

A preliminary report: genistein attenuates cerebral ischemia injury in ovariectomized rats *via* regulation of the PI3K-Akt-mTOR pathway

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Abstract. Stroke is a leading cause of disability and death in the worldwide. Therefore, prevention of stroke is critically important. Genistein, a natural phytoestrogen extracted from soybeans, has been found to be a potential neuroprotective agent for stroke prevention. However, the role of genistein and its underlying mechanism in ovariectomized rats has been rarely evaluated. In this study, ovariectomized rats were treated with genistein (10 mg/kg) or vehicle daily for two weeks before they received middle cerebral artery occlusion (MCAO) and reperfusion. Seventy-two hours after reperfusion, the neurological function was evaluated by Garcia test, infarct volumes were detected by 2,3,5-triphenyltetrazolium chloride staining; and neuronal damage and cell apoptosis were detected by Nissl and Tunel staining in the ischemic penumbra, respectively. In addition, Western blotting was used to detect the activity of PI3K-Akt-mTOR signal pathway in the ischemic penumbra in different groups. And we found that genistein treatment in ovariectomized rats significantly improved neurological outcomes, reduced infarct volumes, decreased neuronal damage and cell apoptosis, and increased the activity of PI3K-Akt-mTOR signal pathway. Our findings indicated that treatment genistein could alleviate neuronal apoptosis induced by cerebral ischemia in ovariectomized rats *via* promoting the activity of PI3K-Akt-mTOR signal pathway, which provides a new molecular mechanism for the neuroprotective effects of genistein against stroke.

Key words: Genistein — PI3K-Akt-mTOR — Apoptosis — Cerebral ischemia — Neuroprotection

Abbreviations: ERT, estrogen replacement treatment; MCAO, middle cerebral artery occlusion; mTOR, mammalian target of rapamycin; rTPA, recombinant tissue-type plasminogen activator; TTC, 2,3,5-triphenyltetrazolium chloride.

Introduction

Stroke is a leading cause of disability and death both in developed countries and in developing countries, and results from artery blockage and vascular occlusion by thrombus that leads to a decrease in blood supply in the affected region

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(Lim et al. 2007; van der Worp et al. 2007). Nearly 80% of strokes are caused by ischemic attack (consequent of thrombosis, embolism and/or hypoperfusion) and the others are the result of hemorrhage (European Stroke Organisation Executive Committee, 2008). Following stroke, a number of disorders, including weakness, dysphasia, sensory loss, ataxia, depression, anxiety, anhedonia, etc. occur which directly influence patient's life style and increase stroke relapse (Sagen et al. 2010; Nabavi et al 2014).

In recent years, diet and dietary components have been regarded as important strategies to prevent the stroke or mitigate stroke injury. A plethora of researches showed the promising effect of soy-based genistein, which is one of

the predominant isoflavone compounds and is a selective estrogen receptor modulator, in the prevention and mitigation of ischemic stroke-induced damages (Liang et al. 2008; Castelló-Ruiz et al. 2011; Wang et al. 2014; Miao et al. 2018). However, the underlying molecular mechanism remains largely unknown.

As an important signal transduction pathway, PI3K-Akt-mTOR is involved in many cellular processes, including cell apoptosis, survival and proliferation (Liu et al. 2017). Phosphatidylinositol 3 kinase (PI3K) is an intracellular phosphatidylinositol kinase (Hennessy et al. 2005). Protein kinase B (Akt), a serine/threonine kinase, is a primary downstream target in the transduction pathway of PI3K signaling, which is a key information molecule that promotes cell survival, inhibits apoptosis (Ouyang et al. 1999) and maintains normal functions (Castaneda et al. 2010). Activated Akt can transmit signals to a variety of downstream substrates including mammalian target of rapamycin (mTOR), which is a serine/threonine kinase that can benefit cell growth, survival, and metabolism (Wullschlegler et al. 2006). A number of researches have demonstrated the critical role of PI3K-Akt-mTOR signal pathway in alleviating cerebral ischemic injury (Kamada 2007; Pastor et al. 2009). However, the role of PI3K-Akt-mTOR signal pathway in genistein's neuroprotection against ischemic stroke has rarely been explored.

Accordingly, we aimed to explore the possible role of PI3K-Akt-mTOR signal pathway in genistein's neuroprotection against cerebral ischemia in ovariectomized rats, which would supply a new molecular mechanism for genistein neuroprotection.

