Neuroprotective Effects of Oxytocin Hormone after an Experimental Stroke Model and the Possible Role of Calpain-1

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Background: Different mechanisms will be activated during ischemic stroke. Calpain proteases play a pivotal role in neuronal death after ischemia damage through apoptosis. Anti-apoptotic activities of the oxytocin (OT) in different ischemic tissues were reported in previous studies. Recently, a limited number of studies have noted the protective effects of OT in the brain. In the present study, the neuroprotective potential of OT in an animal model of transient middle cerebral artery occlusion (tMCAO) and the possible role of calpain-1 in the penumbra region were assessed. *Methods:* Adult male Wistar rats underwent 1 hour of tMCAO and were treated with nasal administration of OT. After 24 hours of reperfusion, infarct size was evaluated by triphenyltetrazolium chloride. Immunohistochemical staining and Western blotting were used to examine the expression of calpain-1. Nissl staining was performed for brain tissue morphology evaluation. *Results:* OT reduced the infarct volume of the cerebral cortex and striatum compared with the ischemia control group significantly ($P < .05$). Calpain-1 overexpression, which was caused by ischemia, decreased after OT administration ($P < .05$). The number of pyknotic nuclei in neurons increased dramatically in the ischemic area and OT attenuated the apoptosis of neurons in the penumbra region ($P < .01$). *Conclusion:* We provided evidence for the neuroprotective role of OT after tMCAO through calpain-1 attenuation. **Key Words:** Stroke—tMCAO—calpain-1—oxytocin.

Introduction

Stroke is a devastating neurological condition and the second leading cause of death in the world.[1-3] The 2 main types of strokes are ischemic and hemorrhagic, and ischemic stroke is more common.[4] Delayed neuronal death often occurs after ischemic insult from a few hours to days following stroke onset, which provides a wide therapeutic window.[5] Use of tissue plasminogen activator such as intravenous alteplase is the only treatment with proven efficacy in patients with ischemic stroke[6]; however, this treatment has a time limit of 4.5 hours after the onset of symptoms and in some cases have contraindications.[7] Neuronal injury in stroke is caused by different mechanisms, including excitotoxicity, inflammation, oxidative stress, and apoptosis.[8,9] One of the dramatic events during ischemia is the increase in intracellular calcium (Ca^{+2}), which leads to the activation of calpain proteases.[5] Two major isoforms of calpains, calpain-1 and calpain-2, are differently sensitive to the amount of calcium required for their activation. Calpain-1 (μ-calpain) and calpain-2 (m-calpain) are activated by approximately 50 μM and approximately 1 mM of calcium concentrations, respectively.[10] Once activated in the cytoplasm of neurons, calpain degrades cytoskeleton elements, leading to the promotion of neuronal apoptosis.[5,11] The role of calpain-1 in the neuronal apoptosis

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Received January 23, 2017; revision received September 13, 2017; accepted October 10, 2017.

Grant support: This study was financially supported by deputy of Research and Technology Kashan University of Medical Sciences (Grant No 9421).

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1052-3057/$ - see front matter

https://doi.org/10.1016/j.jstrokecerebrovasdis.2017.10.020

in response to various stimulatory conditions has been known. Research shows that calpain-1 increases reactive oxygen species levels and inflammatory cytokines.[12] Because of the molecular complexity process[13] and clinical importance of penumbra region management after brain ischemia, the neuroprotection is needed.[14] The oxytocin hormone (OT) is a small peptide with 9 amino acids. OT is synthesized mainly by paraventricular and supraoptic nuclei in the hypothalamus and its receptors are present in many parts of the brain on the neuronal membrane, in the cytoplasm, around the nucleus, and within the nucleus.[15,16] Recently, it has been shown that OT plays an important role in many pathological conditions following ischemia–reperfusion in various regions, including the bladder and cardiomyocytes, and ovarian, liver, and renal tissue. Studies have indicated that the protective effects of OT are related to its anti-inflammatory, anti-apoptotic, and antioxidant properties.[17-23]

Moreover, some recent data suggest that OT has also neuroprotective effects in both males and females, including social neuroprotection, oxygen–glucose deprivation resistance, immune system modulation, and anti-apoptotic and anti-inflammatory functions.[24-29] Altogether, the protective role of OT in pathological conditions has turned it into an attractive candidate for improving the outcomes after cerebral ischemia. However, the neuroprotective effects of OT on cerebral stroke and related signaling pathways are not completely understood. In this study the effect of OT on the ischemic brain and the potential of calpain-1 protease as a hallmark in apoptosis were examined.

Materials and Methods

Ethical Statement

All the experiments were approved by the ethics committee of Kashan University of Medical Sciences (Reference number: IR.KAUUMC.REC.1394.21) and conducted according to the Guide for the Care and Use of Laboratory Animals.

Animals

Because endogenous OT may influence the severity of infarction, male rats are preferable. Thirty Wistar rats weighing 200-300 g (3-4 months old) were used in this experiment. The animals were kept in air-conditioned rooms in a controlled environment at 21 ± 2°C with seasonal lighting conditions (12 hours of light and 12 hours of darkness); food and water were freely available during the experiment. The rats were randomly divided into 4 groups: (1) sham group; (2) ischemia/reperfusion (I/R) group; (3) IR + OT 20 µL group; and (4) IR + OT 40 µL group. The animals received a single dose of OT hormone after transient middle cerebral artery occlusion (tMCAO) through nasal administration and were sacrificed 24 hours after I/R.

