Preconditioning With Morphine Protects Hippocampal CA1 Neurons Against Ischemia/Reperfusion Injury via the Activation of the mTOR Pathway

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Preconditioning With Morphine Protects Hippocampal CA1 Neurons Against Ischemia/Reperfusion Injury via the Activation of the mTOR Pathway

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\textbf{Running Title}

\textbf{mTOR Pathway Is Involved in Morphine Preconditioning in Brain Ischemia}

\textbf{Keywords:} Cerebral ischemia, mTOR, Apoptosis

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ABSTRACT

The signaling pathway of chronic morphine treatment to prevent neuronal damage following transient cerebral ischemia is not clear. In the present study, we examined the role of mammalian target of rapamycin (mTOR) to reveal the neuroprotective effects of chronic morphine preconditioning on the hippocampus following ischemia/reperfusion (I/R) injury. Morphine was administrated for 5 days twice a day before I/R injury induction. The possible role of mTOR was evaluated by the injection of rapamycin (5 mg/kg, ip) before I/R injury induction. The passive avoidance test was used to evaluate memory performance. Neuronal density and apoptosis were measured in the CA1 region, 72 hours after the induction of I/R injury. The expressions of mTOR and phosphorylated mTOR (p-mTOR) and superoxide dismutase (SOD) activity were determined 24 hours after I/R injury induction. Chronic morphine treatment attenuated the apoptosis and neuronal loss in the hippocampus after I/R injury, which led to improvement in memory ($P < 0.05$ vs I/R) and increase in the expression of p-mTOR ($P < 0.05$ vs I/R) and SOD activity ($P < 0.05$ vs I/R) in the hippocampus. Pretreatment with rapamycin abolished all the above-mentioned protective effects. These results describe novel findings whereby chronic morphine preconditioning in hippocampal CA1 neurons is mediated by the mTOR pathway and through increased phosphorylation of mTOR can improve oxidative stress and apoptotic agents and eventually protect the hippocampus against I/R injury.

**Keywords:** Morphine, Preconditioning, Ischemia/reperfusion, mTOR, Apoptosis, Hippocampus, Memory, SOD
INTRODUCTION

Global brain ischemia usually develops in patients subjected to such clinical conditions as myocardial infarction and systemic hypoperfusion state and can lead to the activation of a cascade of cellular and molecular mechanisms that result in neuronal injuries and/or death. In efforts aimed at reducing injuries caused by brain ischemia, the use of immediate interventions and/or preventive approaches has been associated with more satisfying results. Hence, investigators have recently focused on the use of the endogenous ability of the brain to protect against brain stroke (Bhardwaj et al. 2003).

Pharmacologic preconditioning can activate the biochemical pathways and mediators involved in the different types of preconditioning (Riess et al. 2004). Recent studies have shown that opioid receptors are one of the main mediators for the development of preconditioning protective effects (Murry et al. 1990). In addition, releasing endogenous opioids after mild ischemic injuries can exert protective impacts on cardiac and cerebral tissues (Gao et al. 2012; Kunecki et al. 2017). Morphine, as an agonist of opioid receptors, can imitate endogenous opioids and confer protection against ischemic injuries (Dorsch et al. 2016; Rehni et al. 2008). Schultz et al (Schultz et al. 1995) were the 1st to show that intravenous morphine was able to decrease cardiac injuries. It has also been found that the effects of chronic morphine treatment (CM) on reducing cardiac ischemic injuries are more than those of single-dose morphine, and the impact remains for longer periods as well (Iwatsubo et al. 2003). Preconditioning can contribute to substantial changes in cells and the expression of specific genes in neurons. Important genomic responses with protective effects at preconditioning include changes in the expression of anti-inflammatory and anti-apoptotic genes (Kim et al. 2015). Mammalian target of rapamycin (mTOR) is an intracellular key
factor that accounts for the regulation of protein synthesis, cellular growth, and neuronal
plasticity (Pazoki-Toroudi et al. 2016). mTOR is a serine/threonine kinase which can be
inhibited by rapamycin (Rapa) (Asnaghi et al. 2004). mTOR can wield its neuroprotective
effects at preconditioning via phosphatidylinositol 3-kinase and its downstream targets,
including the Akt-pathway (Zemke et al. 2007). Moreover, mTOR through S6K leads to the
inactivation of pro-apoptotic protein BAD and the increase in Bcl-2, and it can consequently
reduce hypoxia-induced apoptosis and hypoglycemia in astrocytes (Javedan et al. 2016; Ma
et al. 2011).

