

Original Article

The mechanism of Let-7a regulating MKP1 involved in neurons cell ischemia-reperfusion injury

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Abstract: To observe the expression of let-7 and MKP1 in ischemia and reperfusion in mice brain tissue, and explore the relationship between let-7 and MKP1 on the basis of animals and cells. The cerebral ischemia and reperfusion model was constructed, let-7a, MKP1 expression levels in brain tissue were detected by RT-PCR and Western blot at different time points. By tail vein injection of let-7a inhibitor, the expression of let-7a was inhibited, then detected MKP1 expression by Western blot using neurological scores evaluated neurological function, and nerve cell apoptosis was observed by TUNEL staining. At the same time RT-PCR was used to detect the expression of inflammatory cytokines IL-6 and TNF- α in. In cultured PC12 cells, MKP1 gene and protein expression were measured and cell apoptosis was observed using TUNEL staining after over-expression or inhibition of let-7a, RT-PCR detected the expression of pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2. The results showed that let-7a highly expressed in the reperfusion brain tissue, Simultaneously, after tail vein injection, MKP1 expression significantly increased, mice neurologic impairment score decreased, brain tissue inflammatory cytokines IL-6 and TNF- α also significantly lower, and the degree of neuronal apoptosis significantly reduced; cell experiments suggested that transfection of let-7a analogs could down regulate the expression of MKP1 gene and protein in PC12 cells, promote the expression of pro-apoptotic protein Bax and inhibit anti-apoptotic protein Bcl-2, thereby promoting apoptosis. Transfection of let-7a inhibitor could promote the MKP1 expression of genes and proteins, inhibit the expression of pro-apoptotic protein Bax and promote anti-apoptotic protein Bcl-2, thereby reducing apoptosis of PC12 cells. Let-7a targeted regulated MKP1, and involved in MAPK signaling pathway, which leads to Inflammation and Apoptosis of Nerve cells reperfusion. This study provided a new direction for the study of cerebral ischemia and reperfusion basic clinical treatment.

Keywords: Let-7a, MKP1, ischemia-reperfusion injury

Introduction

Ischemic stroke is a common and frequently occurring disease in clinic, and showing an increased incidence rate and mortality rate year by year, as well as a trend of youthization. For treatment, blood supply to the ischemic area should be restored as soon as possible, oxygen and nutrients should be provided, and metabolites should be eliminated [1, 2]. However, it was found that the recirculation may aggravate the tissue damage, i.e., the ischemic reperfusion injury (IRI) [3], the mechanism of which has not been fully elucidated at present. Previous experiments showed that IRI may be associated with the generation of oxygen free

radicals, mitochondrial damage induced by calcium overloading, inflammatory reaction and apoptosis, wherein the inflammation reaction and apoptosis play important roles in IRI, and they are the key links of leading IRI nerve cell damage [4]. Therefore, inhibiting the inflammatory reaction and reducing the cellular apoptosis are the key factors to improve the prognosis of ischemic stroke.

In the past, MicroRNAs (miRNAs) is a hot topic in the study of tumor pathogenesis, with its gradual deepening study in the ischemic stroke, the discovery of miRNAs provides a new direction for the study of IRI [5-7]. miRNAs are small molecular non coding single-stranded RNAs

widely spread in the eukaryote, with the length of about 17-25 nucleotides, they can specifically recognize and pairing with target mRNA 3'-non coding region, regulate the post transcriptional gene expression, and participate in the physiological and pathological processes of cell proliferation, differentiation, apoptosis and so on [8]. Previous studies have confirmed that Let-7a is a tumor suppressor, but its expression and mechanism in cerebral IRI are poorly studied at present. Recent studies have found that the expression of let-7a in cerebral ischemic injury is increased, and aggravates the damage of nerve cell [9]. Mitogen activated protein kinase phosphatase 1 (MKP1) is mainly distributed in the nucleus. As a member of mitogen activated protein kinase phosphatases (MKPs) family, it mainly plays an important role in the dephosphorylation of mitogen activated protein kinases (MAPKs), and is involved in cell growth, division, differentiation and apoptosis [10]. It was found in the study that MKP1 is a stress protein. It can inhibit apoptosis by regulating the c-Jun amino terminal kinase (JNK) and p38, and play the protective effect by reducing the nerve cell injury [11].

In this subject group, the expression level of let-7a, and MKP1 were observed by the construction of a murine cerebral IRI model; subsequently, the let-7a expression was inhibited, the MKP1 expression was detected, the nerve function was evaluated, and the cellular apoptosis was observed in the animal model. At the same time, the expressions of inflammatory factors IL-6 and TNF- α were detected. Detected the expression variance of MKP1 and apoptosis-related factors, and observed the cellular apoptosis in PC12 cells with let-7a was over expressed or inhibited. In this study, the interaction between let-7a and MKP1 in the occurrence and development of IRI was studied in vivo and in vitro, which provided the basis for searching for the new target of IRI in clinic.