Transient Focal Cerebral Ischemia

The rats were anesthetized with 3% isoflurane in conjunction with oxygen (Eickemeyer, Germany) first in a chamber, and they remained in this state with 2% isoflurane through a nose cone. Incision in the scalp was made along midline to find the bregma point. The skull was thinned bilaterally 4-5 mm outside and 1-2 mm posterior the bregma; laser Doppler probes (Moor Instruments, VMS-LDF2, Axminster, UK) were then fixed in thinned locations. Each animal was placed in supine position at 37 ± .5°C on a warm pad (Narco Bio-Systems, Houston, TX). Midline skin incision was performed with a 3-cm length in the neck to expose the left carotid sheath. The left common carotid artery (CCA) is charily separated from the vagus nerve and was blocked to stop the backflow of blood. The bifurcation of CCA into the external carotid artery and internal carotid artery was blocked temporarily with a microclip. Then a small incision was made in the wall of CCA (in 5-mm bifurcation) by microscissors. The nylon monofilament with a silicone-coated tip (Doccol, USA) entered into the artery lumen and moved slowly forward until a sudden decrease pertains to the base flow (Fig 1, A). In this condition, the left middle cerebral artery blood flow is barred through the circle of Willis. Animals were kept anesthetized in the ischemic state for 1 hour, and middle cerebral artery (MCA) territory blood flow was recorded every 10 minutes. After 1 hour, the intraluminal monofilament was withdrawn slowly and the artery was ligated. In this situation blood flow returned to the base values, which is defined as reperfusion.

Hormone Therapy

Hormone administration was done immediately after I/R. OT drug from sigma (Cat. Number 06379) was provided and mixed with sterile Ringer's solution.[30] Hormone therapy was done for 2 groups with doses of 20 and 40 µg. Since OT could rapidly access the brain following intranasal administration, the nasal path was used to investigate the effects of OT.[31] In detail, 20 µL solution with different doses was used bilaterally on the rhinarium. The rhinarium refers to the glabrous skin circa, the nostrils that have a large number of free nerve endings. Using the tip of a 100-µL pipette, the total volume of 20 µL was divided similarly and allowed to spread in the squamous epithelium of both sides of the rhinarium for 2 minutes. According to this method, OT increases in the brain and plasma following intranasal administration after only 30-60 minutes at a dose of 20 µL.[31]

Behavioral Testing

The Garcia test for evaluating the behavioral aspects of stroke was performed in a blinded manner 24 hours

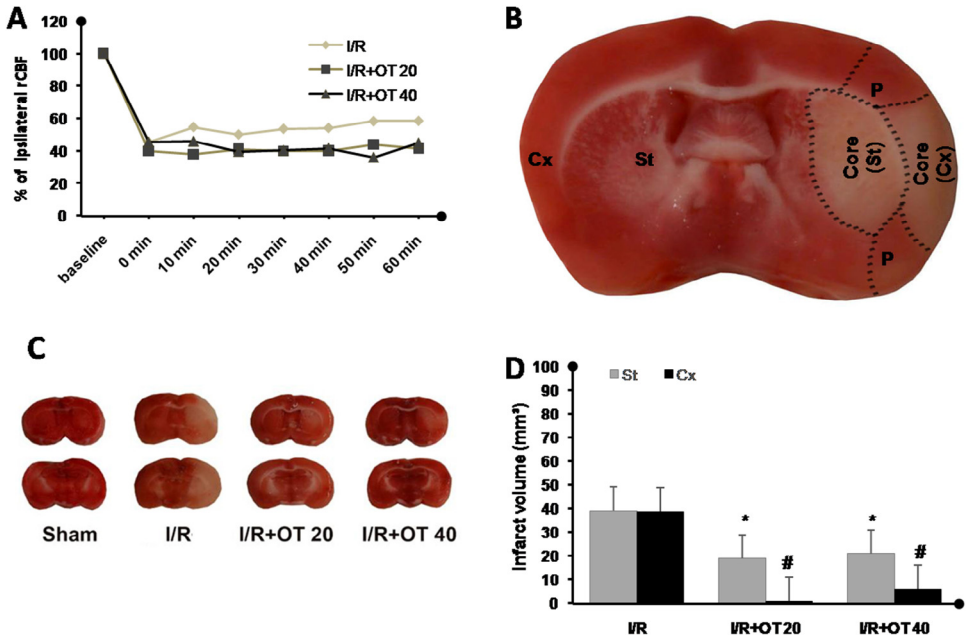

Figure 1. *rCBF measurement of MCA perfusion territory and volumetry of infarct region. (A) Laser Doppler was used for monitoring relative rCBF of MCA in the ipsilateral hemisphere during surgery. rCBF decrease is shown as percentage of baseline value. (B) A central core of severe infarction endangered after ischemia, surrounded by damaged brain tissue but viable and potentially salvageable (penumbra: P). Core infarct volume of rat brains obtained 24 hours after tMCAO with TTC staining. The noninfarct areas which still have metabolism reaction with TTC and produced red stain and dead infarct tissue appears white. (C) Two images of TTC-stained sections are representative of the different groups. (D) Quantitative analysis of infarct volume. OT treatment reduced the infarct volume relative to the I/R group in both St (*P ≤ .05) and Cx (#P < .05) following ischemia. Abbreviations: Cx, cortex; MCA, middle cerebral artery; OT, oxytocin; rCBF, regional cerebral blood flow; St, striatum; tMCAO, transient middle cerebral artery occlusion; TTC, triphenyltetrazolium chloride. (Color version of figure is available online.)*

after stroke.[32] The rats were given different scores through a series of tests, including spontaneous activity and walking, and observing symmetry in limb movement, ability to climb, body proprioception, and response to vibrissae touch. In these tests, the minimum and maximum scores were 3 and 18, respectively. Statistical analysis was performed by Mann–Whitney test by comparing the groups.

Infarct Volumes

Triphenyltetrazolium chloride (TTC) staining was used to evaluate the infarct volume. The animals were anesthetized by 10% chloral hydrate (500 mg/kg body, intraperitoneal) 24 hours after tMCAO induction. The brain was removed after decapitation and was placed in a Brain Matrix (Zivic Instruments, USA) in a freezer for 4 minutes to tighten the soft brain tissue. The brain was sliced into 6 coronal slices in with a thickness of 2 mm. The brain sections were shacked for 10 minutes at 37°C in 1% TTC solution in phosphate-buffered saline. Nonischemic areas turned deep red, whereas damaged areas remained colorless. The sections were arranged from anterior to posterior in a petri dish serially; images of the brain slices were taken by a digital camera with nice separability (Canon, Ohta-ku, Tokyo, Japan); and the stained brain sections were scanned with ImageJ program (version 1.44p, Bethesda, Maryland, USA). Sections were preserved in –80°C for further experiments. The infarct volume in the cerebral cortex (Cx) and striatum (St) region were measured separately (Fig 1, B,C). Total infarct volume was calculated by adding the mean area of each section and multiplied by the thickness of the sections (Fig 1, D).[33]

