The effects of rapamycin on the symptoms of cerebral ischemia due to changing the expression of miR-1 and its target genes, Bad and Bcl-w

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Abstract

Introduction: Stroke is the major cause of long-term disability in adults. The precise role of the mTOR signaling pathway in neural viability due to rapamycin effect in the animal model of middle cerebral artery occlusion (MCAO) remained elusive. Since the relationship between mTOR and miR-1, especially in neurons, is unknown, we have evaluated the effect of rapamycin as a post-ischemic treatment on improving stroke symptoms.

Methods: Rats were divided into three groups including sham, control and rapamycin treatment group. Each contains four subgroups (n=7). One hour after MCAO, rats were received intravenously 0.1ml normal saline or 0.1ml rapamycin in the control and treatment groups, respectively. After 24 hours, neurologic deficit score, infarct volume, brain edema, and blood-brain barrier (BBB) permeability were measured in the control and treatment group. The expression of miR-1, Bcl-w and Bad were analyzed using quantitative RT-PCR in all groups.

Results: Our results indicate that post-treatment with rapamycin, significantly reduces neurological deficits, infarct volume, brain edema and BBB permeability. It also decreases the level of miR-1 and Bad expression and increases the level of Bcl-w expression.

Conclusion: According to our findings, post-ischemic treatment with rapamycin can be effective in improving symptoms of stroke using changing in the expression of the miR-1 gene and consequently, a changing in the expression of the target genes of this miRNA (i.e., Bad and Bcl-w). In summary, we unravel for the first time a link between mTOR, miRNA-1, Bcl-w and Bad in brain ischemia.

Introduction

Stroke is one of the most important causes of death worldwide. Since various neurological disabilities reduce life quality, finding new protective methods to reveal deficits is very necessary (Towfighi and Saver, 2011; Lloyd-Jones et al., 2010). Many studies have proven the role of different genes, RNAs and proteins in brain damages during the stroke (Wang et al., 2013). Although various pathological signaling pathways and molecules are detected, unfortunately,
administration of current treatment for stroke has been limited because of its narrow therapeutic window (Bansal et al., 2013).

Rapamycin is an important mediator of the mammalian target of rapamycin (mTOR) pathway and could be an effective cure for many types of neuronal injuries and related diseases (Fletcher et al., 2013). In addition to, the mTOR signaling pathway plays a vital role in regulating different processes such as immune responses and cell growth, it also has a critical role in the nervous system. Studies have illustrated rapamycin enhances neuronal viability and decreases damages in several animal models (Foster and Fingar, 2010; Hailer, 2008). Dysfunction of the mTOR pathway has been detected in several neurological diseases including brain stroke and neurodegenerative diseases (Lu et al., 2014). Rapamycin has a protective role in the CNS by inhibiting mTOR signaling pathway results in autophagy. As limited autophagy leads to brain injuries, rapamycin administration reduces neurological deficits and apoptosis in a rat model of subarachnoid hemorrhage (Jing et al., 2012). Lu et al. (2014) have demonstrated inhibition of mTOR pathway modulates the immune response and also improves neurobehavioral deficit in the model of intracerebral hemorrhage. Conversely, many studies have revealed mTOR pathway ameliorates neural damages and improves memory function (Gong et al., 2006) and synaptic plasticity (Ma et al., 2010) in Alzheimer disease and also makes a long-term memory in the amygdala (Parsons et al., 2006).

Many studies confirm the mechanism of microRNAs-derived rapamycin. Micro(mi)RNAs are small noncoding molecules (containing about 22 nucleotides) play a vital role in many biological processes such as proliferation, apoptosis and cell differentiation using changing in the expression of target genes (Negrini et al., 2009). Evaluating proteins profile after brain ischemia in numerous studies indicate proteins involvement in ischemic damage (Dharap and Vemuganti, 2010; Lee et al., 2010; Lusardi et al., 2010). Therefore, clarifying the function of the miRNAs could be useful to understand the pathogenesis of cerebrospinal ischemia. MicroRNAs, such as miR-1, are gene expression regulators and control many physiological processes by changing the expression of their target genes (Negrini et al., 2009). It has been reported miR-1 has a critical role in many disorders including ischemia results in cardiomyocytes (Bigdeli et al., 2007) and neuronal hypoxia due to apoptosis (Bigdeli et al., 2008).