Materials and methods

Materials

60 male Balb/C mice each weight at (22 \pm 3) g were purchased from Beijing Hua Fukang Biotechnology Co., In. Rabbit anti mouse MKP1 antibody was purchased from Cell Signaling Technology companies in the United States; β -actin antibody was purchased from Santa Cruz

company; Tunel staining kit was purchased from Wuhan Boshide Biological Engineering Co., Ltd.; Reagents for RT-PCR (RNA extraction reagent, reverse transcription kit and SYBR Kit) were purchased from Takara Co., Ltd.; The RT-PCR primers of MKP1, IL-6, TNF- α and GAPDH were purchased from Shanghai Invitrogen Biotechnology Co., Ltd. PC12 cells were purchased from Shanghai Jimian Biological Technology Co., Ltd., 1640 medium and fetal calf serum were purchased from Hyclone company in the United States, dual-antibiotics of Penicillin and streptomycin and trypsin were purchased from the GIBCO company in the United States; lipofectamine™ 2000 transfection reagent was purchased from Invitrogen company in the United States, Let-7a inhibition agent for animal (micr OFFTM mmu-let-7a-1-3p antagomir), forward and reverse primers of Let-7a and reverse transcriptional primers of RT-PCR, the analogues, inhibitor and the corresponding negative control of Let-7a for cell transfection were purchased from Guangzhou Reibo Biological Technology Co., Ltd.

Animal grouping and Model

Balb/C mice were bred in the cage at (22 \pm 2) $^{\circ}$ C, 12 hours in light and 12 hours in dark, respectively, all of them can drink and eat freely. Mice were randomly divided into sham operation group (n=10), model group (n=34) and Let-7a inhibitor group (n=10). The focal cerebral ischemia model of middle cerebral artery occlusion (MCAO) was constructed according to the reference [12], mice were ischemic treated for 2 h, and reperfused for 12 h, 24 h, 36 h and 48 h. Let-7a inhibitor (20 mg/kg) was injected through the murine tail vein for Let-7a inhibitor group when reperfused, at a dose of 0.1 ml for each time. The normal saline with the same volume to the inhibitor agent was injected through the murine tail vein for the model group. After reperfusion, the nervous system scores were evaluated. Intraperitoneal injected hydrated chloral (3 ml/kg), took the cerebral tissue to carry out the corresponding experiment.

Nervous system ratings

The nervous system was scored with the more general international scoring method Longa [13], including 6 grades 5 points: if there is no neurological deficit symptoms in mice,

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Table 1. Primer sequences of the target gene

Gene	Upstream primer	Downstream primer
Let-7a	CGGAGTCCCATCGGCACCAAGACCGACTGC	TCTGTCCACCGCAGATATTACAGCCACTTC
U6	CTCGCTTCGGCAGCACA	AACGCTTACGAATTTGCGT
MKP1	GAGCTGTGCAGCAAACAGTC	AGCCATGTACGTAGCCATCC
IL-6	GGAGGCTTAATTACACATGTT	TGATTTCAAAGATGAATTGGAT
TNF- α	TTCGAGTGACAAGCCTGTAGC	AGATTGACCTCAGCGCTGAGT
Bax	TGCAGAGGATGATTGCTGAC	GATCAGCTCGGGCACTTTAG
Bcl-2	GTACCTGAACCGGCATCTG	GCTGAGCAGGGTCTTCAGAG
GAPDH	GCAGTGGCAAAGTGGAGATTG	TGCAGGATGCATTGCTGACA

scored 0 point; carry the mouse tail and put the head down, if the right forepaw unable to completely stretch, scored 1 point; When the mice were walking, if they turn round to right side when walking, scored 2 points; if the mice dump to the right side, and difficult in walking, scored 3 points; unable to walk and falls in consciousness scored 4 points; death scored 5 points. Scored 1-3 points represent the model construction is successful.

Cell culture and transfection

PC12 cells were cultured in 1640 medium, all the culture medium contained 10% fetal bovine serum, 1% dual-antibiotics, and were placed in 5% CO₂ incubator at 37°C. Passaged the cells with 1:2 or 1:3 dilution ratio by the routine 0.25% trypsin digestion when the cellular confluence reached 90%, and the cells in the logarithmic growth phase were used for experiment. An in vitro model of cell ischemia reperfusion was constructed with PC12 cell, which was oxidatively damaged by H₂O₂ [14]. Cells were plated in 6-well plates before transfection, and transfected the cells with Let-7a analogs and inhibitors according to the instruction of lipofectamine™ 2000 when the cellular confluence reached 50%-70%. RNA was extracted after transfection for 24 h, and the expression variance of Let-7a were verified by RT-PCR. Cell proteins were extracted after 48 h, the expression of MKP1 was detected by Western Blot; at the same time, Tunel staining was used to observe the cell apoptosis.