RNA Isolation and Real-Time PCR

The isolation of total RNA from the penumbra region of samples was carried out with AccuZol reagent (Bioneer, Daedeok-gu, Daejeon, Korea) and transcribed to complementary DNA (cDNA) using iScript Reverse Transcription Supermix (Bio-Rad, Germany), also the cDNA were diluted 1:10 after serial dilutions were made. Primer3 online software was applied to design polymerase chain reaction (PCR) primers, and annealing temperatures were determined using gradient PCR. In this study, the following primers were used: oxytocin receptor (OTR) forward: 5′CTGGCTTTTGTCCCTTTCCC3′ and OTR reverse: 5′TTCCCAGTGCTTTCCAGGAT3. Real-time reverse transcription-PCR reaction was performed in a reaction mixture consisting of 5 µL of master mix (Bio-Rad), 2 µL of water, .5 µL of each primer (10 µM stock), and 2 µL of diluted cDNA. The results were calculated of the ratio between the amount of target gene and the amount of hypoxanthine phosphoribosyltransferase. The

relative changes were analyzed using the ΔΔCT method as previously described.[34]

Tissue Fixation and Immunohistochemistry

The animals were profoundly anesthetized with 10% chloral hydrate (500 mg/kg body, intraperitoneal) 24 hours after tMCAO. Animals were perfused transcardially first by 50 mL of normal saline solution (.9% NaCl) and then by 250 mL of NBF fixative solution (10% neutral buffered formalin, pH = 7). Brains were carefully dissected out and kept in the same fixative solution for another 48 hours at room temperature. After processing, brains were embedded in paraffin wax. The brains were then sectioned at a thickness of 5 μm by a microtome (Diapath, Martinengo, Italy), and sections of forebrain were placed on silane-coated glass slides. These slides were deparaffinized and rehydrated. Before antibody incubation, antigen unmasking was performed in preheated .1 M citrate buffer (pH = 6). Sections were blocked with a blocking solution obtained from kit (UltraTek HRP Anti-Polyvalent, ScyTek Laboratories, Logan, USA) to prevent nonspecific binding of the antibody for 7 minutes at room temperature. Blocking solution was removed and each section was incubated with calpain-1 primary antibody (ab28258, Abcam, Germany) at 1:100 dilution in a humid chamber for 90 minutes. The endogenous peroxidase activity was blocked using 10% hydrogen peroxide–methanol for 10 minutes at room temperature. After washing, sections were incubated with a secondary antibody (Anti-Polyvalent Biotinylated Antibody, ScyTek) and then with horseradish peroxidase in a humid chamber for 10 minutes. Visualization occurred after the reaction with diaminobenzidine. Coverslips were mounted on sections and the slides were examined by a Nikon microscope (Nikon Eclipse Ti-U, Hokkaido, Japan). Calpain–1-positive cells were counted and calculated as number of cells per mm^2.[35]

Western Blotting

The penumbra region of ischemic tissues was taken out and homogenized. After protein quantification, 80 μg of total protein was loaded from each sample. Using sodium dodecyl sulfate polyacrylamide gel (10% gel), proteins were separated by molecular weight and transferred onto polyvinylidene difluoride papers using a semidry transfer method. The membranes were blocked in 5% skim milk for 1 hour and incubated with primary antibodies against β-actin (abcam, 1:5000) and calpain-1 (abcam, 1:2000) at 4°C overnight. After washing, blots were incubated for 1 hour with horseradish peroxidase–conjugated secondary antibody (abcam, 1:3000). Protein bands were illuminated using Clarity western enhanced chemiluminescence substrate kit (BioRad) and visualized on x-ray films. Images were analyzed through densitometry software (ImageJ version 1.44).

Cresyl Violet (Nissl) Staining

For cresyl violet staining, 5-μm coronal sections were used. Deparaffinized and rehydrated tissue sections were stained with .1% cresyl violet solution for 10 minutes. Histological changes were observed by a light microscope (Nikon Eclipse Ti-U) and counted by the Camera Control Unit DS-L2 software. Neuronal count and survival (normal or pyknotic nuclei) in Nissl-stained sections was presented as cells per mm^2 and estimated as follows: (number of surviving neurons/total number of neurons) × 100.

Nitrate Analysis

Griess reagent was applied for nitrite measurement.[36] Reduction of nitrate to nitrite was done using vanadium chloride (VCl3). Tissue lysates were precipitated with trichloroacetic acid (10%). After that, supernatants were collected and used for nitric oxide (NO) measurement. One hundred microliters of each sample and standard (0-100 μM) was pipetted into a 96-well plate. One hundred microliters of VCl3 was added to each well and then mixed with 50 μL of 2% sulfanilamide and 50 μL of .1% N-(1-Naphthyl) ethylenediamine dihydrochloride. Plate was incubated at 37°C for 45 minutes. Absorption was read at a wavelength of 540 nm and concentrations were calculated using the gained standard curve.

Statistical Analysis

In this study, a total of 30 rats were used. Seventeen of them were used for western blot and Nitrate analysis and 13 were subjected to perfusion and immunohistochemistry (IHC) or Nissl staining. SPSS software (version 16.0) was used for data analysis. The one-way analysis of variance and Tukey post hoc tests for the difference among the groups were applied, except for behavioral analysis. In this study, a P value of less than .05 was considered statistically significant.

Results

Cerebral Blood Flow Measurement

Laser Doppler monitoring was used to ensure a successful blockage of MCA. Laser Doppler measurement indicated that in all rats, regional cerebral blood flow values dropped at least more than 55% compared with periischemic basal values significantly after tMCAO induction. Values should also remain stable during MCA occlusion (Fig 1, A). Regional cerebral blood flow values showed no significant differences among the groups.