Although the role of microRNAs-derived rapamycin has been confirmed in different biological processes (Jin et al., 2013; Martin et al., 2014; Sun et al., 2010), its neuroprotective function still needs to be clarified. On the other hand, the role of rapamycin as an apoptosis inhibitor or inducer has been challenged in many studies (Foster and Fingar, 2010). Bcl-w and Bad are anti-apoptotic and pro-apoptotic genes, respectively, are regulated with miro-R1-3p. Members of the bcl2 family can act either as anti-apoptotic or pro-apoptotic molecules based on the presence of their conserved regions which termed homology domains (Van Delft and Huang, 2006). Thus in the future, investigating the pathway of apoptosis could be an appropriate target to be studied in evaluation the possible effect of miR-1 derived rapamycin during brain ischemia. According to what has been mentioned so far and also the effect of Bcl-w and Bad derived miR-1 in the apoptotic pathway in many studies (Tang et al., 2009; Ouyang and Giffard, 2014; Wang et al., 2010), as well as the rapamycin role in stroke improvement due to changes in various pathways including apoptosis, the goal of this study was to investigate the unknown effect of miR-1-derived rapamycin on the improvement of stroke due to changing of Bcl-w and Bad expression. Our results indicated that post-treatment with rapamycin significantly ameliorates neurological deficits and also reduces brain edema, blood-brain barrier permeability and infarct volume. The expression of miR-1 and Bad are decreased while the expression of Bcl-w is increased during our investigation. Hence, according to the results of this study in the mechanism of micro-RNA-derived rapamycin, it is hoping that a step forward would be taken to improve stroke.

Materials and methods

Animals

All laboratory methods performed in this study have been approved by the Bioethics Committee of Shahid Beheshti University, Iran. All male rats (250–350g) were purchased from Pastor Institute and were provided with standard food and water. All animals were purchased from Pastor Institute and were provided with standard food and water. All animals were purchased from Pastor Institute and were provided with standard food and water.
were kept in the similar condition of 12:12 hours of a dark-light cycle at 25°C.

**Ethical guidelines**
The care of experimental animals conforms to National Institutes of Health guidelines for the humane use of animals and has been affirmed by our Institute committee.

**Experimental protocol**
Rats were divided into three experimental groups. Group 1 (intact): a group that had no surgical or treatment in which using the RT-PCR method, the expression of miR-1, Bcl-w and Bad in penumbra is investigated (n=7). Group 2 (control): middle cerebral artery occlusion (MCAO) was performed. One hour later, we were treated with 0.1 ml sterile saline (intravenous injection into the tail vein). Subgroup 1: infarct volume assessment (n=7); Subgroup 2: assessment of blood brain barrier (BBB) integrity (n=7); Subgroup 3: assessment of brain water content (n=7); and subgroup 4: evaluation of expression of miR-1, Bcl-w and Bad in penumbra (sampling 24 hours after injection, n=7). In each of the four subgroups, first we investigated the neurological defects (Table 1).

Group 3 (treatment): MCAO was performed. One hour later, we were treated with 0.1ml rapamycin (intravenous injection into the tail vein). Subgroups in the treatment group were as subgroups in the control group.

**Focal cerebral ischemia**
MCAO surgery was done as described by Longa et al. (1989). Briefly rats were anesthetized with chloral hydrate (400mg/kg; Merck, Germany) and under a microscopic surgery, a 3-0 silicone-coated nylon suture was introduced through the external carotid artery stump. The occluder was advanced into the internal carotid artery 20 to 22mm from the carotid bifurcation until a mild resistance occurred indicating that the tip was lodged in the anterior cerebral artery, thus blocking the blood flow to the MCA. After 60 minutes of ischemia, the suture was extracted and reperfusion was started. During surgery, temperature was monitored and maintained at 37.0°C (Citizen-513w, CITIZEN) by surface heating and cooling.

**Neurobehavioral evaluation**
After the suture was withdrawn, the rats were returned to their separate cages. Twenty-four hours later, the rats were assessed neurologically by an observer who was blinded to the animal groups. The neurobehavioral scoring was performed using the 6-point scale previously described by Longa et al. (1989): the number 0 does not show any neurological complications. No.1, complete failure at the end of the front cuffs (which is considered a mild focal neurological defect). No.2, to the left of the animal (medium focal neurological deficiency). No.3, falling

