal and external carotid arteries were exposed. The ischemia-reperfusion model was successfully established using suture embolization in the other two groups. After modeling, rats in sham group and model group were intraperitoneally injected with normal saline. However, rats in miR-199a mimics group were injected with miR-199a mimics. Following intervention for 3 d, sampling was conducted. Neurological deficit was evaluated in rats based on the Zea-Longa scoring system. Hematoxylin-eosin (HE) staining was performed to observe neuronal morphology. The expression of mTOR was detected using immunohistochemistry, and the relative expression level of tau protein was determined via Western blotting (WB). Besides, the messenger RNA (mRNA) expressions of mTOR and tau were detected by quantitative Polymerase Chain Reaction (qPCR). Finally, inflammatory factor content was measured through enzyme-linked immunosorbent assay (ELISA).

RESULTS: Model group and miR-199a mimics group exhibited a substantially higher Zea-Longa score than sham group ($p<0.05$). Compared with model group, the Zea-Longa score rose prominently in miR-199a mimics group ($p<0.05$). According to the results of HE staining, the structure of neurons in sham group was clear and intact, while the structure of neurons in model group was disordered. Meanwhile, neuronal morphology in miR-199a mimics group was significantly worse than that in model group ($p<0.05$). Immunohistochemistry results demonstrated that the positive expression level of mTOR was considerably upregulated in both model group and miR-199a mimics group in comparison with sham group ($p<0.05$). Moreover, its positive expression level in miR-199a mimics group was markedly higher that in model group ($p<0.05$). Based on the results of WB, model and miR-199a mimics groups exhibited a remarkably higher relative expression level of tau protein than sham group ($p<0.05$). However, the relative expression level of tau protein in miR-199a mimics group was prominently higher than that in model group ($p<0.05$). QPCR results manifested that the relative mRNA expression levels of mTOR and tau in model group and miR-199a mimics group were dramatically higher than those in sham group ($p<0.05$). Compared with those in model group, the relative mRNA expression levels of mTOR and tau increased significantly in miR-199a mimics group ($p<0.05$). ELISA results revealed that model group and miR-199a mimics group had prominently higher content of inflammatory factors than sham group ($p<0.05$). In addition, content of inflammatory factors in miR-199a mimics group was considerably higher than that in model group ($p<0.05$).

CONCLUSIONS: MiR-199a modulates mTOR expression to exert important regulatory effects on the autophagy and inflammation in rats with cerebral infarction.

Key Words:
Cerebral infarction, MiR-199a, mTOR, Autophagy, Inflammation.

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Introduction

Cerebral infarction is a clinically common cardio-cerebrovascular disease in the middle-aged and elderly. It is also one of the leading causes of limb motor dysfunction, speech disorders and even death in affected patients[1,2]. Due to the worsened aging of population and changes in people's lifestyles, the morbidity rate of cerebral infarction is increasing annually. Therefore, it is of great importance to explore the pathogenesis, pathological reactions and clinical treatments of cerebral infarction[3,4].

Numerous studies have confirmed that post-cerebral infarction pathological reactions are a series of complex cascade reactions. Meanwhile, a train of pathological changes in ischemic and hypoxic brain exert important effects on nerve repair and physiological function recovery after cerebral infarction[5,6]. Autophagy, one of the critical pathological reactions, plays a vital role in the repair of the nerve system after cerebral infarction. Mammalian target of rapamycin (mTOR) is a substance mediating autophagy, which has shown an important regulatory effect on cell autophagy after injury. Furthermore, it has been considered to play a pivotal role in the pathological process of autophagy after cerebral infarction[7,8].

Micro ribonucleic acid (miR)-199a is a crucial miRNA family member with important regulatory effects on multiple pathological reactions[9,10]. Therefore, the aim of this study was to explore the effects of miR-199a on rats with cerebral infarction by regulating mTOR expression.