Materials and Methods

Animals

One hundred and forty adult female Sprague-Dawley rats weighing 300 g were obtained from the Laboratory Animal Center of the Mudanjiang Medical University. These rats were divided into the following 4 groups ($n = 35$): 1) Sham group, sham operation group; 2) Con group, control group consisted of female rats with intact ovaries who received middle cerebral artery occlusion (MCAO-R); 3) OVX group, the rats received ovariectomy surgeries, vehicle (sesame oil) treatment and MCAO-R injury; 4) Gen group, the rats received ovariectomy surgeries, genistein treatment and MCAO-R injury. All the animals were maintained under the following standard conditions: 12:12-h light-dark cycle, 50–60% environmental humidity, a temperature of $25 \pm 1^\circ\text{C}$ and *ad lib* access to food and water. All animal experimental procedures followed a protocol approved by the Ethics Committee for Animal Experimentation of the Mudanjiang Medical University, China.

Ovariectomy and drug treatment

Ovariectomy was performed through dorsolateral incisions as previously described (Marcondes et al. 2002) ($n = 35$). The animals in the sham group were subjected to the same operation; however, their ovaries were kept intact. Following ovariectomy, vaginal smears were performed for 5 days to confirm the success of the ovariectomy and the cessation of the estrous cycle. In the OVX group, only vehicle (sesame oil) was administered. In the Gen group, genistein (TOCRIS, Catalog: 1110/50; diluted in sesame oil solution) was administered *intraperitoneally* (*i.p.*) at a dose of 10 mg/kg once daily for two weeks, which was based on the dosage used in a previous study (Castelló-Ruiz et al. 2011).

Middle cerebral artery occlusion and reperfusion (MCAO-R)

The intraluminal filament model of MCAO was used to induce transient focal cerebral ischemia as described previously (Wang et al. 2009) ($n = 35$). In brief, after the rats were anesthetized with 10% chloral hydrate (0.3 ml/100g weight, *i.p.*), a heat-blunted 3–0 nylon suture was inserted into the right common carotid artery to obstruct the middle cerebral artery. The right external carotid artery and the common carotid artery were both simultaneously ligated. After 1.5 h of transient occlusion, cerebral blood flow was restored by removing the nylon suture for 72 h. Regional cerebral blood flow was measured *via* transcranial laser Doppler flowmetry (PeriFlux 5000, Perimed AB, Sweden). Rats with >80% flow reduction during the ischemic period and >70% flow recovery within the first 10 min of reperfusion were included in the study. Physiological variables monitored included rectal temperature, blood pressure, heart rate, blood gas, and glucose levels and were monitored and data are shown in Supplementary Materials, Table S1. The animals in the sham group were subjected to the same operation; however, no suture was inserted into the right common carotid artery to obstruct the middle cerebral artery. The rats in Con, OVX and Gen group were all received obstruction of the middle cerebral artery.

Neurological score

Neurological deficits were evaluated 24 h after reperfusion by a blinded observer based on the Garcia Test (Garcia et al. 1995), as shown in the Table 1 ($n = 12$). There are six scoring items in Garcia Test. Each scoring item includes 0 point, 1 point, 2 points and 3 points and rats were scored by all these six items. The higher the scores, the better the neurological function of the rat.

Table 1. Garcia Test

	Score			
	0	1	2	3
Spontaneous activity (in cage for 5 min)	No movement	Barely moves	Moves but does not approach at least three sides of cage	Moves and approaches at least three sides of cage
Symmetry of movements (four limbs)	LS: no movement	LS: slight movement	LS: moves slowly	Both sides: move symmetrically
Symmetry of forelimbs (out stretching while held by tail)	LS: no movement, no outreaching	LS: slight movement to outreach	LS: moves and outreaches less than right	Symmetrically outreach
Climbing wall of wire cage	–	Fails to climb	LS is weak	Normal climbing
Reaction to touch on either side of trunk	–	No response on LS	Weak response on LS	Symmetrical response
Response to vibrissae touch	–	No response on LS	Weak response on LS	Symmetrical response

LS, left side.

Assessment of infarct volume

After neurological scoring, infarct volume was assessed via 2,3,5-triphenyltetrazolium chloride (TTC) staining as previously described (Wang et al. 2009) ($n = 12$). Briefly, after the rats were anesthetized with 10% chloral hydrate (0.3 ml/100 g weight, *i.p.*), their brains were rapidly removed and cooled in ice-cold saline for 10 min. Six slices of brain were obtained at 2-mm intervals from the intersection of the lambdoidal suture to the front using a brain matrix. The slices were stained in a 2% solution of TTC at 37°C for 30 min and then transferred to 4% paraformaldehyde in 0.01 M phosphate-buffered saline (PBS, pH 7.4) for a 24-h fixation period. The brain slices were photographed (Canon IXUS 220HS), and infarct volume (the unstained areas) was determined using image analysis software (Adobe Photoshop CS3). Corrections were made for swelling, and relative infarct size was determined based on the following equation: relative infarct size = (contralateral area – ipsilateral non-infarct area)/contralateral area.