Quantitative RT-PCR (RT-PCR)

The mice in each group were treated at the corresponding time points after modeling or transfection. After PBS washing, 1 ml Trizol was added to the cerebral tissues and fully

ground, the total RNA was extracted by phenol chloroform method, and the purity and concentration of tissue RNA were detected by ultraviolet spectrophotometry. RNA was reverse transcribed into cDNA with reverse transcription kit. Finally, the expression of target genes of Let-7a, MKP1, IL-6, TNF- α , Bax, Bcl-2 and GAPDH were detected by RT-PCR kit. The specific primer sequences were shown in **Table 1**. RT-PCR reaction was completed by the United States ABIstepone type real-time fluorescence quantitative PCR instrument, the Ct value and copy number were calculated by the software automatically. RT-PCR reaction conditions: 95°C for 20 sec pre denaturation, 95°C 5 sec, 60°C for 30 sec, amplified for 45 cycles. Taking GAPDH as the internal reference, the relative expression of the target gene and the internal reference were analyzed, and the results were represented with $2^{-\Delta\Delta Ct}$.

Western blot detection

The brain tissue was cut into pieces or the cells were collected, lysate was added in for complete lysis, and the total protein in the supernatant was extracted after 15 minutes of centrifugation at 4°C, stored at -80°C for subsequent usage. BCA method was used to determine the concentration, and 20 μ g of supernatant protein was taken for SDS-PAGE electrophoresis, then the protein was transferred to the NC membrane with 300 mA constant current. The film was blocked with 5% skim milk powder for 2 h, and incubated with the first antibody MKP1 (1:1000), GAPDH (1:2000), respectively, at 4°C over night. After TBST rinsing, the corresponded HRP second antibody (1:5000) was added onto the membrane, the membrane was washed after incubation at room temperature for 2 hours, ECL color development on target protein, the optical density of the band

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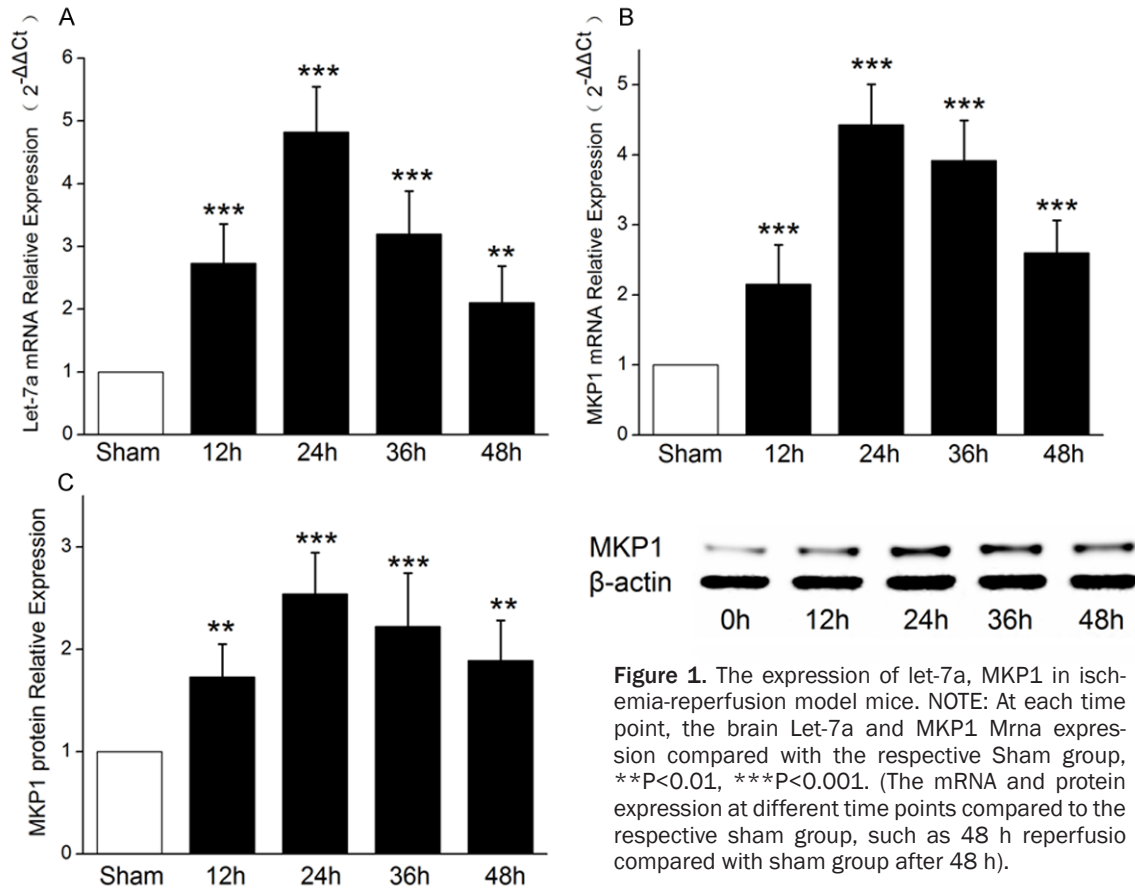


Figure 1. The expression of let-7a, MKP1 in ischemia-reperfusion model mice. NOTE: At each time point, the brain Let-7a and MKP1 Mrna expression compared with the respective Sham group, **P<0.01, ***P<0.001. (The mRNA and protein expression at different time points compared to the respective sham group, such as 48 h reperfusion compared with sham group after 48 h).

was analyzed with the software of gel imaging system. Taking actin as an internal reference, the relative expression of MKP1 protein was calculated.