Materials and Methods

Laboratory Animals

A total of 36 specific pathogen-free laboratory Sprague-Dawley (SD) rats aged 1 month old [Shanghai Laboratory Animal Co., Ltd., certificate No.: SCXK (Shanghai, China) 2014-0003)] were fed with normal diet and sterile filtered water daily under a 12/12 h light-dark cycle, and normal room temperature and humidity in the Laboratory Animal Center. This investigation was approved by the Animal Ethics Committee of Wuhan Hospital of Traditional Chinese Medicine Animal Center.

Experimental Reagents and Instruments

MiR-199a mimics (MCE, Monmouth Junction, NJ, USA), anti-mTOR and anti-tau primary antibodies and secondary antibodies (Abcam, Cambridge, MA, USA), immunohistochemistry and enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA), fluorescence quantitative polymerase chain reaction (qPCR) instrument (ABI 7500, Foster City, CA, USA), optical microscope (Leica DMI 4000B/DFC425C, Wetzlar, Germany), and qPCR kits (Vazyme Biotech Co., Ltd., Nanjing, China).

Animal Grouping, Treatment and Modeling

All rats were first randomly assigned into three groups using a random number table, including: sham group (n=12), model group (n=12) and miR-199a mimics group (n=12). Then, the rats were adaptively fed in the Laboratory Animal Center for 7 d for experiments.

Only the common carotid artery, external carotid artery, and internal carotid artery were exposed in sham group. Meanwhile, the cerebral infarction model was prepared in model group and miR-199a mimics group. After operation, 3 μM of miR-199a mimics were intraperitoneally injected daily in rats of miR-199a mimics group. Similarly, an equal amount of normal saline was intraperitoneally injected daily in rats of sham and model groups. Each group of rats was sampled after intervention for 3 d.

The cerebral infarction model was established as follows: the rats were first anesthetized through intraperitoneal injection of 2% pentobarbital sodium at a concentration of 0.2 mL/100 g. After successful anesthesia, the rats were fixed in the supine position. The neck was depilated, disinfected and covered with a sterile towel. Then, a 2 cm-long longitudinal incision was made at the anterior midline of the neck to carefully separate and expose the common carotid artery, external carotid artery and internal carotid artery. Subsequently, the common carotid artery and external carotid artery were ligated using silk threads, while the internal carotid artery was clamped by vascular forceps. Next, a thread was inserted from the ligation site of the common carotid artery. After releasing the vascular forceps, the thread was pushed slowly to the middle cerebral artery branch. Afterwards, the internal carotid artery was ligated again, and the thread was fixed. After washing with normal saline, the incision was sutured, followed by timing. Finally, the vessels were blocked for 90 min, and the thread was slowly drawn out.

Sampling

Following successful anesthesia, 6 rats in each group were directly sampled. Cerebral tissues

were directly taken out, rinsed with normal saline, and stored in Eppendorf (EP) tubes at -80°C for use. Besides, the remaining 6 rats in each group were fixed through perfusion for sampling. Briefly, the thoracic cavity of rats was first sheared open to expose the heart. Subsequently, 400 mL of 4% paraformaldehyde was perfused into the left atrial appendage. Next, cerebral tissues were obtained, soaked and fixed in 4% paraformaldehyde.

Zea-Longa Scoring

After intervention for 3 d, the neurological deficit of rats was evaluated using the Zea-Longa scoring method according to symptoms and performance. The Zea-Longa scoring criteria was shown in Table I.

Hematoxylin-Eosin (HE) Staining

Paraffin-embedded tissues were first made into 5 μm-thick sections, extended in 42°C warm water, mounted, baked, and prepared into paraffin-embedded tissue sections. Then, the sections were immersed successively in xylene solution for routine de-paraffinization and in gradient ethanol for hydration. Next, the resulting sections were stained in accordance with the HE staining kit. The sections were immersed in hematoxylin solution and stained for 5 min. Then, they were soaked in pure water for 10 min, and in 95% alcohol for differentiation for 5 s. Finally, the sections were transparentized with xylene for 10 s and sealed in neural resin.