In the present study, we sought to evaluate the role of the mTOR pathway in the development
of the neuroprotective effects of CM as a pharmacologic preconditioning method on
hippocampal CA1 neurons in the setting of I/R injury to the brain.

**METHODS**

Seventy-two male *BALB/c* mice (Razi Institute, Tehran, Iran), weighing 25 to 30 g, were
housed in standard condition with free access to tap water and chow. All the experimental
procedures were approved by the Ethics Committee of Tehran University of Medical
Sciences, Iran (TUMS 90–770), and they were conducted in accordance with the Guide for
the Care and Use of Laboratory Animals, published by the National Academy Press (National
Research Council Institute for Laboratory Animal 1996).

The animals were equally categorized into 6 groups comprised of 12 mice each as follows
(table 1):

1. Sham: subcutaneous normal saline administration 5 days prior to surgery without the
   occlusion of the bilateral common carotid arteries

2. CM+Sham: subcutaneous administration of morphine 5 days prior to sham surgery
3- I/R: subcutaneous administration of normal saline for 5 days followed by 30 minutes of ischemia

4- CM+I/R: subcutaneous administration of morphine for 5 days followed by 30 minutes of ischemia

5- Rapa+I/R: subcutaneous administration of normal saline for 5 days followed by 30 minutes of ischemia along with Rapa (5 mg/kg) injection before the surgical procedure

6- CM+Rapa+I/R: subcutaneous administration of morphine for 5 days followed by 30 minutes of ischemia along with Rapa (5 mg/kg) injection 30 minutes after the last morphine dose

In all the groups, ischemia was followed by a 24-hour or 72-hour reperfusion period.

**Chronic Morphine Preconditioning Induction**

Chronic preconditioning with morphine was induced via subcutaneous administration of morphine sulfate for 5 consecutive days. The dose of the regimen was 10 mg/kg/d on the 1st and 2nd days and 15 mg/kg/d on the 3rd and 4th days. All the daily doses were administered in 2 equally divided injections at 9:00 AM and 5:00 PM. A final dose of 30 mg/kg was given on the 5th day, 4 hours before ischemia. This method for morphine preconditioning induction has been validated already in a CM-treated mice model (Habibey et al. 2010). The Control Group received saline alone instead of morphine sulfate. Rapa (5 mg/kg)(Zare Mehrjerdi et al. 2013) was also administered 30 minutes after the last dose of morphine and 3.5 hours before ischemia induction (table 1).

**Ischemia/Reperfusion Protocol and Procedures**
Four hours after the last morphine dose, the mice were deeply anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg). During the surgical procedure, the body temperature was monitored and maintained at 36 ± 0.5 °C by using heating pads and rectal probes. The left and right common carotid arteries were exposed through a neck incision and were then released from the vagus nerve and the surrounding tissues. The bilateral carotid arteries were occluded with a microsurgery clamp and 30 minutes later, the arterial clamps were removed and reperfusion condition was performed for either 24 hours for the assessment of protein expression associated with enzymatic activity or 72 hours for histological evaluation (Arabian et al. 2015).