Western blot

In brief, 72 h after MCAO, the rats were rapidly anesthetized with 10% chloral hydrate (0.3 ml/100g weight, *i.p.*) ($n = 6$). Then the rats were decapitated, the skull and meninges were removed and the whole brain tissue was taken out. The region of the ischemic penumbra was microdissected according to established protocols in rodent models of unilateral proximal MCAO (Ashwal et al. 1998). The brain tissue extracted from each rat was independently homogenized on ice in an RIPA lysis buffer containing 1 mM PMSF and performed protein electrophoresis. The extracted proteins were separated using 10% SDS-PAGE and then electrically transferred to polyvinylidene difluoride membranes (the specific conditions of

electrophoresis and transfer varied according to the molecular weight of the target protein). Subsequently, the membranes were blocked in 5% nonfat dry milk diluted in TBST for 1 h at room temperature. The membranes were then incubated with the primary antibodies: rabbit anti-PI3K (1:1000, Cell Signaling Technology, #4292), rabbit anti-Phospho-Akt (1:1000, Cell Signaling Technology, #4060), rabbit anti-Akt (1:1000, Cell Signaling Technology, #4685), rabbit anti-Phospho-mTOR (1:1000, Cell Signaling Technology, #2971), rabbit anti-mTOR (1:1000, Cell Signaling Technology, #2972), and mouse β -actin (1:1000, Cell Signaling Technology). After incubation overnight at 4°C, the membranes were washed with Tris-buffered saline and incubated with a secondary antibody for about 2 h at room temperature. Protein bands were visualized using the LI-COR Odyssey System (LI-COR Biotechnology, USA) and densitometrically analyzed by automated ImageJ software (NIHImage, Version 1.61).

Nissl staining

Nissl staining was performed to observe morphological changes in cells within the ischemic penumbra 72 h following MCAO ($n = 6$). The rats were anesthetized with 10% chloral hydrate (0.3 ml/100g weight, *i.p.*), the brains were perfused with cold 4% paraformaldehyde in 0.01 M PBS. After post-fixation, the brains were successively placed into 20% and 30% sucrose solutions at 4°C. After the brains were equilibrated with the sucrose, 12- μ m-thick sections were prepared using a Leica CM1900 frozen slicer. Then, the frozen sections were stained with 0.1% cresyl violet for 20 min, rinsed with PBS, dehydrated in a graded alcohol series, cleared with xylene, and mounted with neutral gum. The sections were observed using light microscopy. The total number of damaged neurons in the penumbra was counted in 5 different fields of view for each section by an observer

blinded to the treatment group manner *via* light microscopy at $\times 400$ magnification (BX51; Olympus, Tokyo, Japan).

Tunel staining

To detect *in situ* DNA fragmentation, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (Tunel) staining was performed using an In Situ Cell Death Detection Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions ($n = 6$). TUNEL staining was performed on 5- μm -thick paraffin-embedded coronal brain sections. The sections were treated with 0.3% (v/v) H_2O_2 for 20 minutes and then incubated in a TUNEL reaction mixture for 1 hour at 37°C . The sections were then incubated in converter-peroxidase for 30 minutes at 37°C . After 3 washes in PBS, sections were developed with 3,3'-diaminobenzidine for 5 minutes at room temperature. The total number of TUNEL-positive neurons in the region of the penumbra was counted in 5 different fields of view for each section by an observer blinded to the treatment group manner *via* light microscopy at $\times 400$ magnification (BX51; Olympus, Tokyo, Japan).

Statistical analyses

Data was presented as mean \pm standard deviation (SD), and statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) Version 16.0 (SPSS Inc, Chicago, IL, USA) for windows, except for neurological scores which were expressed as median with interquartile range and analyzed by Kruskal-Wallis test followed by the Mann-Whitney U test. Comparison between two groups was performed using Student's *t*-test. $p < 0.05$ was considered statistically significant.

Results

Genistein treatment significantly alleviated cerebral ischemia injury

As shown in Figure 1A, there was no difference in the regional cerebral blood flow among the three groups prior to ischemia, during ischemia or after reperfusion.

To evaluate the neuroprotective effect of genistein against ischemia-reperfusion injury induced *via* MCAO, we performed neurological deficit score tests and assessments of infarct volume 72 h after MCAO. As shown in Figure 1B, compared with the Con group, the rats in OVX group had significantly lower neurological deficit scores (** $p < 0.01$) which represented worse neurological situation. Genistein treatment significantly improved the neurological deficit scores compared with the OVX group (# $p < 0.05$). There was no significant difference between the Con group and Gen group.