Assessment of Neurological Function

Behavioral aspects of tMCAO were evaluated in each rat 24 hours after ischemic induction in a blinded manner. Sham-operated animals without tMCAO showed a maximum score of 18 points. The ischemia group showed

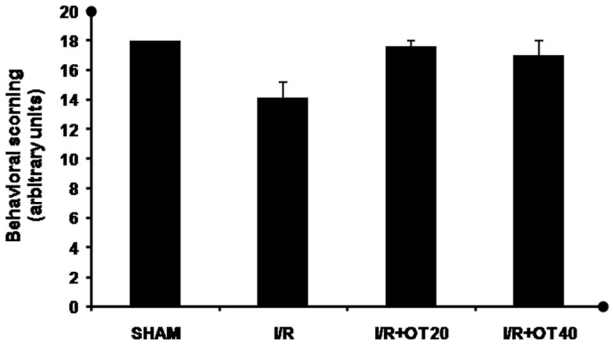

Figure 2. *Neurological behavior score. Garcia test scores in the sham group and I/R, I/R + OT 20, and I/R + OT 40 groups were not significant. Abbreviations: I/R, ischemia–reperfusion; OT 20, oxytocin 20 μg; OT 40, oxytocin 40 μg.*

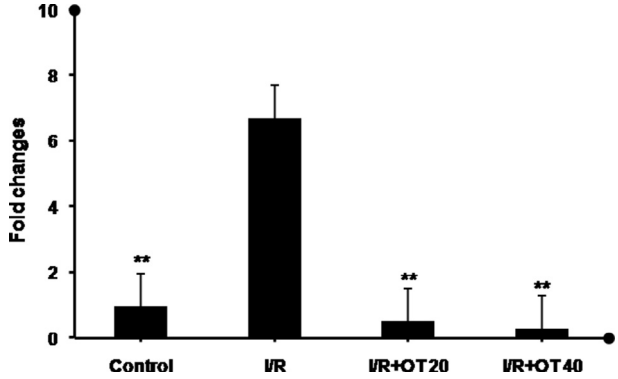

Figure 3. *RT-PCR analysis of OTR gene expression after cerebral ischemia. Ischemia significantly increased the expression of OTR (P < .01) and treatment of oxytocin significantly reduced it (P < .01). Abbreviations: I/R, ischemia–reperfusion; OT 20, oxytocin 20 μg; OT 40, oxytocin 40 μg RT-PCR, reverse transcription polymerase chain reaction.*

sensory–motor deficiency, and a better behavioral condition was observed in all hormone-treated groups, although these data were not statistically significant (Fig 2).

Effects of Oxytocin on the Infarct Volume

The extent of brain damage after tMCAO was determined by evaluating the infarct volume. All animals were sacrificed 24 hours after surgery. TTC-stained brain sections for analysis of infarct volume were examined throughout the ipsilateral Cx and St. Representative TTC-stained brains of the various groups are illustrated in Figure 1, C. All hormone-treated IR/OT 20 and IR/OT 40 groups showed a significant reduction of the infarct region in both St and Cx compared with the I/R group ($P < .05$) (Fig 1, D).

Expression of OTR mRNA

We used real-time PCR to observe the effect of tMCAO on OTR expression in the penumbra region and possible effects of OT on them. The OTR mRNA level in I/R was significantly increased in comparison with control ($P < .01$). The effect of OT treatment on levels of OTR mRNAs was examined 24 hours after I/R, and the hormone-treated IR/OT 20 and IR/OT 40 groups were significantly decreased in comparison with the I/R group ($P < .01$) (Fig 3).

Effects of Oxytocin on Calpain-1 Activation

The expression of calpain-1 protease was evaluated by IHC and Western blot technique (Fig 4). In the ischemia group, the penumbra region of the ipsilateral hemisphere revealed a significant increase in the number of calpain–1-positive cells compared with the contralateral hemisphere (control group). Treatment by OT could decrease the calpain–1-positive cells compared with the ischemia group significantly ($*P < .05$) (Fig 4, B). IHC results were confirmed with immunoblot analysis with a prominent elevation in the levels of calpain-1 (76 kDa fragment) protease after ischemia induction that is attenuated by hormone therapy ($*P < .05$) (Fig 4, C,D).

Effects of Oxytocin on Neuronal Damage

The number of Nissl-stained cells and also neuronal vacuolation and nuclear pyknosis were evaluated and compared. Figure 5 shows representative examples of Nissl staining in the contralateral hemisphere (control group) and the ipsilateral hemisphere (ischemia group) by light microscopic evaluations. In the hormone-treated IR/OT 20 and IR/OT 40 groups, the number of injured neurons after ischemia was significantly lower than in the control group ($**P < .01$).

Effects of Oxytocin on Nitric Oxide Metabolites

Considering that the short half-life of NO makes NO measurement difficult, we evaluated NO metabolites, namely nitrite and nitrate (NOx), which are more stable. The level of NOx in brain tissue can be an indicator of NO production in the region. The concentration of NOx was increased in the ischemic hemisphere compared with that in the nonischemic hemisphere. However, there was no significant change in the levels of NOx in the groups treated with 20 μg and 40 μg OT compared with the ischemia group (I/R) (Fig 6).

Discussion

Our study indicated that the protective effect of OT in ischemic stroke may be associated with calpain–1-dependent mechanisms. Ischemic stroke is the most common cause of adult neurological disorder and 1 of 10 causes of the global burden of disease and premature death worldwide. Therefore, understanding how the brain responds to damage in addition to recovery processes after stroke will provide insight into prevention and new therapeutic strategies.[37-39] Neuroprotective effects