**Neurobehavioral Assessments**

Learning and memory performances were evaluated using the passive avoidance test with a shuttle box, a detailed protocol of which has been described previously (Arabian et al. 2015; Zarrindast et al. 2011). Briefly, before ischemia, in the habituation trial, the animals were adapted to the shuttle box conditions. Each animal was then placed in the light compartment and after 5 sec, the guillotine door was opened and the animal was allowed to pass to the dark compartment. Those animals that waited for more than 100 seconds in the light compartment were excluded from the study. When the animal crossed with all four paws to the next compartment, the guillotine door was closed and after 10 sec the animal returned to the cage. Thirty minutes later, the acquisition trial was performed by exposing the animals to foot shock in the dark compartment. In this trial the animal was placed in the light compartment and 5 sec later the guillotine door was retracted. When the animal crossed to the dark compartment, the door was closed and immediately a foot shock (50 Hz, 1 s, 0.5 mA) was induced and after 20 sec, the animal was removed and placed into its home cage. All the animals were trained with a maximum of three trials. Twenty-four hours after ischemia, the retention trial was performed without applying electric shock. In this trial, a cut-off time of
300 sec was considered for those animals that remained in the light compartment and the test session terminated when the animal entered to the dark compartment. In this trial the latency time was evaluated by measuring the length of time the animal remained in the light compartment before crossing into the dark compartment and compared between different groups (Quillfeldt 2016).

**Histological Assessment**

Seventy-two hours after ischemia (Bendel et al. 2005), the animals were anesthetized and perfused transcardially with phosphate buffered saline (PBS) (20 mL, 0.1 M) followed by paraformaldehyde in PBS (50 mL, 4%). The mice’s brains were then removed and fixed overnight in the same solution. After having been dehydrated and embedded in paraffin, the tissues were cut into 7-µm thick sections. The Nissl staining with cresyl violet was done to assess neuronal cell loss in the CA1 region of the hippocampus. The CA1 neurons were counted under a light microscope at 400 × magnification (Olympus, Hamburg, Germany) (Arabian et al. 2015).

Terminal deoxynucleotidyl transferase dUTP-end- labeling (TUNEL) staining was done by using a cell death detection kit (Roche Molecular Biochemicals kit, Germany) to detect apoptosis and DNA fragmentation in the degenerating neurons. Briefly, the brain sections were deparaffinized and dehydrated with embedding in ethanol and xylene. After being washed with PBS, the sections were treated with proteinase K. All the sections were incubated in 3% H₂O₂ in methanol for 10 minutes in order to block endogenous peroxidase activity. After being re-washed with PBS and re-incubated in the TUNEL reaction buffer for 10 minutes, the brain slides were incubated again in the TUNEL reaction mixture (consisting of enzyme and label solution) for 1 hour in a humidified chamber at 37 °C to 40 °C. Thereafter the brain sections were labeled with fluorescent antibody conjugated with
horseradish peroxidase for 30 minutes. Finally, the sections were incubated with 0.05% 3, 3-diaminobenzidine (DAB) substrate for 1 to 2 minutes to visualize the apoptotic cells and counterstained with the Gill hematoxylin for 30 seconds to stain the cells’ background (Mehrjerdi et al. 2015). Positive controls were provided by incubating the tissue sections with DNAase (3000 U/mL in 50 mM Tris-HCl) for 10 minutes at 15 °C to 25 °C to break the DNAs before the process of labeling. For negative controls, the brain samples were exposed only to the label solution (without terminal transferase) (Mehrjerdi et al. 2015). A light microscope at 400 × magnification was applied to observe the apoptotic cells (Olympus, Hamburg, Germany).

Protocol for the Assessment of Enzyme Activity

Superoxide dismutase (SOD) activity was evaluated by lysing the hippocampal tissues in an ice-cold RIPA buffer (#9806, Cell Signaling Technology, Italy) and a protease inhibitor. After 30 minutes, the tissues were centrifuged at 13000 g for 20 minutes at 4 °C. The auto-oxidation of hematoxylin is inhibited by the SOD enzyme; accordingly, the tissue level of SOD is proportional linearly to the inhibition of hematoxylin auto-oxidation. Enzyme activity is detected by spectrophotometer at 560 nm absorbance and expressed as U/mg protein.