As shown in Figure 2B, the infarct volume in the Con group was $20.1 \pm 4.9\%$. The ovariectomized surgery significantly increased infarct volume to $29.7 \pm 5.1\%$ (** $p < 0.01$ vs. the Con group), and the genistein treatment significantly decreased the infarct volume to $24.3 \pm 4.1\%$ (# $p < 0.05$ vs. the OVX group). There was no significant difference between the Con and Gen group.

Genistein treatment attenuated neuronal damage and cell apoptosis

Next, we used Nissl and Tunel staining to examine the neuronal damage and cell apoptosis in the ischemic penumbra 72 h after MCAO. As shown in Figure 3, the injured neurons

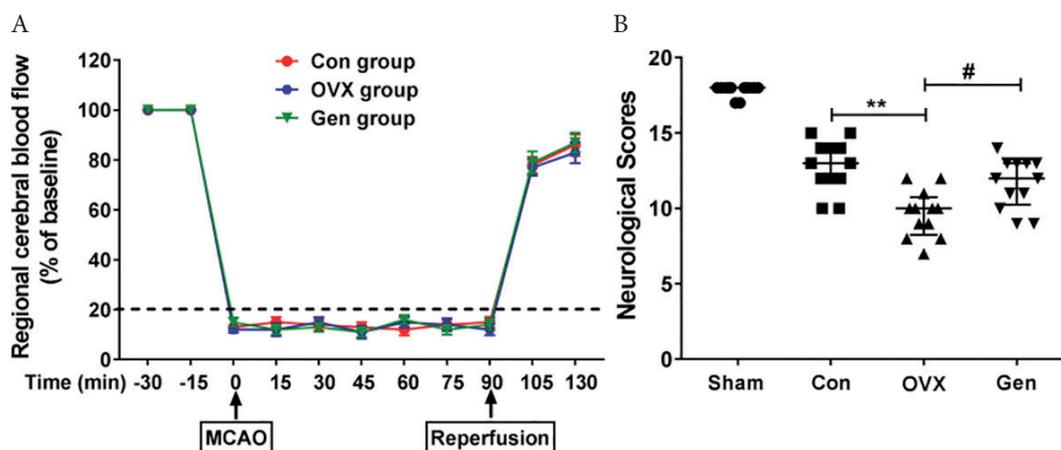


Figure 1. A. Regional cerebral blood flow in ischemic hemispheres of rats during MCAO surgery. B. Neurological scores after ischemia-reperfusion in the different groups. The Garcia Test was applied to determine neurological deficits in the rats undergoing ischemia-reperfusion injury in the different groups. Data are expressed as median \pm interquartile range (** $p < 0.01$, # $p < 0.05$, $n = 12$). Con, Control; OVX, ovariectomy; Gen, genistein; MCAO, middle cerebral artery occlusion.