Tunel staining to detect cell apoptosis

The model mice were sacrificed and the brain tissue was paraffin embedded and sectioned. Select 3 sections from each specimen for fluorescent staining; Operate strictly according to the instructions of the kit, and dewax the paraffin sections by soaking them in different solutions. Add TdT enzyme buffer and reaction solution for 1 h, washed the sections and terminated the reaction, washed the sections with PBS three times, developed with DAB solution for 5 min at room temperature, methyl green counter stain for 5 min, 100% n-butyl alcohol washed three times, finally, the sections were dehydrated, mounted and dried. Observed under the optical microscope and performed statistics, the percentage of positive Tunel staining cells (%) were calculated.

Statistical methods

The experimental results were analyzed by using SPSS 20 statistical analysis software, and were represented with mean ± standard deviation ($\bar{x} \pm s$). T test was used for inter-group comparison, and the difference was considered to be of statistical significance when P<0.05.

Results

The expression of let-7a and MKP1 in model mice

RT-PCR showed that the expression of Let-7a in brain tissue of mice with cerebral ischemia and reperfusion varied with the extension of time, with a certain trend; the let-7a gene expression reached its peak (4.82 ± 0.73) at 24 h after reperfusion, then decreased gradually, and was still higher than that in the sham operation group (1.03 ± 0.20) after 48 h (2.10 ± 0.58) (Figure 1A). Similarly, the expression of MKP1

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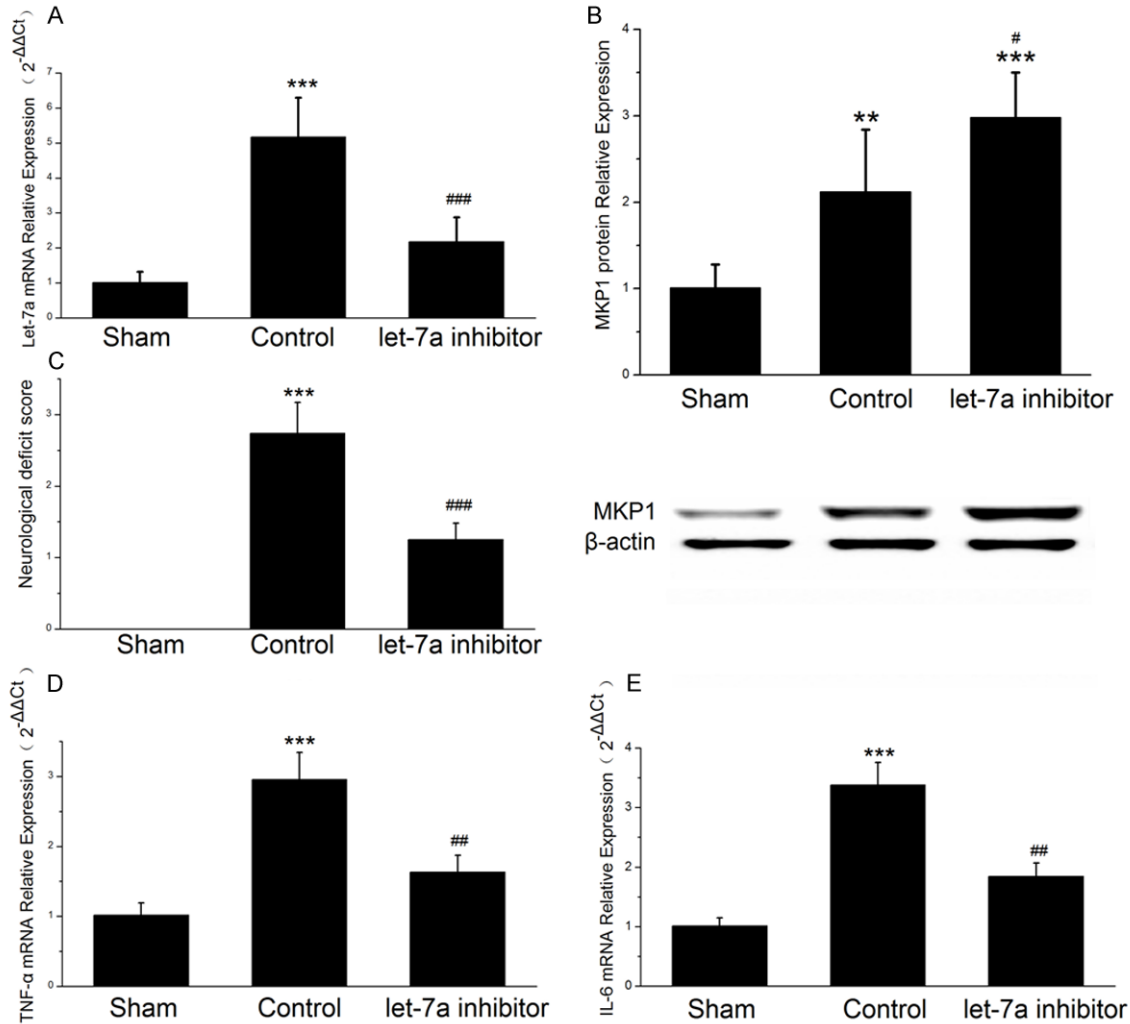