Immunohistochemistry

Paraffin-embedded tissues were first made into 5 μm-thick sections, extended in warm water at 42°C, mounted, and baked. Then, paraffin-embedded tissue sections were collected, routinely deparaffinized in xylene solution, and hydrated in gradient ethanol. Subsequently, the sections were immersed in citrate buffer and subjected to complete antigen retrieval by heating repeatedly using a micro-wave oven for 3 times. During the process, they were heated for 3 min and braised for 5 min per time. After rinsing, the sections were reacted with endogenous peroxidase blocker added dropwise for 10 min. Next, the sections were rinsed, added with goat serum in drops and sealed for 20 min. After removing the goat serum blocking solution, the tissue sections were incubated with anti-mTOR primary antibody (1:200) in a refrigerator at 4°C overnight. On the next day, the sections were rinsed and added dropwise with the secondary antibody solution, followed by reaction for 10 min. After fully rinsing, the sections were reacted with streptavidin-peroxidase solution for 10 min. DAB was added dropwise for color development. Cell nucleic were counterstained using hematoxylin, and the sections were finally sealed and observed.

Western Blotting (WB)

Cryopreserved cerebral tissues were first added with lysis buffer. After 1 h of ice-bath, cerebral tissues were centrifuged at 14,000 g for 10 min. The concentration of extracted protein was quantified using the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Subsequently, the proteins were separated *via* sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After sealing with blocking buffer for 1.5 h, the membranes were incubated successively with anti-tau primary antibody (1:1,000) and secondary antibody (1:1,000). Finally, the proteins were reacted with enhanced chemiluminescent reagent for 1 min in dark to fully develop images.

Quantitative Polymerase Chain Reaction (QPCR)

Total RNA in cerebral tissues was first extracted using TRIzol reagent. Subsequently, extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA). QPCR was completed through 35 cycles of reaction at 53°C for 5 min, pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 s and annealing at

Table I. Zea-Longa scoring.

Score	Symptom
0	No neurological deficit.
1 point	Mild: unable to fully extend the right front paw when the tail is raised.
2 points	Moderate: to circle rightwards while walking.
3 points	Severe: to tumble rightwards while walking.
4 points	Unable to spontaneously walk with unconsciousness.

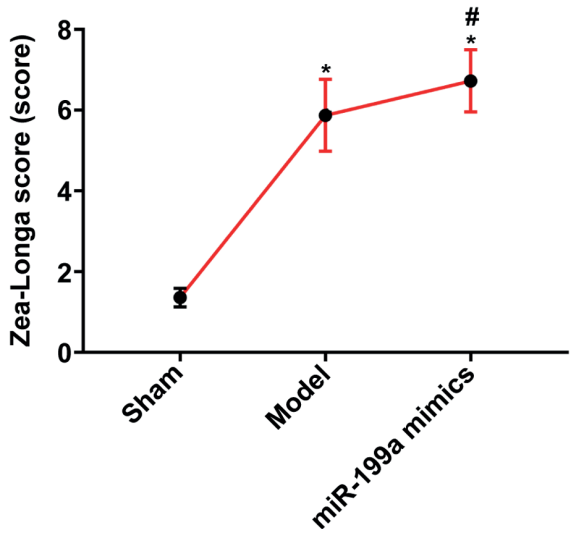

Figure 1. Zea-Longa score in each group. Note: $^{*}p<0.05$ *vs.* sham group, and $^{\#}p<0.05$ *vs.* model group.

62°C for 30 s in a 20 μL reaction system. Finally, the value of ΔCt was calculated, and differences in the expressions of target genes were analyzed. Primers used in this study were shown in Table II.