### Table 1: Schematic timeline of experiments

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<thead>
<tr>
<th>Group</th>
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<th>An hour later</th>
<th>24 hours later</th>
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<td>Investigate the neurological</td>
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<td>defects</td>
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<td>2</td>
<td>MCAO Surgery</td>
<td>IV injection of saline</td>
<td>Infarct volume assessment</td>
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<td>Investigate the neurological</td>
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<td>Estimate of BBB permeability</td>
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<td>Assessment of brain water</td>
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<tr>
<td>3</td>
<td>MCAO Surgery</td>
<td>IV injection of rapamycin</td>
<td>Infarct volume assessment</td>
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to the left (intense focal flaw). No.4, they cannot walk by themselves and have a low level of consciousness. Also, rats die within 24 hours of surgery; if they have been damaged by a large portion of their brains after they have been stained, and death is exclusive to stroke, they will be given No. 5

Assessment of brain water content
After separating the head of the animal, rat brains were removed and dissected into hemispheres through the inter-hemispheric fissure. Then, wet weights (WW) of them measured. Dry weight (Dw) was measured after 24 hours in a dry autoclave at 120°C. Finally, the contents of brain water based on the formula [(WW − DW)/WW]×100 Measured.

Infarct volume assessment
To measure and determine the size and extent of the lesion of the brain after the end of the test, the animal is sacrifice under deep anesthesia, and immediately the animal's head is removed, and the brain is carefully removed. Then, in order to stiffen the brain and prepare for cutting, place it in 4°C saline for 5 minutes. Then, seven cuts of 2mm diameter from the brain are prepared by the Brain Matrix (Brain Matrix, Tehran, Iran). For staining, the slices are placed in a 2% solution of trinyl tetrazolium chloride for 15 minutes. By this method of staining, the area is damaged in white, and the normal area of the brain is red brick. The sections are then placed in buffered formalin for 10 hours to stabilize the tissue for 24 hours. At the end, with the aid of a digital camera, photographs are taken of each of the slices and after transferring to the computer, the surface area of the lesion is measured using a software called Image Tools (National Institutes of Health) and then summating all of the 8 slices according to the method of Datta et al. (1997) corrected infarct volume 5 left hemisphere volume – (right hemisphere volume - infarct volume).

Assessment of blood brain barrier integrity
The BBB strength was measured by the Evans blue (EB, Sigma Chemicals, USA) extravasation. First, the rats received 4ml/kg of 2% EB solution in saline via tail vein injection 30 min after surgery. Twenty-four hour after reperfusion, anesthetized rats were opened from the chest area. Then the rats were perfused with 250ml of the saline transcardially to have intravascular EB washed out until a colorless perfusion fluid was obtained from the atrium. The brains were removed and the hemispheres were weighed separately. To measure EB extraction, brain tissue was homogenized in 2.5ml of phosphate-buffered saline. For protein precipitation, 2.5ml of 60% trichloroacetic acid was added and mixed by vortexing for 3min. The samples were then stored in a refrigerator at 4°C for 30 minutes and then centrifuged at 1000g for 30 min. The EB amounts of the supernatants were measured at 610nm through spectrophotometry (Perkin-Elmer, Illinois, USA). Its concentration was measured (as µg/g of brain tissue) according to the standard curve (Bigdeli et al., 2007).

Quantitative RT-PCR
All RNAs were extricated from brain hemispheres of rats, tests utilizing the Ytzol Pure RNA buffer (Yekta Tajhiz Iran). After that, their immaculateness was recognized by the nanodrop estimation. Lastly, RNA was subjected to reverse transcription utilizing the
was performed to evaluate the expression level of the genes measured on the sample cDNAs. Primers were designed in the form of exon junction using allele ID software according to the sequence of genes archives in NCBI (table 2). Then, qRT-PCR was performed using the SYBR Green PCR Master Mix (Takara Bio Inc). To study the quantitative level of gene expression by Real-Time PCR first, the technical accuracy was evaluated using the melting curve. Then the amount of CT was determined using the amplification diagram. The relative amount of the expression of miR-1 (GenBank accession number: MI0003489), Bcl-w (GenBank accession number: NM_001199839.1) and Bad (GenBank accession number: NM_032989.2) genes in groups in comparison with GAPDH internal control was carried out using Corbet device. Finally, the data were analyzed by the Rest 2009 software.

**Statistical analysis**

Infarct volume, brain water content, and EB extravasation were compared using a one-way ANOVA test (post hoc LSD). The neurologic deficits scores (NDS) were analyzed using the Mann-Whitney U test. Data were expressed as means±SEM. P<0.05 was considered significant.
**Results**

**Effects of rapamycin on neurological deficit score (NDS) and infarct volume**

Median NDS was decreased by intravenous injection of drugs, being 0, 5 and 1 in the sham, control (temporary middle cerebral artery occlusion) and rapamycin, respectively (Table 3). The useful effects of rapamycin were affirmed by a decrease in infarct volume versus the control group (Fig. 1). The neuroprotection exerted by rapamycin was mainly seen in the penumbra (cortex) and subcortex.