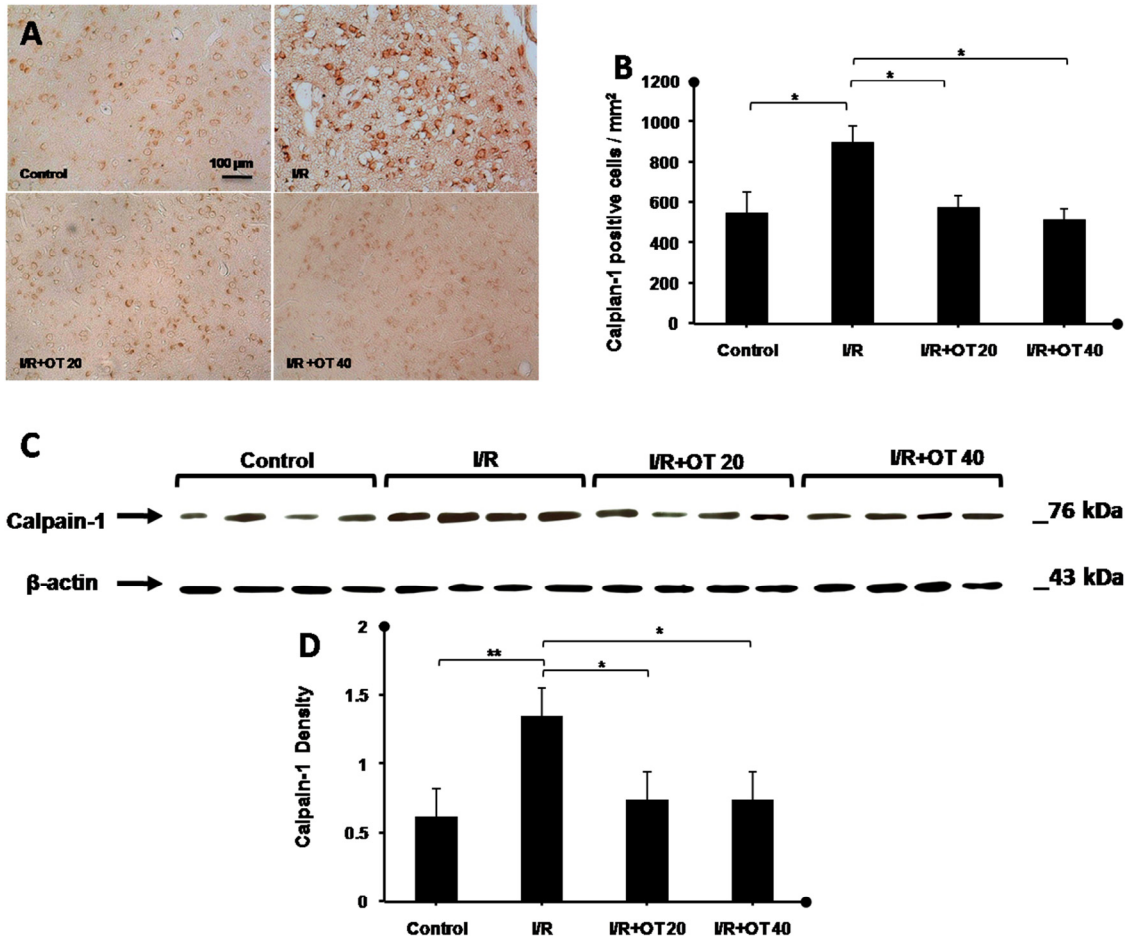

Figure 4. *Calpain-1 analysis after focal cerebral ischemia. Calpain-1 protein was evaluated by IHC and western blot analysis. (A) Photomicrographs of IHC staining of rat brain after ischemia using a polyclonal antibody against calpain-1. The quantity of calpain-positive cells in Cx is shown in (B). The increased number of calpain–1-positive neurons decreased after oxytocin treatment (*P < .05). (C) Photomicrographs of Western blot analysis in Cx (penumbral zone). β-actin (43 kDa) was used as a loading control for analysis of calpain-1 (76 kDa). (D) Densitometry determination was determined by densitometry of calpain-1 band. *P < .05; **P < .01. Abbreviations: Cx, cortex; IHC, immunohistochemistry; I/R, ischemia–reperfusion; OT 20, oxytocin 20 µg; OT 40, oxytocin 40 µg. (Color version of figure is available online.)*

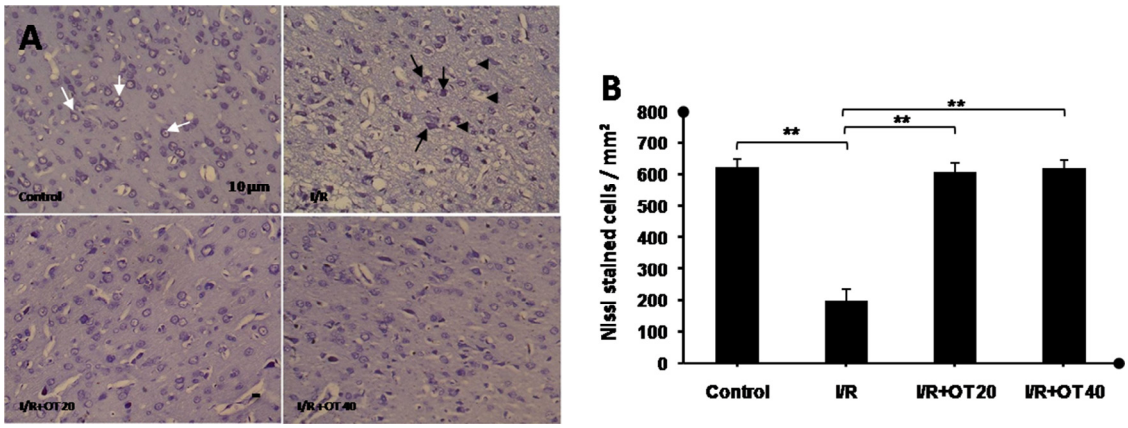

Figure 5. *Nissl staining after focal cerebral ischemia. (A) Photomicrographs of Nissl staining of the brain Cx in the control group and I/R, I/R + OT 20, and I/R + OT 40 groups. Black arrows indicate nuclear pyknosis and arrowheads show neuronal vacuolation as a result of MCAO in the ispilateral hemisphere, which is differentiated from intact neurons in the contralateral hemisphere of the same region with a pale and round nucleus and a prominent nucleolus (white arrows). (B) Neuronal survival rate in the penumbra decreased in animals treated with OT compared with the I/R group (**P < .01). Abbreviations: Cx, cortex; I/R, ischemia–reperfusion; MCAO, middle cerebral artery occlusion; OT 20, oxytocin 20 µg; OT 40, oxytocin 40 µg. (Color version of figure is available online.)*