Enzyme-linked Immunosorbent Assay (ELISA)

Preserved tissues were ground and subjected to ELISA according to the instructions of the kit. Briefly, the sample and standard were first loaded into a plate. Then, the plate was added with biotinylated antibody working solution and enzyme-conjugated substance working solution, followed by washing. Finally, the standard and sample were detected at 450 nm by a micro-plate reader.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA) was adopted for statistical analysis. Measurement data were presented as mean ± standard deviation. *t*-test was performed in data meeting normal distribution and homogeneity of variance, corrected *t*-test in those fulfilling normal distribution and heterogeneity of variance, and nonparametric test in those not conforming to normal distribution or homogeneity of variance. Ranked data and enumeration data were subjected to rank sum test and chi-square test, respectively. $p<0.05$ was considered statistically significant.

Results

Zea-Longa Score

Compared with that in sham group, the Zea-Longa score was markedly elevated in the other two groups, with a statistically significant difference ($p<0.05$). Meanwhile, the Zea-Longa score increased remarkably in miR-199a mimics group compared with model group ($p<0.05$) (Figure 1).

Neuronal Morphology Observed Via HE Staining

Neurons in sham group had a clear and intact structure and abundant and more Nissl bodies, while those in model group had a disorderly structure and fewer Nissl bodies, with dissolution in some Nissl bodies. Meanwhile, neurons in miR-199a mimics exhibited a more disorderly structure and severer dissolution of Nissl bodies (Figure 2).

Immunohistochemistry Results

Positive tissues were tan. Sham group had fewer mTOR-positive tissues, while the other two groups showed more mTOR-positive tissues (Figure 3A). According to the statistical data (Figure 3B), the mean optical density of tissues positive for mTOR in model and miR-199a mimics groups was dramatically higher than that in the sham group ($p<0.05$). Moreover, the mean optical density in miR-199a mimics group was prominently higher than model group, displaying a statistically significant difference ($p<0.05$).

Table II. List of primer sequences.

Name	Primer sequences
MTOR	Forward: 5' GTTTACCGGGACATAGTCAGCA 3' Reverse: 5' CCCATTCCTTATTACAGCTTGGA 3'
Tau	Forward: 5' AACCTTCCTTGGAACGCTTGAG 3' Reverse: 5' TGCTGTTCCTCTTAACCGGAACG 3'
GAPDH	Forward: 5' ACGGCAAGTTCAACGGCACAG 3' Reverse: 5' GAAGACGCCAGTAGACTCCACGAC 3'

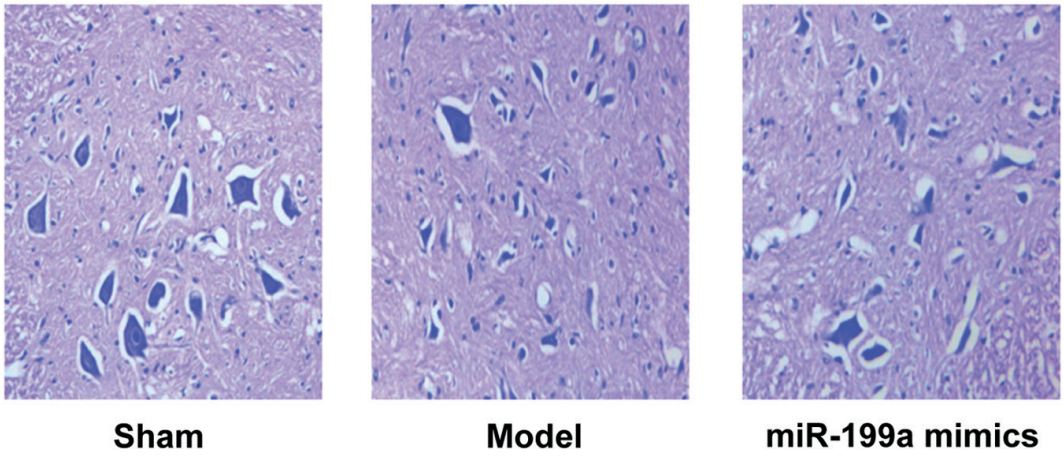

Figure 2. HE staining results in each group (magnification: 200×).