Protein Expression Analysis

The expressions of mTOR and p-mTOR proteins were evaluated by deeply anesthetizing the mice 24 hours after I/R injury induction. The brain was removed, and the hippocampal tissue was immediately dissected. The hippocampus was rapidly frozen by liquid nitrogen and stored in sealed vials at -70 °C. The hippocampal tissues were lysed in an ice RIPA lysis buffer for 30 minutes and centrifuged at 13000 g for 20 minutes at 4 °C. The concentration of protein was measured by spectrophotometer. Samples with loading buffers were boiled for 5 minutes at 95 °C. The proteins were separated on polyacrylamide sodium dodecyl sulfate gels.
(10%) and transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in Tris-buffered saline for 1 hour and incubated with primary antibodies (Cell Signaling Technology, Italy) at 4 °C overnight. Primary antibodies were β-actin as loading control (ab8226), rabbit polyclonal antibody for mTOR (#2972), and rabbit polyclonal antibody for Phospho-mTOR (#2974). The membranes were thereafter washed and incubated with the horseradish peroxidase-conjugated anti-mouse secondary antibody (#7072) for 1 hour at room temperature. The protein bands were scanned, and the level of each protein expression to the β-actin expression was analyzed with Lab Work software.

**Statistical Analysis**

The statistical analyses were performed using SPSS, version 16.0 (IBM Co, USA). All the data were analyzed by one-way ANOVA and the post-hoc Tukey test was done for further analysis for paired group comparisons. Two-sided $P$ values were calculated. The results were presented as mean ± SEM. A $P$ value less than 0.05 was considered statistically significant.
RESULTS

Effects of chronic morphine treatment on memory

The passive avoidance test was done to investigate the effects of CM and ischemic injury on the learning and memory functions of all the experimental groups. In the Sham Group, the mean latency time in the retention trial was 185.9 ±18.5 s. I/R injury decreased the latency time significantly (80.33 ± 10.3 s; \( P < 0.01 \) vs the Sham Group) (Fig. 1). CM increased the latency time post ischemia (147.89 ± 15.03 s; \( P < 0.05 \) vs the I/R Group) (Fig. 1) but did not change it in the Sham animals. Pretreatment with Rapa in the morphine-treated group decreased the latency time (73.78 ± 12.98 s; \( P < 0.05 \) vs the CM+I/R Group) (Fig. 1).

Chronic morphine attenuated apoptosis and neuronal loss

The apoptotic index was evaluated by the ratio of the apoptotic cells to the total cell number. In the Sham Group TUNEL-positive cells in the hippocampal CA1 subregion were detected very rarely (3.65 ± 1.5%) (Fig. 2A and Fig. G). I/R injury induced a significant increase in apoptotic cell death (75.6 ± 4.1%; \( P < 0.001 \) vs the Sham Group) (Fig. 2C and Fig. G). CM significantly decreased the percentage of the apoptotic/total cells post I/R injury (35 ± 2.3%; \( P < 0.001 \) vs the I/R Group) (Fig. 2D and Fig. G). Rapa pretreatment abolished the protective effects of CM and increased neuronal cell apoptosis (70.9 ± 2.6%; \( P < 0.01 \) vs the CM+I/R Group) (Fig. 2F and Fig. G).

Seventy-two hours post ischemia, the number of viable neurons in the CA1 subregion was significantly decreased and the neurons were shrunken (56.1 ± 8.2; \( P < 0.001 \) vs the Sham Group with 142.2 ± 10 cells) (Fig. 3C and Fig. G), while in the Sham Group the CA1 neurons had normal shapes and arrangements (Fig. 3A). In the CM Group compared to the I/R Group, the number of the viable cells was significantly higher (110.5 ± 5.8%; \( P < 0.01 \) vs the I/R Group) (Fig. 3B and Fig. G).
Group) (Fig. 3D and Fig. G). Rapa decreased the viable cells in the CM mice (52.2 ± 8.6%; P < 0.01 vs the CM+I/R Group) (Fig. 3F and Fig. G).