Figure 2. Let-7a inhibitor effect MKP1, the nervous system rates and inflammatory response. NOTE: A. Let-7a mRNA relative expression; B. MKP1 protein relative expression; C. Neurological deficit score; D. TNF- α mRNA relative expression; E. IL-6 mRNA relative expression. Control group compared with the Sham group, ** $P < 0.01$, *** $P < 0.001$; Let-7a inhibitor group compared with Control group, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$.

gene also showed a certain trend, reaching its peak (4.43 ± 0.58) at 24 h, and then gradually decreased (Figure 1B). Western Blot confirmed the same result: the MKP1 protein expression reached its peak (2.54 ± 0.40) at 24 h after reperfusion, and then decreased gradually (Figure 1C). Therefore, mice experienced ischemic and reperfusion treatment for 24 h were selected to carry out the late stage experimental study in this subject group.

The effect on MKP1, nervous system score and inflammatory factors after Let-7a inhibited

Comparing with the model group mice (5.17 ± 1.12), RT-PCR showed that the expression of

let-7a (2.18 ± 0.69) in brain tissue was significantly reduced (Figure 2A) when the mice were injected let-7a inhibitor through the tail vein; Western blot showed that the expression of MKP1 protein (2.98 ± 0.52) was significantly increased (Figure 2B) compared with that of the model group (2.12 ± 0.72); The nerve function scores of mice decreased significantly (Figure 2C) compared with that of the model group (2.74 ± 0.43) when the let-7a was inhibited (1.25 ± 0.23); RT-PCR showed that the expression level of IL-6 and TNF- α inflammatory factors in brain tissue decreased significantly (Figure 2D, 2E) after the injection of let-7a inhibitors through the tail.

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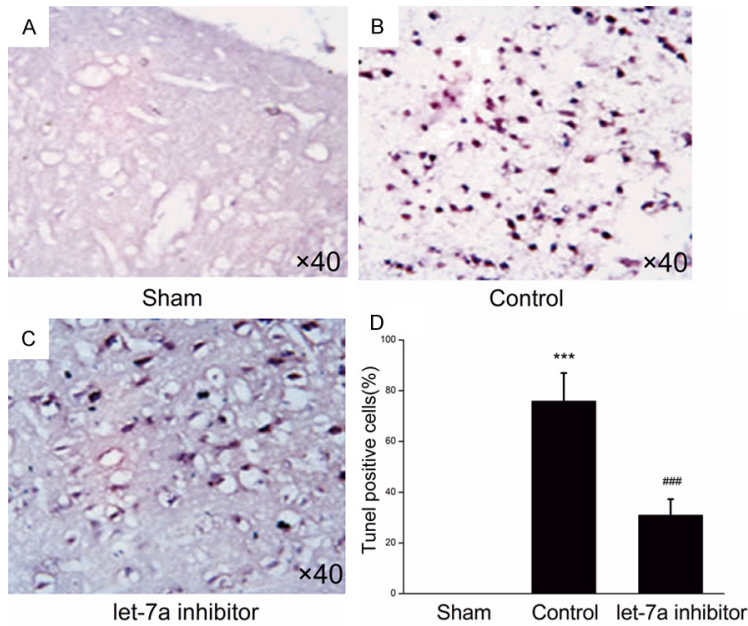


Figure 3. Let-7a inhibitor alleviates reperfusion neuron apoptosis. NOTE: Control group compared with the Sham group, *** $P < 0.001$; Let-7a inhibitor group compared with the Control group, ### $P < 0.001$.

Let-7a inhibitor lightens neuronal apoptosis in ischemia-reperfusion

No obvious positive TUNEL cells (Figure 3A) were found in brain tissue of sham operated mice group, however, a large number of positive TUNEL staining cells, namely the apoptotic nerve cells (Figure 3B, 3C) were found in the mice cortex, hippocampus and basal ganglia of model group and let-7a inhibitor group. The percentage of positive TUNEL cells (%) was calculated. The results showed that the percentage of positive TUNEL cells in the model group (75.92 ± 11.09) was significantly higher than that in the sham operation group, while they (30.89 ± 6.37) were significantly reduced compared with those in the model group (Figure 3D) when the let-7a was suppressed.