Relative Expression Levels of Related Proteins Determined Via WB

There were fewer expressed tau proteins in sham group, but more in the other two groups (Figure 4A). Statistical results revealed that the relative protein expression level of tau in model and miR-199a mimics groups was considerably higher than that in sham group, and the difference was statistically significant ($p<0.05$). Compared with that in the model group, the relative protein

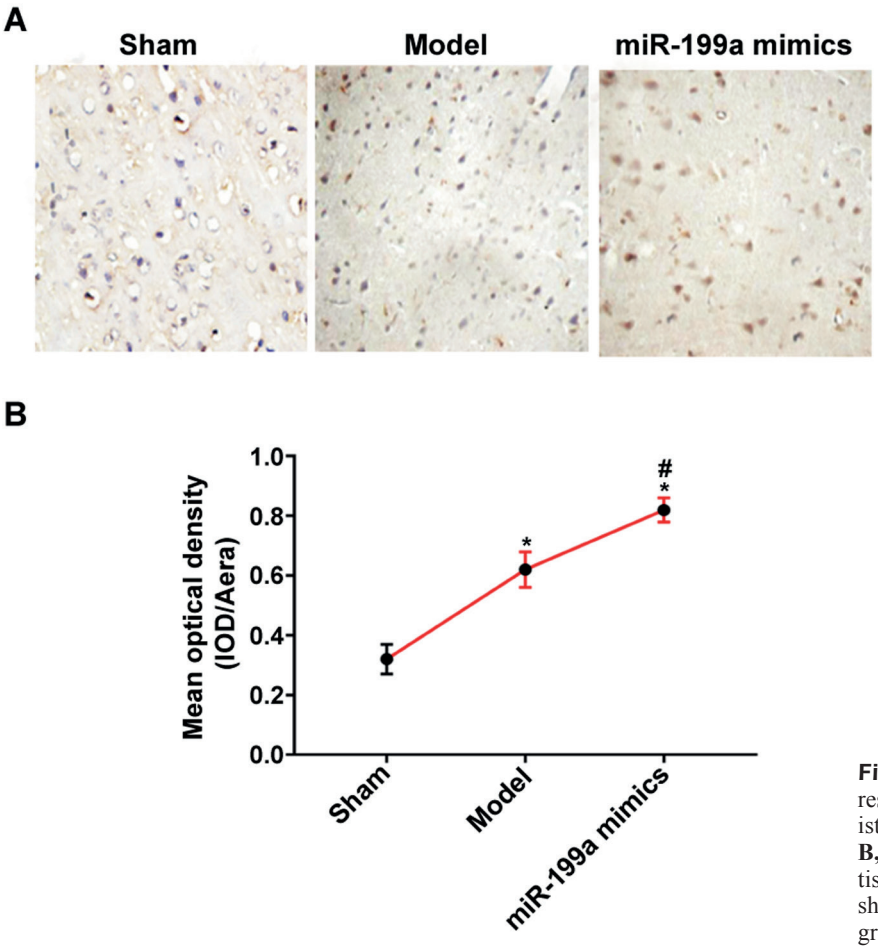

Figure 3. Immunohistochemistry results. Note: **A,** Immunohistochemistry results, (magnification: 200×). **B,** Mean optical density of positive tissues in each group. $^{*}p<0.05$ *vs.* sham group, and $^{\#}p<0.05$ *vs.* model group.