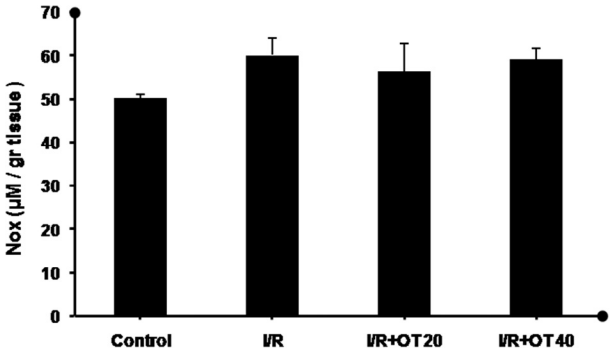

Figure 6. *Levels of NOx after focal cerebral ischemia. The ischemic hemisphere had higher NOx concentrations compared with the nonischemic hemisphere. No significant differences were found between the OT-treated animals and the ischemia group (I/R). Abbreviations: I/R, ischemia–reperfusion; NOx, nitrite and nitrate; OT 20, oxytocin 20 µg; OT 40, oxytocin 40 µg.*

of OT and the underlying molecular mechanisms are under discussion after different ischemia–reperfusion models.[17-23] Our aim here is to investigate the protective effect of OT in a tMCAO rat model and its possible protective mechanisms by investigating calpain expression in the brain.

Determination of infarct size is the primary measure to find out the outcome of ischemia.[28] Our findings show a significant interaction between OT treatment and infarct size (Fig 1). Infarct regions were significantly decreased after 20 µg and 40 µg OT nasal administrations. So far, there have been some reports of reduction in infarct size following treatment with OT after ischemia. Jankowski et al reported that treatment with OT after cardiac ischemic induction in rat can reduce myocardial infarct size and improve heart function.[40] A single research noted that the effects of the OT hormone after cerebral ischemia improved social neuroprotection. Karelina et al concluded that endogenous increase in OT level could decrease infarct size.[27] Different mechanisms such as regulating and balancing of anti-inflammatory and proinflammatory cytokines have been considered as the cause of decreasing infarct volume after OT treatment.[40]

Our findings have shown significant changes in the infarct volume, although no significant difference was found among behavioral variables (Fig 2). These results are consistent with the findings of Desland et al, which is likely due to the small number of animals in their study (n = 6).[41]

It is well known that ischemia causes an increase in inflammatory response.[42] Although Szeto et al showed that in vitro modeling of lipopolysaccharide-induced inflammation, OTR expression increases,[43] for the first time, the present study reports that OTR expression increases after cerebral ischemia. Several studies have indicated that OT has anti-inflammatory activity.[22,44] Here, we observed a decrease in OTR gene expression after OT treatment in the peri-infarct area, which seems to be due to the anti-inflammatory activity of OT.

Our results show a significant decrease in calpain-1 expression after OT administration in the tMCAO model (Fig 4). High levels of glutamate and excessive activation of glutamate receptors (N-methyl-D-aspartate, Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, and kainate receptors) lead to influx of Ca2+ through these receptors immediately after the ischemic insult.[45] Influx of calcium into cells known as a signaling cascade, with overactivation of Ca2+-dependent proteases such as calpain-1, is located mainly in the neuronal cell bodies and dendrites. OT plays a key role in neurons. The presence of OTRs has been shown in several brain regions, such as the bed nucleus of the stria terminalis, lateral septum, anterior olfactory nucleus, pyriform cortex, olfactory bulbs, amygdale, and hippocampus.[46] Indeed, neurons express OTRs in all brain regions.[47] OT decreases the number of functional glutamatergic synapses at the cellular level and the activity of N-methyl-D-aspartate receptors, inhibiting release of the main excitatory amino acids such as glutamate and glutamate activation.[48-50] This might be related to the ability of OT in decreased expression of calpain. As we reported previously, the activity of calpain-1 protease in neurons following focal ischemia causes neural apoptosis.[35] Apoptosis occurs through 2 pathways in cerebral ischemia: mitochondrial (intrinsic pathway) and the activation of death receptors (extrinsic pathway). In the intrinsic apoptotic pathway, an increase in the intracellular Ca^{2+} level leads to overactivation of calpain proteases.[51] This pathway is initiated by cleavage of Bid to truncated Bid (tBid), which leads to conformational changes in the other proapoptotic members such as Bak, Bax, Bad, and BclXS; extracellular release of cytochrome c; activation of caspase-3; and finally DNA damage and apoptosis.[52] Previous studies have shown that pretreatment with OT after cardiac ischemia insert a protection by reducing Bax and p53 gene expression and increasing Bcl-2.[53,54] It has also been supposed that pretreatment with OT in ischemia could provide neuroprotection through Type A gamma-aminobutyric acid receptors modulation.[25] Type A gamma-aminobutyric acid receptors—major inhibitory neurotransmitter receptors in the central nervous system—are downregulated after ischemia in in vitro conditions and causes calpain-dependent neuronal apoptosis.[55] By and large, these data suggest that OT administration has a protective role following the ischemia induction in different organs.[17-23] Our study confirms the beneficial effects of OT treatment in the brain after ischemia by preventing calpain overexpression.

Previous studies have indicated that the involvement of NO after the stroke leads to the activation of calpain and caspase-3.[56,57] On the other hand, a beneficial effect of NO has been shown in some studies through the release of NO. After determining the neuroprotective effect of OT through calpain-1 regulation, we examined the role of OT in NOx production.[20,58] Our findings indicate that treatment with 20 µg and 40 µg doses do not change the level of NO production (Fig 6). Our findings seem to be

consistent with the results of other studies that showed OT function depends on its dose.[59-61]

Biochemical events such as apoptosis, oxidative stress, and inflammatory reaction occur during ischemia–reperfusion and lead to permanent injury of proteins, DNA, and cellular organelles that eventually causes the death of neurons.[51] Markers other than Nissl staining are commonly used for the detection of neuronal cell death.[62] Calpain has been implicated in cell death processes, and inhibition of calpain provides neuroprotection.[63] In this study, many neurons in the I/R group showed an aberrant morphology, including shrunken cell bodies and nuclear pyknosis. OT treatment improved morphology damage in neurons (Fig 5). This refers to the neuroprotective role of OT, which could result in the inhibition of calpain.

Overall, these findings will add to our knowledge of the positive effects of OT on the outcomes of stroke. There is a need for further research to ascertain which factors are involved in OT-induced interactions during or after ischemic stroke.