**Effects of chronic morphine treatment on the tissue levels of superoxide dismutase**

Based on the results, I/R decreased SOD activity in the hippocampal tissue (2.12 ± 0.18; P < 0.05 vs 3.81 ± 0.33 in the Sham Group) (Fig. 4). CM increased SOD activity compared to the ischemia group (3.30 ± 0.24; P < 0.05 vs the I/R Group) (Fig. 4). Pretreatment with Rapa decreased SOD activity in the morphine-dependent mice post ischemia (2.18 ± 0.19; P < 0.05 vs the CM+I/R Group) (Fig. 4).

**Effects of chronic morphine treatment on the expressions of the mTOR and p-mTOR proteins**

The expressions of mTOR or p-mTOR in each sample were normalized to β-actin expression. The results of the mTOR expression showed that there were no significant differences between all the experimental groups. However, the expression of p-mTOR after 30 minutes of global cerebral ischemia significantly increased (92.80 ± 9.41% vs 48.10 ± 6.2% in the Sham Group; P ≤ 0.05) (Fig. 5). Although CM, by itself, did not change the p-mTOR expression (52.92 ± 8.30%), it augmented the effects of I/R injury on the expression of p-mTOR (131.67 ± 8.02 vs I/R; P ≤ 0.05) (Fig. 5). Pretreatment with Rapa inhibited the effects of CM and significantly decreased the p-mTOR expression in the I/R Group (41.51 ± 6.7; P ≤ 0.05 vs the I/R Group) and the CM+I/R Group (37.51 ± 6.58; P ≤ 0.01 vs the CM+I/R Group).
DISCUSSION

In the present study, we evaluated the effects of CM on injuries developed after brain I/R. We found that the implementation of CM before I/R injury induction was able to protect against apoptosis and cell necrosis following the injury. In addition, CM increased the density of the intact neurons in the CA1 region of the hippocampus and consequently improved memory as well.