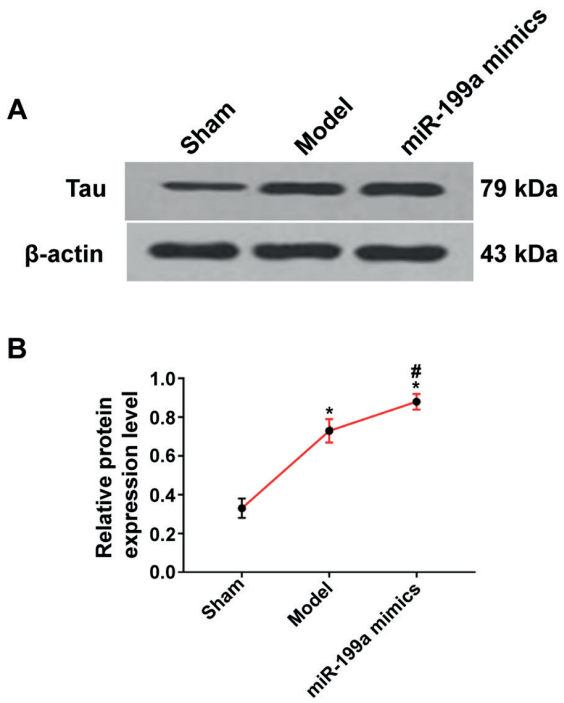

Figure 4. Protein expression detected *via* WB. Note: **(A)** WB results, **(B)** Relative protein expression levels in each group. $^{*}p<0.05$ *vs.* sham group, and $^{\#}p<0.05$ *vs.* model group.

expression level of tau remarkably increased in miR-199a mimics group compared with the model group ($p<0.05$) (Figure 4B).

Messenger RNA (mRNA) Expression Level Determined Via qPCR

The relative mRNA expression levels of mTOR and tau in model and miR-199a mimics groups were markedly higher than those in the sham group, with statistically significant differences ($p<0.05$). Furthermore, their expression levels in miR-199a mimics group were remarkably higher than those in model group ($p<0.05$) (Figure 5).

ELISA Results

In comparison with sham group, the content of interleukin-1 beta 1 (IL-1β) and IL-18 rose dramatically in the other two groups, showing a statistically significant difference ($p<0.05$). Moreover, their content in miR-199a mimics group was significantly higher than that in model group ($p<0.05$) (Figure 6).

Discussion

Cerebral infarction is a severe ischemia-hypoxia-induced cardio-cerebrovascular disease clini-

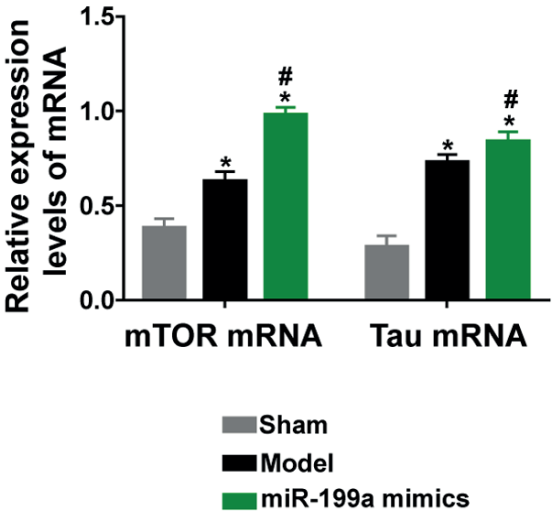

Figure 5. Relative expression levels of relevant mRNAs in each group. Note: $^{*}p<0.05$ *vs.* sham group, and $^{\#}p<0.05$ *vs.* model group.

cally, which is characterized by high morbidity, disability and mortality rates. Patients with this disease tend to suffer from sequelae of limb motor dysfunction, limb sensory disorders and speech disorders[11,12]. At present, the clinical treatment after cerebral infarction remains difficult. In recent years, continuous efforts have been made to facilitate the repair of nervous system and the recovery of limb motor function, limb sensory function and speech function after cerebral infarction. A series of complex cascade reactions, including in-