Let-7a was over expressed in PC12 cells

The neuronal cell line PC12 cells were transfected with let-7a analogues, the RT-PCR result confirmed that the expression of let-7a (521.17 ± 103.39) in transfected cells increased significantly (Figure 4A) compared with the control group (1.00 ± 0.23); RT-PCR showed that compared with the negative control group, the MKP1 gene expression was significantly decreased (Figure 4B) after transfection; the

result of Western blot showed that compared with the negative control group (1.06 ± 0.19), the MKP1 protein expression (0.62 ± 0.22) decreased significantly (Figure 4C) when the let-7a was over expressed; TUNEL staining showed that compared with the negative control group, apoptosis was remarkably increased (Figure 4D, 4E) when the let-7a was over expressed in PC12 cells. RT-PCR results suggested that, compared with the negative control group, overexpression of let-7a could promote the expression of pro-apoptotic protein Bax, inhibit anti-apoptotic Bcl-2 protein expression (Figure 4F).

Let-7a was suppressed in PC12 cells

PC12 cells were transfected with let-7a inhibitor, RT-PCR showed that the expression of let-7a (0.12 ± 0.04) in the transfected cells was significantly lower (Figure 5A) than that of the control group (1.00 ± 0.17). RT-PCR confirmed that compared with the negative control group, after transfection, the expression of MKP1 mRNA was significantly increased (Figure 5B); Western blot also showed the same results that compared with the negative control group (1.00 ± 0.24), MKP1 protein (2.12 ± 0.32) increased significantly (Figure 5C) when let-7a was suppressed. TUNEL staining showed that compared with the negative control group, the apoptosis was significantly decreased when let-7a was suppressed in PC12 cells (Figure 5D, 5E) RT-PCR results suggested that compared with the negative control group, the inhibition of let-7a could inhibit the expression of pro-apoptotic protein Bax and promote anti-apoptotic proteins Bcl-2 (Figure 5F).

Discussion

In clinic, ischemic cerebral stroke is a common and frequently occurring disease in the department of internal neurology. At present, its mortality rate is about 10%, being one of the three main causes of death among human beings [15]. With the improvement of living standards and the development of the aging

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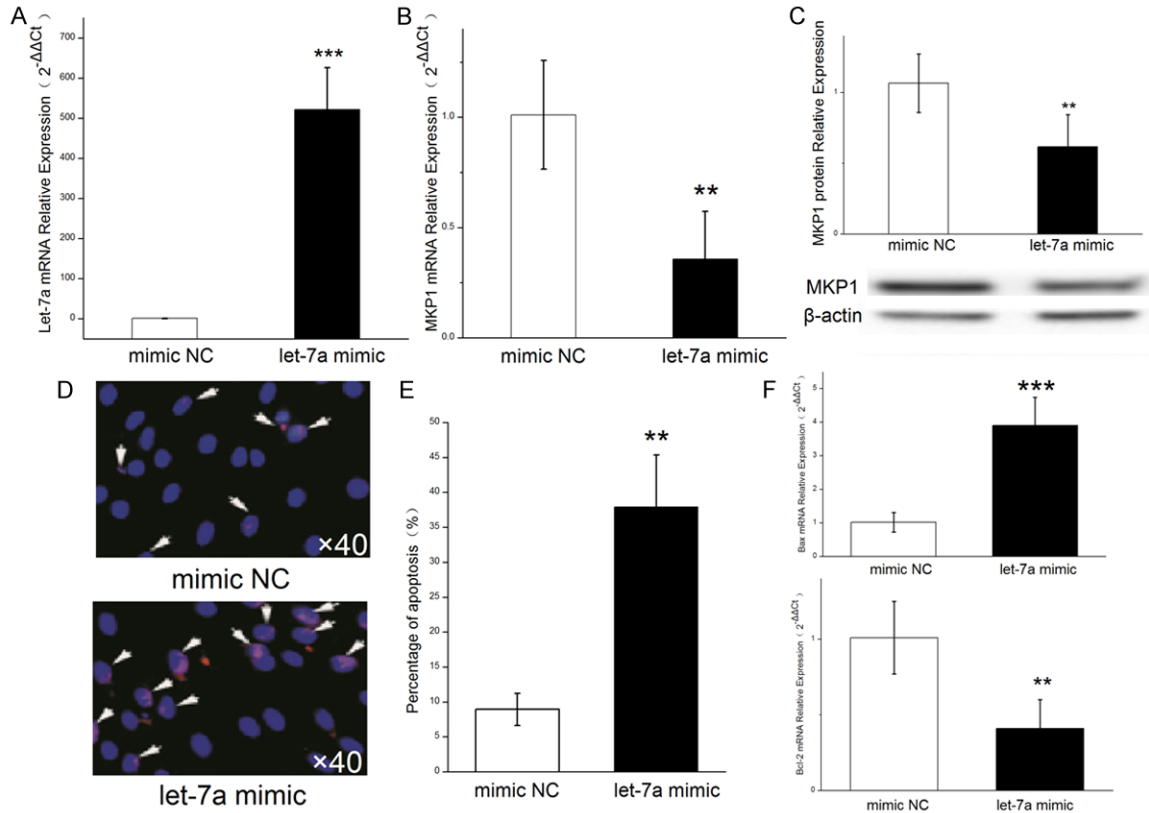