Conclusion

OT could have an important role in protecting neurons against cerebral ischemia. However, little attention has been paid to the mechanisms of OT action after ischemia. This study confirms that the protective effect of OT is associated with calpain–1-dependent apoptotic pathway.

References

1. Kamat S, Michelson A, Benoit S, et al. Tissue plasminogen activator for acute ischemic stroke. N Engl J Med 1996;1996:1405-1406.
2. Organization WH. The top 10 causes of death. 2014.
3. Feigin VL, Forouzanfar MH, Krishnamurthi R, et al. Global and regional burden of stroke during 1990-2010: findings from the Global Burden of Disease Study 2010. Lancet 2014;383:245-255.
4. Del Zoppo GJ, Poeck K, Pessin MS, et al. Recombinant tissue plasminogen activator in acute thrombotic and embolic stroke. Ann Neurol 1992;32:78-86.
5. Neumar RW, Meng FH, Mills AM, et al. Calpain activity in the rat brain after transient forebrain ischemia. Exp Neurol 2001;170:27-35.
6. Emberson J, Lees KR, Lyden P, et al. Effect of treatment delay, age, and stroke severity on the effects of intravenous thrombolysis with alteplase for acute ischaemic stroke: a meta-analysis of individual patient data from randomised trials. Lancet 2014;384:1929-1935.
7. Jauch EC, Saver JL, Adams HP, et al. Guidelines for the early management of patients with acute ischemic stroke a guideline for healthcare professionals from the American Heart Association/American Stroke Association. Stroke 2013;44:870-947.
8. Won S-J, Kim D-Y, Gwag B-J. Cellular and molecular pathways of ischemic neuronal death. BMB Rep 2002;35:67-86.
9. Wang Q, Tang XN, Yenari MA. The inflammatory response in stroke. J Neuroimmunol 2007;184:53-68.
10. Zheng D, Wang G, Li S, et al. Calpain-1 induces endoplasmic reticulum stress in promoting cardiomyocyte apoptosis following hypoxia/reoxygenation. Biochim Biophys Acta 2015;1852:882-892.
11. Momeni HR. Role of calpain in apoptosis. Cell J 2011;13:65-72.
12. Yamada KH, Kozlowski DA, Seidl SE, et al. Targeted gene inactivation of calpain-1 suppresses cortical degeneration due to traumatic brain injury and neuronal apoptosis induced by oxidative stress. J Biol Chem 2012;287:13182-13193.
13. Hori M, Nakamachi T, Rakwal R, et al. Unraveling the ischemic brain transcriptome in a permanent middle cerebral artery occlusion model by DNA microarray analysis. Dis Model Mech 2011;doi:10.1242/dmm.008276. dmm. 008276.
14. Singhal AB. A review of oxygen therapy in ischemic stroke. Neurol Res 2007;29:173-183.
15. Bethlehem RA, van Honk J, Auyeung B, et al. Oxytocin, brain physiology, and functional connectivity: a review of intranasal oxytocin fMRI studies. Psychoneuroendocrinology 2013;38:962-974.
16. Boccia M, Petrusz P, Suzuki K, et al. Immunohistochemical localization of oxytocin receptors in human brain. Neuroscience 2013;253:155-164.
17. Senturk GE, Erkanli K, Aydin U, et al. The protective effect of oxytocin on ischemia/reperfusion injury in rat urinary bladder. Peptides 2013;40:82-88.
18. Ondrejcakova M, Ravingerova T, Bakos J, et al. Oxytocin exerts protective effects on in vitro myocardial injury induced by ischemia and reperfusion this article is one of a selection of papers from the NATO Advanced Research Workshop on Translational Knowledge for Heart Health (published in part 1 of a 2-part Special Issue). Can J Physiol Pharmacol 2009;87:137-142.
19. Akdemir A, Erbas O, Gode F, et al. Protective effect of oxytocin on ovarian ischemia-reperfusion injury in rats. Peptides 2014;55:126-130.
20. Tas Hekimoglu A, Toprak G, Akkoc H, et al. Oxytocin ameliorates remote liver injury induced by renal ischemia-reperfusion in rats. Korean J Physiol Pharmacol 2013;17:169-173.
21. Faghihi M, Alizadeh AM, Khori V, et al. The role of nitric oxide, reactive oxygen species, and protein kinase C in oxytocin-induced cardioprotection in ischemic rat heart. Peptides 2012;37:314-319.
22. Tuğtepe H, Şener G, Bıyıklı NK, et al. The protective effect of oxytocin on renal ischemia/reperfusion injury in rats. Regul Pept 2007;140:101-108.
23. Gonzalez-Reyes A, Menaouar A, Yip D, et al. Molecular mechanisms underlying oxytocin-induced cardiomyocyte protection from simulated ischemia–reperfusion. Mol Cell Endocrinol 2015;412:170-181.
24. Ceanga M, Spataru A, Zagrean A-M. Oxytocin is neuroprotective against oxygen–glucose deprivation and reoxygenation in immature hippocampal cultures. Neurosci Lett 2010;477:15-18.
25. Kaneko Y, Pappas C, Tajiri N, et al. Oxytocin modulates GABAAR subunits to confer neuroprotection in stroke in vitro. Sci Rep 2016;6.
26. Brown CH, Grattan DR. Does maternal oxytocin protect the fetal brain? Trends Endocrinol Metab 2007;18:225-226.
27. Karelina K, Stuller KA, Jarrett B, et al. Oxytocin mediates social neuroprotection after cerebral ischemia. Stroke 2011;42:3606-3611.
28. Karelina E. Mechanisms of social neuroprotection after cerebral ischemia. The Ohio State University, 2010.