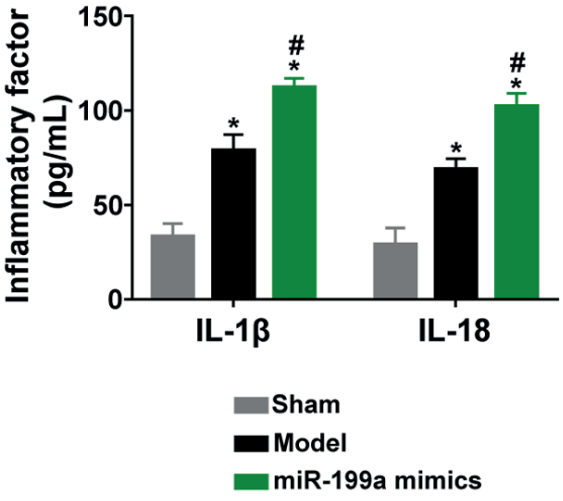

Figure 6. Inflammatory factor content in each group. Note: $^{*}p<0.05$ *vs.* sham group, and $^{\#}p<0.05$ *vs.* model group.

flammation, apoptosis, necrosis, oxidative stress responses, ion imbalance and autophagy, occur as the pathological reactions after cerebral infarction[13,14]. Stimulated by massive inflammatory factors and cytokines in ischemia-hypoxia and injury, autophagy and inflammatory responses in large numbers of neurons play important roles in nervous system repair after cerebral infarction. Inflammatory responses induce autophagy in numerous neurons to further damage the nervous system in cerebral tissues. This may lead to apoptosis and even necrosis in plenty of neurons, seriously affecting the repair of nervous system after cerebral infarction and the recovery of various physiological functions in patients[15,16]. Therefore, it is of great significance to explore the pathological mechanisms of post-cerebral infarction inflammation and autophagy for elucidating the related mechanisms of pathological reactions after cerebral infarction.

mTOR is one of the important substances modulating and mediating autophagy in organisms. The formed mTOR signaling pathway serves as a pivotal player in autophagy as well[17,18]. Injuries and ischemia-hypoxia enable large numbers of inflammatory factors and cytokines to activate mTOR in the mTOR signaling pathway and substantially up-regulate its expression level. This may further activate the downstream autophagy-associated tau protein, ultimately promoting autophagy[19,20]. The results of the present study corroborated that mTOR had a notably aberrant expression in the injured zone after cerebral infarction. This suggests that the mTOR signaling pathway is activated, and abnormally high expressed mTOR is implicated in related pathological processes after cerebral infarction. In the meantime, significantly increased tau expression and inflammatory factors were observed after cerebral infarction. All these findings imply that autophagy and inflammation occur in massive neurons after cerebral infarction. Furthermore, the abnormal expression of mTOR may have an important regulatory effect on the aberrantly high expression of tau protein and inflammatory factors.

Conclusions

Based on the findings in the present study, miR-199a considerably facilitated the expressions of mTOR, tau protein, and inflammatory factors in cerebral tissues of rats with cerebral infarction and weakened the nerve function of limbs. It can be inferred that miR-199a has an important regulatory effect on mTOR. Shortly, miR-199a regulates mTOR expression to modulate the autophagy and inflammation in rats with cerebral infarction.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Yokokawa R, Akino H, Ito H, Zha X, Yokoyama O. Nerve growth factor release from the urothelium increases via activation of bladder C-fiber in rats with cerebral infarction. Neurourol Urodyn 2017; 36: 1448-1455.
2) Sveinsson OA, Kjartansson O, Valdimarsson EM. [Cerebral ischemia/infarction - epidemiology, causes and symptoms]. Laeknabladid 2014; 100: 271-279.
3) Zhang C, Zhao S, Zang Y, Gu F, Mao S, Feng S, Hu L, Zhang C. The efficacy and safety of Dl-3n-butylphthalide on progressive cerebral infarction: a randomized controlled STROBE study. Medicine (Baltimore) 2017; 96: e7257.
4) Yamamoto N, Satomi J, Yamamoto Y, Shono K, Kanematsu Y, Izumi Y, Nagahiro S, Kaji R. Risk factors of neurological deterioration in patients with cerebral infarction due to large-artery atherosclerosis. J Stroke Cerebrovasc Dis 2017; 26: 1801-1806.
5) Zhang J, Wang H, Yang S, Wang X. Comparison of lipid profiles and inflammation in pre- and post-menopausal women with cerebral infarction and the role of atorvastatin in such populations. Lipids Health Dis 2018; 17: 20.
6) Isahaya K, Yamada K, Yamatoku M, Sakurai K, Takaishi S, Kato B, Hirayama T, Hasegawa Y. Effects of edaravone, a free radical scavenger, on serum levels of inflammatory biomarkers in acute brain infarction. J Stroke Cerebrovasc Dis 2012; 21: 102-107.
7) Jung CH, Ro SH, Cao J, Otto NM, Kim DH. mTOR regulation of autophagy. FEBS Lett 2010; 584: 1287-1295.
8) Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, Zlotchenko E, Scrimgeour A, Lawrence JC, Glass DJ, Yancopoulos GD. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nat Cell Biol 2001; 3: 1014-1019.
9) Lee YB, Bantounas I, Lee DY, Phylactou L, Caldwell MA, Uney JB. Twist-1 regulates the miR-199a/214 cluster during development. Nucleic Acids Res 2009; 37: 123-128.
10) Ding G, Huang G, Liu HD, Liang HX, Ni YF, Ding ZH, Ni GY, Hua HW. MiR-199a suppresses the hypoxia-induced proliferation of non-small cell lung cancer cells through targeting HIF1alpha. Mol Cell Biochem 2013; 384: 173-180.