Figure 4. The role of let-7a overexpression on PC12 cells. NOTE: After PC12 cells transfected with let-7a mimic, let-7a mRNA expression was significantly increased (A), MKP1 mRNA expression significantly decreased (B); Western blot results showed that, MKP1 protein significantly reduced after let-7a overexpression (C); TUNEL staining confirmed that cell apoptosis was significantly increased after let-7a overexpression (D, E); RT-PCR found that overexpression of let-7a promoted pro-apoptotic protein Bax, inhibited anti-apoptotic protein Bcl-2 expression (F). Compared with mimic NC group, ** $P < 0.01$, *** $P < 0.001$.

society, the incidence of this disease showed a trend of youthization, and the incidence rate is increasing year by year, bringing a heavy burden to the family and society [16]. For cerebral blood supply interruption, the main therapeutic principle is to restore the cerebral blood flow as soon as possible, reaccess to oxygen and nutrients, scavenging the metabolites. However, it is found that the blood flow reperfusion in the ischemic region can lead to further aggravation of tissue injury and dysfunction, which is also known as IRI (the focus of attention of clinicians), but the specific mechanism is unclear at present. Previous studies have indicated that it is associated with oxygen free radicals, calcium overloading, inflammatory response and apoptosis [4]. In the inflammatory reaction mechanism, cytokines closely related to IRI include TNF- α , TNF- β , IL-1 and IL-6, in which the TNF- α appeared to rise earlier in IRI [17]. In our subject group, RT-PCR

results showed that the TNF- α and IL-6 inflammatory factors were significantly increased in the model group, which confirmed the presence of inflammatory reaction mechanism in the IRI. What's more, the positive TUNEL staining results in the model group showed that apoptosis was involved in IRI.

MiRNAs is an important factor involved in the regulation of gene level. With the deepening study of ischemic cerebral stroke, the studies of the relationship between miRNAs and ischemic stroke are also increased [5-7]. Let-7a, as a tumor suppressor, was found to be involved in the occurrence and development of a variety of tumors, but there is little research on the expression and role of let-7a in IRI. Recent studies found that the expression of let-7a would increase in cerebral ischemic injury, which can aggravate the injury of nerve cells [9]. By detecting the gene and protein expression levels of

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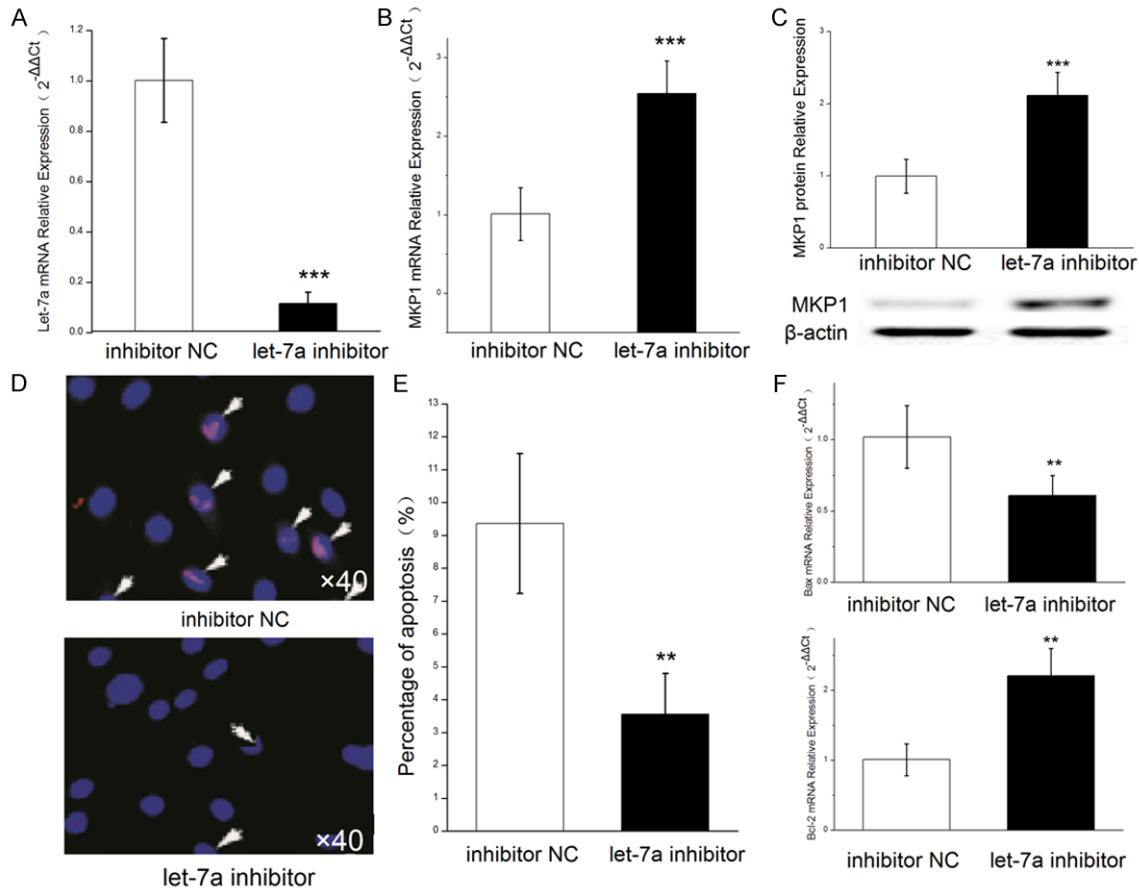