The neuroprotective effects of opioids on the brain have received more attention in recent years because the response of the neuronal system to hypoxic/ischemic stress is a unique and complicated process and ischemic preconditioning in the brain tissue is different from that in other organs such as the heart (Feng et al. 2009; Yang et al. 2015a). However, information achieved from study in the CNS may give some insight into the mechanisms behind chronic opioid preconditioning. Ischemia can damage hippocampal CA1 neurons and lead to learning and memory deficiency. Preconditioning through the protection of the cholinergic neurons in the CA1 part of the hippocampus can boost learning and memory performances (Hu et al. 2013). In addition, the activation of the δ receptors can enhance the growth of neurons and improve learning and memory performances via the extracellular signal-regulated kinase pathway, which is a known pathway in ischemic preconditioning (Yan et al. 2007). Several evidences have been shown that opioids may protect neurons during ischemic injury via activation of opioid receptors and inhibition of apoptosis. It has been shown that delta opioid peptide had neuroprotective effect in CA1 neurons against ischemia reperfusion injury in a dose-dependent manner (Su et al. 2007). On the other hand, some studies showed that chronic morphine treatment could increase apoptosis in hippocampus and impaired spatial memory and long-term potentiation (Lu et al. 2010). There are several conflicting studies which reveal
the effects of morphine on the CNS and learning and memory. Evidences have been shown that morphine can induce spatial learning deficiency in morris water maze task (Bodnar 2017). On the other hand, there are numerous studies which have been shown that morphine can improve retention performance in the behavioral tests (Motamedi et al. 2010). Furthermore, it has been shown that high dose and average dose of morphine improve memory in the passive avoidance test (Jafari-Sabet et al. 2005) and repeated administration of morphine does not impair memory performance in water maze task (Motamedi et al. 2010). We found that chronic morphine treatment in mice with increasing doses for five days led to improvement of memory following I/R injury. It has been found that ischemic preconditioning augments primarily the production of free O$_2$ radicals in the target organ, which can initiate anti-oxidative enzyme activity, enhance resistance against ischemia, improve neurologic function, and decrease tissue damage (Dong et al. 2010). The effects of CM on I/R injury were demonstrated in our previous study (Arabian et al. 2015). In the present study, we showed that the amount of SOD enzyme significantly decreased in the I/R Group compared with the Control Group. Nevertheless, CM increased SOD enzyme in the hippocampus, which probably enhanced resistance against ischemia and improvement in memory. mTOR, sensor of energy and metabolism of the cells, is activated by insulin, growth hormone, and oxidative stress and is involved in protein synthesis, cell growth, and neuronal plasticity (Chi et al. 2017). Rapa inhibits mTOR through the FK506-binding protein (Zarogoulidis et al. 2014). Gross et al demonstrated that mTOR was an essential factor for morphine-induced preconditioning in the heart. The authors found that morphine decreased cardiac infarct size via the PI3K and mTOR pathways (Zhang et al. 2007). In order to determine the mechanism of the neuroprotective effects of morphine, we assessed the expression of mTOR and p-mTOR in the hippocampal tissue 24 hours after global ischemia. We found no significant differences in the mTOR expression between the study groups;
however, p-mTOR in the I/R Group increased significantly compared with the Control Group. It seems that this early activation of the mTOR pathway during reperfusion, as reflected by increased levels of p-mTOR, is a compensatory response to ischemia injury. In the previous studies showed that the activation of the mTOR pathway was occurred 3 hours after ischemia injury and may be induced by the reflow of blood and glucose to the ischemic tissue (Yang et al. 2015b). Moreover, CM amplified the amount of p-mTOR in the ischemic hippocampus. Our results showed that level of mTOR expression was not changed obviously. Some pharmacological agents such as; melatonin (Koh et al. 2008) and bpv, a potent inhibitor of PTEN (phosphatase and tensin homolog deleted on chromosome 10) (Shi et al. 2011) protected brain tissue against focal cerebral ischemia via up-regulation of Akt and its downstream targets mTOR and p70S6 kinase. Notably, bpv could not affect the mTOR expression after ischemia, but it increased phosphorylation of mTOR. So, these results emphasized the role of mTOR phosphorylation in neuroprotection which is in agreement with our obtained results in the CM induced protection.

As the roles of the mTOR-related pathways in both preconditioning and neuroprotection have been previously discovered (Yang et al. 2015b), we postulated that CM through the mTOR pathway could protect the hippocampal tissue against I/R injury and decrease neuronal necrosis and apoptosis as well. Furthermore, to further identify the role of mTOR in morphine-induced preconditioning, we used Rapa to inhibit mTOR. We found that neuronal injury and apoptosis in the CA1 subregion increased and the density of intact neurons decreased significantly in the Rapa Group compared to the CM Group. We believe that the administration of Rapa blocked the phosphorylation of mTOR and consequently inhibited the protective effects of morphine on reducing tissue injury and improving memory performance.