29. Yuan L, Liu S, Bai X, et al. Oxytocin inhibits lipopolysaccharide-induced inflammation in microglial cells and attenuates microglial activation in lipopolysaccharide-treated mice. J Neuroinflammation 2016;13:1.
30. Lukas M, Neumann ID. Nasal application of neuropeptide S reduces anxiety and prolongs memory in rats: social versus non-social effects. Neuropharmacology 2012;62:398-405.
31. Neumann ID, Maloumby R, Beiderbeck DI, et al. Increased brain and plasma oxytocin after nasal and peripheral administration in rats and mice. Psychoneuroendocrinology 2013;38:1985-1993.
32. Garcia JH, Wagner S, Liu K-F, et al. Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats statistical validation. Stroke 1995;26:627-635.
33. Lammerding L, Slowik A, Johann S, et al. Poststroke inflammasome expression and regulation in the peri-infarct area by gonadal steroids after transient focal ischemia in the rat brain. Neuroendocrinology 2016;103:460-475.
34. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 2001;25:402-408.
35. Vahidinia Z, Alipour N, Atlasi MA, et al. Gonadal steroids block the calpain-1-dependent intrinsic pathway of apoptosis in an experimental rat stroke model. Neurol Res 2016;1-11.
36. Miranda KM, Espey MG, Wink DA. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide 2001;5:62-71.
37. Murray CJ, Lopez AD. Measuring the global burden of disease. NEJM 2013;369:448-457.
38. Mann GE. Molecular mechanisms underlying neurovascular protection in stroke. J Physiol 2011;589:4103-4104.
39. Tu X-K, Yang W-Z, Chen J-P, et al. Curcumin inhibits TLR2/4-NF-κB signaling pathway and attenuates brain damage in permanent focal cerebral ischemia in rats. Inflammation 2014;37:1544-1551.
40. Jankowski M, Bissonauth V, Gao L, et al. Anti-inflammatory effect of oxytocin in rat myocardial infarction. Basic Res Cardiol 2010;105:205-218.
41. Desland FA, Afzal A, Warraich Z, et al. Manual versus automated rodent behavioral assessment: comparing efficacy and ease of Bederson and Garcia neurological deficit scores to an open field videotracking system. J Cent Nerv Syst Dis 2014;6:7.
42. Ji J, Su L, Liu Z. Critical role of calpain in inflammation (Review). Biomed Rep 2015;5:647-652.
43. Szeto A, Sun-Suslow N, Mendez AJ, et al. Regulation of the macrophage oxytocin receptor in response to inflammation. Am J Physiol Endocrinol Metab 2017;312:E183-E189.
44. Düşünceli F, İşeri SÖ, Ercan F, et al. Oxytocin alleviates hepatic ischemia–reperfusion injury in rats. Peptides 2008;29:1216-1222.
45. Huang Y, Wang KK. The calpain family and human disease. Trends Mol Med 2001;7:355-362.
46. Febo M, Shields J, Ferris CF, et al. Oxytocin modulates unconditioned fear response in lactating dams: an fMRI study. Brain Res 2009;1302:183-193.
47. Meddle SL, Bishop VR, Gkoumassi E, et al. Dynamic changes in oxytocin receptor expression and activation at parturition in the rat brain. Endocrinology 2007;148:5095-5104.
48. Qi J, Yang J-Y, Wang F, et al. Effects of oxytocin on methamphetamine induced conditioned place preference and the possible role of glutamatergic neurotransmission in the medial prefrontal cortex of mice in reinstatement. Neuropharmacology 2009;56:856-865.
49. Caruso S, Agnello C, Campo M, et al. Oxytocin reduces the activity of N-methyl–D–aspartate receptors in cultured neurons. J Endocrinol Invest 1993;16:921-924.
50. Breton J-D, Veinante P, Uhl-Bronner S, et al. Oxytocin-induced antinociception in the spinal cord is mediated by a subpopulation of glutamatergic neurons in lamina I-II which amplify GABAergic inhibition. Mol Pain 2008;4:19.
51. Peng Z, Wang S, Chen G, et al. Gastrodin alleviates cerebral ischemic damage in mice by improving anti-oxidant and anti-inflammation activities and inhibiting apoptosis pathway. Neurochem Res 2015;40:661-673.
52. Broughton BR, Reutens DC, Sobey CG. Apoptotic mechanisms after cerebral ischemia. Stroke 2009;40:e331-e339.
53. Alizadeh AM, Faghihi M, Khori V, et al. Oxytocin protects cardiomyocytes from apoptosis induced by ischemia–reperfusion in rat heart: role of mitochondrial ATP-dependent potassium channel and permeability transition pore. Peptides 2012;36:71-77.
54. Mostafa DG, Khaleel EF, Abdel-Aleem GA. Mechanism of action of oxytocin as cardioprotection in rat model of myocardial infarction. IOSR J Dent Med Sci 2015;14:25-36.
55. Mele M, Ribeiro L, Inácio AR, et al. GABA A receptor dephosphorylation followed by internalization is coupled to neuronal death in in vitro ischemia. Neurobiol Dis 2014;65:220-232.
56. Sun M, Zhao Y, Gu Y, et al. Inhibition of nNOS reduces ischemic cell death through down-regulating calpain and caspase-3 after experimental stroke. Neurochem Int 2009;54:339-346.
57. Sun M, Zhao Y, Gu Y, et al. Neuroprotective actions of aminoguanidine involve reduced the activation of calpain and caspase-3 in a rat model of stroke. Neurochem Int 2010;56:63441.
58. Moghimian M, Faghihi M, Karimian SM, et al. Upregulated Hsp27 expression in the cardioprotection induced by acute stress and oxytocin in ischemic reperfused hearts of the rat. Chin J Physiol 2014;57:329-334.
59. Houshmand F, Faghihi M, Zahediasl S. Biphasic protective effect of oxytocin on cardiac ischemia/reperfusion injury in anaesthetized rats. Peptides 2009;30:2301-2308.
60. Gutkowska J, Jankowski M, Antunes-Rodrigues J. The role of oxytocin in cardiovascular regulation. Braz J Med Biol Res 2014;47:206-214.
61. Anvari MA, Imani A, Faghihi M, et al. The administration of oxytocin during early reperfusion, dose-dependently protects the isolated male rat heart against ischemia/reperfusion injury. Eur J Pharmacol 2012;682:137-141.
62. Zhou M, Baudry M. Developmental changes in NMDA neurotoxicity reflect developmental changes in subunit composition of NMDA receptors. J Neurosci 2006;26:2956-2963.
63. Tsubokawa T, Yamaguchi-Okada M, Calvert JW, et al. Neurovascular and neuronal protection by E64d after focal cerebral ischemia in rats. J Neurosci Res 2006;84:832-840.