**Effects of miR-1 rapamycin on brain edema**

Cerebral ischemia significantly enhanced the brain edema in the ischemic hemisphere in MCAO groups. The rapamycin injection decreased the post-ischemic brain edema (Fig. 2).

**Effects of rapamycin on BBB permeability**

Creating cerebral edema was an accomplice with enhanced BBB permeability at 24h. Details of BBB permeability in the right hemisphere of experimental groups come in Figure 3.

**Measurement of gene expression**

After qRT-PCR, the results were analyzed. Based on the results of REST program, from the statistical point of view, the level of miR-1, Bad and Bcl-w gene expression in the right hemisphere of experimental
groups comes in Figures 4, 5 and 6, respectively. The level of miR-1 gene expression in control (MCAO) group significantly increased versus the sham group and the expression of this gene in rapamycin group significantly decreased versus the control group (P≤0.05).

The aim of this study was to investigate regulatory pathways which alter neuroprotective processes in an animal model of MCAO, by focusing gene expression which is regulated by miRNAs. One of the main causes of disability around the world is cerebral ischemia. Ischemic preconditioning caused a temporary alternation in the genes expression and protective proteins during a subsequent ischemic injury (Dirnagl et al., 2009; Dhodda et al., 2004).

Since current treatment for brain ischemia is limited by many factors, the molecular study mechanism of new treatments can help to ameliorate ischemic outcomes.

Although the role of rapamycin as an inhibitor of mTOR is evident in the improvement of neurodegenerative diseases, its specific function in brain ischemia is unclear. (Yin et al., 2012). It has...
been proven that rapamycin has specific neuroprotective features in a various model of neuronal damage. For example, some studies have demonstrated that rapamycin improves brain injury (Erlich et al., 2007) and protects against neurodegenerative diseases (Malagelada et al., 2010; Santos et al., 2011). mTOR pathway is the specific modulator, in the cellular response to nutrient accessibility and stress caused by cerebral ischemia (Foster and Fingar, 2010). A study has shown that intravenous Rapamycin injection does not protect the injury of brain ischemia (Sharkey and Butcher, 1994; Bochelen et al., 1999). On the other, Chauhan et al. (2011) demonstrated that intraperitoneal rapamycin treatment protects the symptoms of MCAO by reversing the changes in levels of glutathione and nitric oxide in the brain. The contradictory results may be due to different circumstances such as treatment time and rapamycin treatment methods. Lauren Fletcher and colleagues have shown that rapamycin improves neuronal survival following oxygen glucose deprivation (OGD). Treatment with rapamycin can reduce cell survival by decreasing mTORC1 signaling activity from each other (Fletcher et al., 2013) while Chong et al. (2010) have shown that rapamycin decreases neuronal survival during OGD and increases apoptotic injuries. It has been seen rapamycin administration one hour after MCAO in a rat has a neuroprotective effect (Chauhan et al., 2011). The study has revealed the injection of rapamycin into the hippocampus twenty hours before ischemia reduces the neurological deficits, infarct volume and brain edema. It is also declared levels of NF-κB, TNFα and Bax are reduced while no change in Bcl2 level has been seen (Yin et al., 2012). In recent studies have reviewed the effects of rapamycin in biology and pathology processes through a variety of miRNAs. The miRNAs, contain 19-24 nucleotides, are non-coding RNA molecules. The miRNAs usually regulate gene expression through rudimentary coupling with the target mRNAs, thus may mediate different biological processes containing cell proliferation, differentiation, cellular response to stress, cell cycle and apoptosis (Bartel, 2004; Zhao et al., 2007; Carleton et al., 2007). It has been anticipated that about 40 percent of mammalian mRNAs could be regulated by microRNAs (Jeyaseelan et al., 2008). In mammals, specific miRNAs have been diagnosed to control processes including differentiation, hematopoiesis, exocytosis, neuronal cell fate, apoptosis, development, proliferation as well as in diseases (Kloosterman and Plasterk, 2006).

The mechanism various miRNAs effect on rapamycin pathway has been studied recently. MicroRNA-21 mediates the rapamycin-induced suppression of endothelial proliferation and migration (Jin et al., 2013). The miRNA-155 mediates alteration in mTOR, and estrogen receptor alpha signaling establishes a new mechanism for altering estrogen responses independent of growth factor stimulation (Martin et al., 2014). Mammalian target of rapamycin regulates miRNA-1 and follistatin in skeletal myogenesis. The results of this study showed for the first time that miR-1 activity is up-regulated during skeletal muscle differentiation and this activity is inhibited by rapamycin (Sun et al., 2010), but in the neurons, the relationship between rapamycin and miR-1, especially in vivo, has not yet been studied.