11) Vergouwen MD, Ilodigwe D, Macdonald RL. Cerebral infarction after subarachnoid hemorrhage contributes to poor outcome by vasospasm-dependent and -independent effects. Stroke 2011; 42: 924-929.

12) Culebras A, Rotta-Escalante R, Vila J, Dominguez R, Abiusi G, Famulari A, Rey R, Bauso-Tosselli L, Gori H, Ferrari J, Reich E. Triflusal vs aspirin for prevention of cerebral infarction: a randomized stroke study. Neurology 2004; 62: 1073-1080.

13) Zhang Y, Liu G, Wang Y, Su Y, Leak RK, Cao G. Procalcitonin as a biomarker for malignant cerebral edema in massive cerebral infarction. Sci Rep 2018; 8: 993.

14) Dore S, Otsuka T, Mito T, Sugo N, Hand T, Wu L, Hurn PD, Traystman RJ, Andreasson K. Neuronal overexpression of cyclooxygenase-2 increases cerebral infarction. Ann Neurol 2003; 54: 155-162.

15) Zhang C, Zhao S, Zang Y, Gu F, Mao S, Feng S, Hu L, Zhang C. The efficacy and safety of Dl-3n-butylphthalide on progressive cerebral infarction: a randomized controlled STROBE study. Medicine (Baltimore) 2017; 96: e7257.

16) He X, Li DR, Cui C, Wen LJ. Clinical significance of serum MCP-1 and VE-cadherin levels in patients with acute cerebral infarction. Eur Rev Med Pharmacol Sci 2017; 21: 804-808.

17) Osborne LR. Caveat mTOR: aberrant signaling disrupts corticogenesis. J Clin Invest 2010; 120: 1392-1395.

18) Fu L, Huang L, Cao C, Yin Q, Liu J. Inhibition of AMP-activated protein kinase alleviates focal cerebral ischemia injury in mice: interference with mTOR and autophagy. Brain Res 2016; 1650: 103-111.

19) Tu L, Wang Y, Chen D, Xiang P, Shen J, Li Y, Wang S. Protective effects of Notoginsenoside R1 via regulation of the PI3K-Akt-mTOR/JNK pathway in neonatal cerebral hypoxic-ischemic brain injury. Neurochem Res 2018; 43: 1210-1226.

20) You H, Li T, Zhang J, Lei Q, Tao X, Xie P, Lu W. Reduction in ischemic cerebral infarction is mediated through golgi phosphoprotein 3 and Akt/mTOR signaling following salvianolate administration. Curr Neurovasc Res 2014; 11: 107-113.