Recent studies on Rapa have yielded contradicting findings. Some investigators have demonstrated that Rapa decreases neuronal death by reducing macrophage and/or microglia
activities and resultant inflammatory pathways and have highlighted it as a creator of the preconditioning state (Erlich et al. 2007). In contrast, some other authors have shown that Rapa by inhibiting mTOR—as a regulator of protein synthesis and cell growth—can increase I/R injury (Chen et al. 2012). A study published in 2012 showed that Rapa inhibited the effects of ischemic preconditioning in the brain and also increased neuronal death, which was mediated by inhibition in mTOR phosphorylation and decrease in p-mTOR. This may be explained by the notion that p-mTOR can increase hypoxia-inducible factor 1α and vascular endothelial growth factor and subsequently inhibit apoptosis (Chen et al. 2012). In addition to these findings, according to the neurobehavioral assessment in our study, we also found that Rapa through inhibiting p-mTOR led to memory impairment, which was confirmed by the results obtained in our histological assessment and passive avoidance test. Investigations have shown that learning and memory performances at the level of the hippocampus need the mTOR pathway activity as mTOR phosphorylation increases in the hippocampus during the learning process. Moreover, the administration of Rapa before learning can impair this process (Bekinschtein et al. 2007). Additionally, we assessed the tissue level of SOD, as an anti-oxidant enzyme, in the group of mice that received Rapa and found that Rapa plus CM decreased SOD activation in the hippocampus. It has been postulated that morphine via mTOR phosphorylation is able to activate some pathways to decrease oxidative stress and it also protects the hippocampus against ischemic injury. It is probable that after chronic morphine preconditioning, SOD is activated subsequently or in parallel with the phosphorylation of mTOR. In the preconditioning state, the levels of antioxidant enzymes such as catalase, glutathione peroxidase, and SOD are increased and these elevations interestingly depend on the preconditioning method (Gasche et al. 2001; Hu et al. 2012). SOD acts as a convertor of superoxide into H₂O₂ and leads to PTEN removal and modulation of the PI3-kinase/AKT signaling pathway (Lee et al. 2002; Leslie 2006). With regard to the
previous studies, the activity of catalase and SOD enzymes is correlated with PI3K-Akt, nuclear factor kappa-B (NF-κB), and c-Jun NH2-terminal kinase (JNK), which are the main pathways in the mTOR signaling pathway (Kennedy and Lamming 2016; Xue et al. 2011). Therefore, the inhibition of mTOR by Rapa can influence the activation of these factors and enzymes (Aiken et al. 2008). In the present study, we did not determine the downstream and upstream pathways, which could contribute to mTOR effects. Be that as it may, an increase in the SOD level in the CM-treated mice and its reduction by Rapa administration may suggest that the phosphorylation of mTOR could protect neurons against ischemic injury through interaction with other signaling pathways.

CONCLUSIONS

This study is the 1st of its kind to show that CM can improve memory and learning performances via the mTOR pathway by inhibiting the neuronal loss in the CA1 region of the hippocampus. The paramount advantage of using CM as a pharmacological preconditioning method is that it can be translated into clinical practice and applied for susceptible patients with a history of ischemia because in pharmacologic preconditioning, a non-ischemic and non-hypoxic stimulus is used to induce protection and there are no clinical limitations associated with ischemic preconditioning or remote preconditioning. Further pharmacokinetic studies on the evaluation of the neurofunctional effects of CM and more basic and clinical studies with long-term follow-ups are required to confirm the potential of CM. In addition, the effectiveness of CM in large animal models, particularly in females and aged animals, should be also studied. Ultimately, we believe that our study underscores the need for further studies to evaluate the applicability of morphine to protect the brain against stroke in a clinical setting and improve the neurofunctional outcomes of patients.
The present study has some limitations, which should be taken into consideration when interpreting the results. All the experiments were conducted only on male rats, whereas we know that the neuroprotection effects of drugs and agents are gender-dependent. Furthermore, we could have bolstered our results had we performed the procedure on a larger sample size.

Future Perspectives

It has been identified that the role of different cellular signaling pathways is interestingly complex in cell fate. These findings indicate that the survival rate after the induction of global ischemia in the CA1 region of the rat hippocampus is associated with the activation of the mTOR pathway. There is no doubt that these results are of great significance; nevertheless, since mTOR is in close association with autophagy, it seems that morphine administration confers neuroprotection in coping with global ischemia through the activation of mTOR and the subsequent suppression of acute autophagy. It is clear now that many pharmaceutical compounds may inhibit mTOR and consequently lead to neural survival via the induction of protective autophagy, while other therapeutic agents activate mTOR, which in turn results in neural preservation through the inhibition of acute autophagy.