On the other hand, many studies have examined the role of various miRNAs in various neurodegenerative diseases. The miRNA expression has been detected in stroke (Jeyaseelan et al., 2008; Dharap et al., 2009), Down’s syndrome (Kuhn et al., 2008), Alzheimer’s disease (Hébert et al., 2008) and Parkinson’s disease (Kim et al., 2007). Many types of

<table>
<thead>
<tr>
<th>No</th>
<th>Experimental group</th>
<th>Neurological deficit score</th>
<th>Total</th>
<th>Median</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sham</td>
<td>7 0 0 0 0 7 0</td>
<td>0 1</td>
<td>2</td>
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<tr>
<td>2</td>
<td>Control</td>
<td>0 1 1 0 1 4 7 5</td>
<td>2:3 significant</td>
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<tr>
<td>3</td>
<td>Rapamycin</td>
<td>3 1 2 0 0 1 7 1</td>
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</table>

Table 3: Distribution neurologic deficit score in each experimental group
researches have demonstrated changes in the number of miRNA in cerebral ischemia. Therefore, miRNAs may be initiated in the development of neurodegenerative diseases (Wang et al., 2014). Alternation in the miRNA expression in humans and rodents following stroke has been proven in previous studies (Kocerha et al., 2009; Tan et al., 2009). Increasing the level of miR-1 is detected in Parkinson diseases and stroke (Wang et al., 2014). A study has shown that intracerebroventricular injection of anti-miR1 reduces cortical infarct volume in compared to the control group (Selvamani et al., 2012). MicroRNA-1 has various protective mechanisms to protect neuronal and brain injuries (Selvamani et al., 2012; Tang et al., 2009).

According to what has been said before, our assumption in this study was that probably in neurons, rapamycin might also affect stroke symptoms by altering the expression of miR-1. To confirm this hypothesis, the effect of rapamycin on the expression of miR-1 was evaluated. We showed for the first time that rapamycin decreases the expression of miR-1 simultaneously with decreasing infarct volume, edema, BBB permeability and neurological defects.

The contradictory effects of rapamycin in apoptosis were studied in a 2002 study (Castedo et al., 2002). It has been shown that apoptosis plays a key role in physiological neurogenesis in neural development (Okouchi et al., 2007). The role of rapamycin as an inducer of apoptosis is obvious in many studies. Rapamycin, without a specific cytotoxic effect, destroys dendritic cells (Woltman et al., 2001). Apoptosis induction by cis-platin with treatment rapamycin can make cancerous cells susceptible (Shi et al., 1995). Rapamycin can selectivity destroys fibroblast by the p53 deficiency (Huang et al., 2001).

On the other, the role of rapamycin inhibition on apoptosis has also been studied in several investigations (Peiretti et al., 2001; Harada et al., 2001). Since the two Bad and Bcl-w genes are pro-apoptotic and anti-apoptotic genes respectively, both of which play a direct role in the regulation of apoptosis, and also considering that both genes are the target of miR-1(miR-1 up-regulate Bad and down-regulate Bcl-w). In completing the study, the effect of rapamycin on change the expression of these two genes were also evaluated. We detected that rapamycin increased and decreased the expression of Bcl-w and Bad, respectively. Regarding the effect of rapamycin on the reduction of miR-1 expression, it can be said that the reduction of miR-1 expression by the effect on the expression of its two target genes is likely to set the pathway for apoptosis to improve the symptoms of stroke. Certainly, in subsequent studies, the precise molecular study of the rapamycin, miR-1, Bad and Bcl-w pathway will increase the hope for effective treatment of stroke.

**Conclusion**

Improvement of brain edema, BBB permeability, neurological deficits and reduction of infarct volume are clearly shown in this study. It also demonstrates that the expression level of Bcl-w in rapamycin treatment rat is significantly increased versus MCAO group. In reverse, the expression level of Bad and miR-1 is significantly decreased in compared to the MCAO group. Therefore, according to the obtained results, it seems that the neuroprotective effect of rapamycin in up-regulation of Bcl-w expression and down-regulation of Bad expression is due to decrease of miR-1 expression. This findings can be an essential point in the molecular understanding of the positive effects of rapamycin in treating stroke.

**Acknowledgments**

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**Conflict of interest**

There is no conflict of interest.

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