Figure 5. The role of let-7a inhibition on PC12 cells. NOTE: After PC12 cells transfected with let-7a inhibitor, let-7a mRNA expression was significantly decreased (A), MKP1 mRNA expression significantly increased (B); Western blot results showed that, MKP1 protein significantly increased after let-7a overexpression (C); TUNEL staining confirmed that cell apoptosis was significantly increased after let-7a was suppressed (D, E); RT-PCR found that suppression of let-7a inhibited pro-apoptotic protein Bax, promoted anti-apoptotic protein Bcl-2 expression (F). Compared with inhibitor NC group, ** $P < 0.01$, *** $P < 0.001$.

let-7a in the ischemic and perfused brain tissue of mouse, our research group found that the expressions varied with the time extension of reperfusion, the let-7a expression reached its peak at 24 h after ischemia and reperfusion, then decreased gradually, and was still higher than that in the sham operation group after 48 h. The mouse nervous system scores were reduced by injection of let-7a inhibitor through the mouse tail vein, the expressions of inflammatory factors of TNF- α , IL-6 were inhibited, and the percentage of positive TUNEL cells was reduced. Therefore, we speculated that the let-7a can aggravate the inflammatory response and apoptosis, which leads to the nerve damage.

MAPK signaling pathway widely exists in many kinds of cell signal transduction pathways, and

has important roles in cell proliferation, differentiation and apoptosis as well as other biological effects [18]. MKP1 is one of the most important and most studied MAPK phosphatases, mainly distributed in the nucleus. It is a member of the MKPs family, and it mostly play roles in the dephosphorylation of MAPKs. In the past, it was considered that MKP1 was the growth factor for cells transfer from G0 into G1 phase, and the recent study found that MKP1 as a stress protein can inhibit apoptosis. P38, JNK1/2 and ERK1/2 protein kinase signaling pathways involved in cell growth, differentiation and apoptosis and other cellular processes, while MKP1 phosphatase is a major negative regulator [19]. Studies have confirmed that MKP1 can inhibit the apoptosis by inhibiting JNK and p38 [11]. The studies

have found that MKP1 not only is expressed in microglia, but also has protective effect on the neurons in the nervous system inflammation, which can inhibit the inflammatory response and provide a target for the treatment of inflammatory diseases of the nervous system [20]. MKP1 is highly expressed in the cerebral IRI, and the promotion of the expression of MKP1 can inhibit the ERK1/2 signal pathway, which can reduce the nerve damage [21]. Our research group found that the expression of MKP1 in the model group also showed a certain trend, which was consistent with the expression of let-7a, and reached its peak at 24 h. The expression of MKP1 increased more obviously when let-7a was inhibited, which indirectly confirmed that IRI can be alleviated by over expression of MKP1.

PC12 cell is pheochromocytoma cell line and can synthesize, store the neurotransmitters, its differentional phenotype is similar to the primary sympathetic neurons when nerve growth factors were added into the culture medium, and is widely used in the research of neural cell differentiation, apoptosis and function [22]. In this study, the effect of PC12 cell line was observed when Let-7a was inhibited or over expressed, the expressions of MKP1 gene and protein were detected, and the role of let-7a and MKP1 in the apoptosis of PC12 cells was further discussed. The results showed that the MKP1 gene and protein expressions of PC12 cells were decreased when the Let-7a was over expressed, while the expression of MKP1 in PC12 cells was significantly increased when Let-7a was inhibited. Apoptosis detected by Tunel staining showed that the apoptosis of PC12 cells were significantly increased when Let-7a was over expressed, and the apoptosis was significantly decreased when Let-7a was inhibited. RT-PCR confirmed the same view, the overexpression of Let-7a prompted pro-apoptotic protein Bax expression, inhibited the expression of anti-apoptotic protein Bcl-2. In summary, the animal and cellular experimental studies in our subject group confirmed that the expression of let-7a up-regulated in ischemic and reperfused brain tissue, and the MKP1 expression was also significantly up-regulated, the inhibition of let-7a could act on target gene MKP1, reduced the inflammatory factors of TNF- α , IL-6 in cerebral ischemia and reperfusion, reduced the neural cell apoptosis, and improved the nervous sys-

tem injury. The study in this subject group complemented the mechanism of cerebral ischemia reperfusion, provided a new target therapy pathway for clinical treatment, and had significant clinical value.

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Disclosure of conflict of interest

None.

Authors' contribution

Qun Wang edited and reviewed the manuscript, Jingjing Fan, Qinlan Xu, Anchen Guo, Yilong Zhao and Huajun Yang conducted the experiments. All authors read and approved the manuscript.

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