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Compliance with Ethical Standards

Disclosure of potential conflicts of interest: All authors declare that there are no conflicts of interest.
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**Ethical approval:** All experimental procedures were approved by the Ethics Committee of Tehran University of Medical Sciences, Iran (TUMS 90–770), and they were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Academy Press (National Research Council Institute for Laboratory Animal 1996).
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**Table 1.** Experimental timetable. IR: ischemia reperfusion; Rapa: rapamycin.

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<tr>
<th>1 to 4 Days before IR or Sham Operations (mg/kg)</th>
<th>Day 1 and 2</th>
<th>Day 3 and 4</th>
<th>4hr before IR</th>
<th>3.5hr before IR</th>
<th>45 min before IR</th>
<th>24hr interval</th>
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<td>Morphine (30)</td>
<td>saline</td>
<td>Learning</td>
<td>Sham</td>
<td>Sham</td>
<td>Learning</td>
<td>Sham</td>
<td>Learning</td>
<td>Sham</td>
</tr>
</tbody>
</table>

Morphine (10-10) Morphine (15-15) saline
Morphine (10-10) Morphine (15-15) Morphine (30) Rapa (5) Learning IR
Morphine (10-10) Morphine (15-15) Morphine (30) Rapa (5) Learning IR
Morphine (10-10) Morphine (15-15) Morphine (30) Rapa (5) Learning IR

* indicates a significant difference.
Figure legends

**Figure 1.** Latency time in the passive avoidance test (mean ± SEM, n = 12) in the different groups.

I/R, Ischemia/reperfusion; CM, Chronic morphine treatment; Rapa, Rapamycin

** P < 0.01 vs the Sham Group, # P < 0.05 vs the I/R Group and ^ P < 0.05 vs the CM+I/R Group

**Figure 2.** Effects of chronic morphine treatment on CA1 neuron apoptosis (mean ± SEM). Representative pictures of the Sham (A), CM+Sham (B), I/R (C), CM+I/R (D), Rapa+I/R (E), and CM+Rapa+I/R (F) 72 hours after ischemia (400×). Black arrows indicate intact cells and the red arrows indicate apoptotic cells (scale bar = 100 µm). To compare the mean percentage of apoptosis between the groups, we applied one-way ANOVA followed by the Tukey test.

*** P < 0.001 vs the Sham Group, ### P < 0.001 vs the I/R Group, ^^ P < 0.01 vs the Sham Group, and ££ P < 0.01 vs the CM+I/R Group (G)

**Figure 3.** Effect of chronic morphine treatment on the number of Nissl-stained neurons in the CA1 subregion.

Representative pictures of the Sham (A), CM+Sham (B), I/R (C), CM+I/R (D), Rapa+I/R (E), and CM+Rapa+I/R (F) 72 hours after ischemia (400×). Black arrows indicate intact cells and the red arrows indicate necrotic cells (scale bar = 100 µm). Data are expressed as the
number of the counted live hippocampal CA1 neurons. One-way ANOVA followed by the Tukey test was done to express the results (mean ± SEM) (G).

*** $P < 0.001$ vs the Sham Group, ###$P < 0.01$ vs the I/R Group, and ^^$P < 0.01$ vs the CM+I/R Group

**Figure 4.** Effect of chronic morphine treatment on superoxide dismutase (SOD) activity in the different groups.

One-way ANOVA followed by post-hoc Tukey test was used to analyze the data. The data are shown as mean ± SEM.

* P<0.05 vs the Sham Group, #P < 0.05 vs the I/R Group, and ^ $P < 0.05$ vs the CM+I/R Group

**Figure 5.** Expression of mTOR and phosphorylated mTOR (p-mTOR) in the different groups.

B-Actin served as loading control. The mean values of mTOR/β-actin and P-mTOR/β-actin are presented. The data are shown as mean ± SEM.

* $P < 0.05$ vs the Sham Group, # $P < 0.05$ vs the I/R Group, £ $P < 0.05$ vs the I/R Group, and ^^$P < 0.01$ vs the CM+I/R Group
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