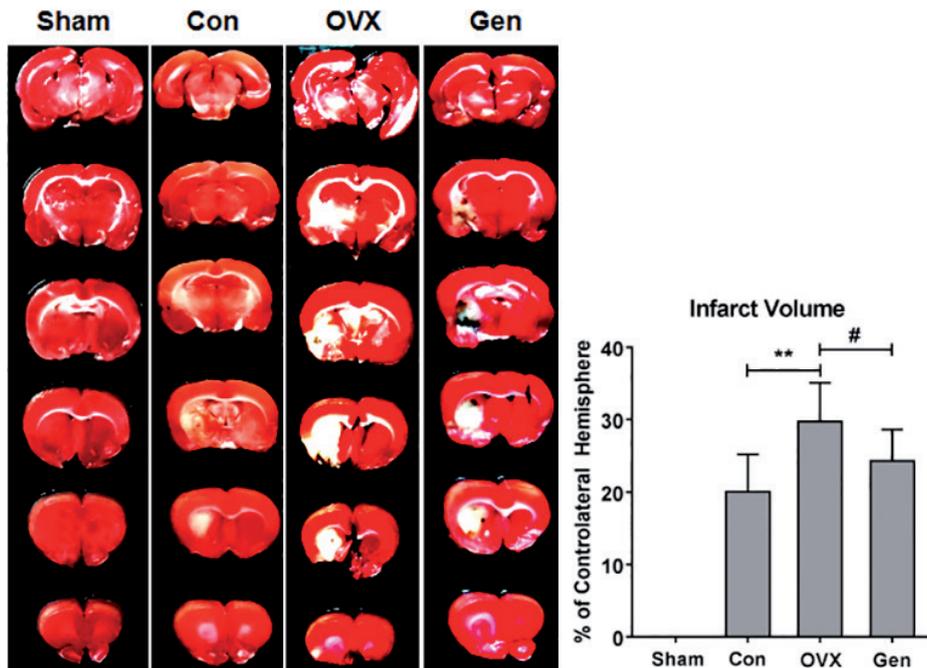


Figure 2. Results of TTC staining after ischemia-reperfusion in the different groups. **A.** Representative photographs showing infarct volumes in the different group following ischemia-reperfusion. **B.** Infarct volume in rats undergoing ischemia-reperfusion in the different groups. (** $p < 0.01$, # $p < 0.05$; $n = 12$). For abbreviations, see Fig. 1.

showed shrunken cell bodies accompanied by shrunken and pyknotic nuclei. The ratio of intact neurons in the Con group was $71.2 \pm 7.0\%$, the ovariectomy treatment significantly decreased the ratio of intact neurons to $59.6 \pm 6.4\%$ (** $p <$

0.01 vs. the Con group). And genistein treatment significantly increased the ratio of intact neurons to $65.4 \pm 6.9\%$ (# $p < 0.05$ vs. the OVX group). There was no significant difference between the Con and Gen group.

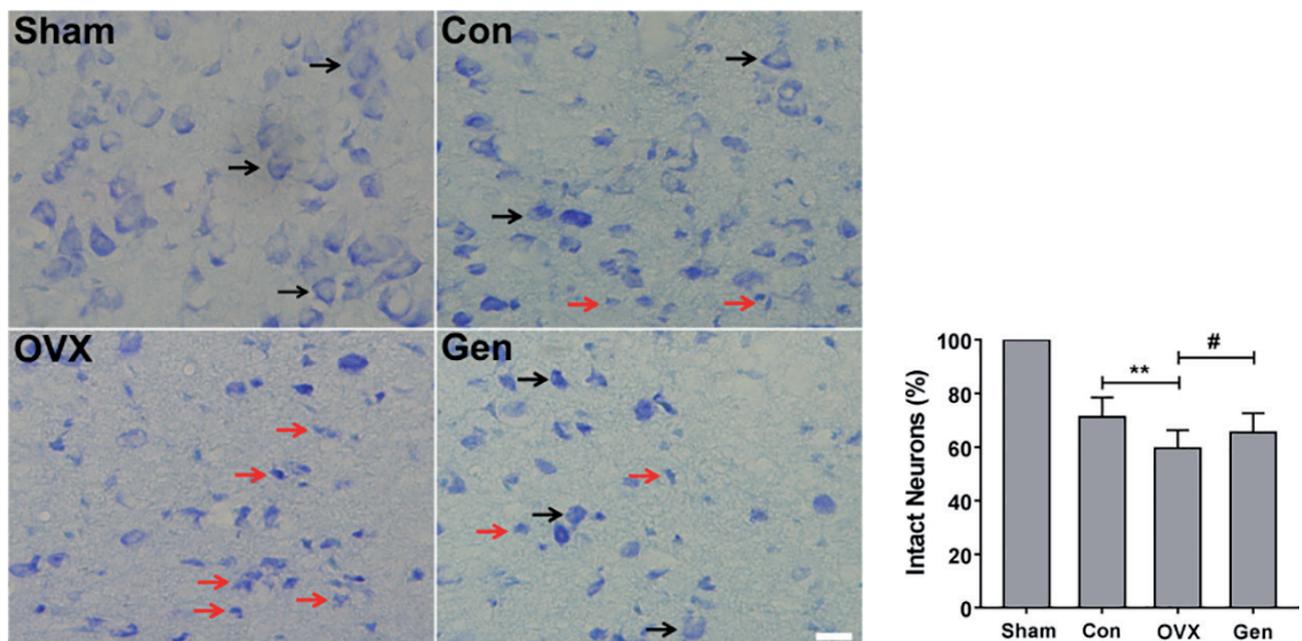


Figure 3. Nissl staining of morphological changes to neurons in ischemic penumbras after ischemia-reperfusion. **A.** Representative photographs showing morphological changes to neurons in ischemic penumbras after ischemia-reperfusion. Black arrowhead, normal neurons; red arrowhead, damaged neurons. Bar = $10 \mu\text{m}$. **B.** Graph of the number of intact neurons in the ischemic penumbras of the different groups. (** $p < 0.01$, # $p < 0.05$; $n = 6$). For abbreviations, see Fig. 1. (See online version for color figure.)

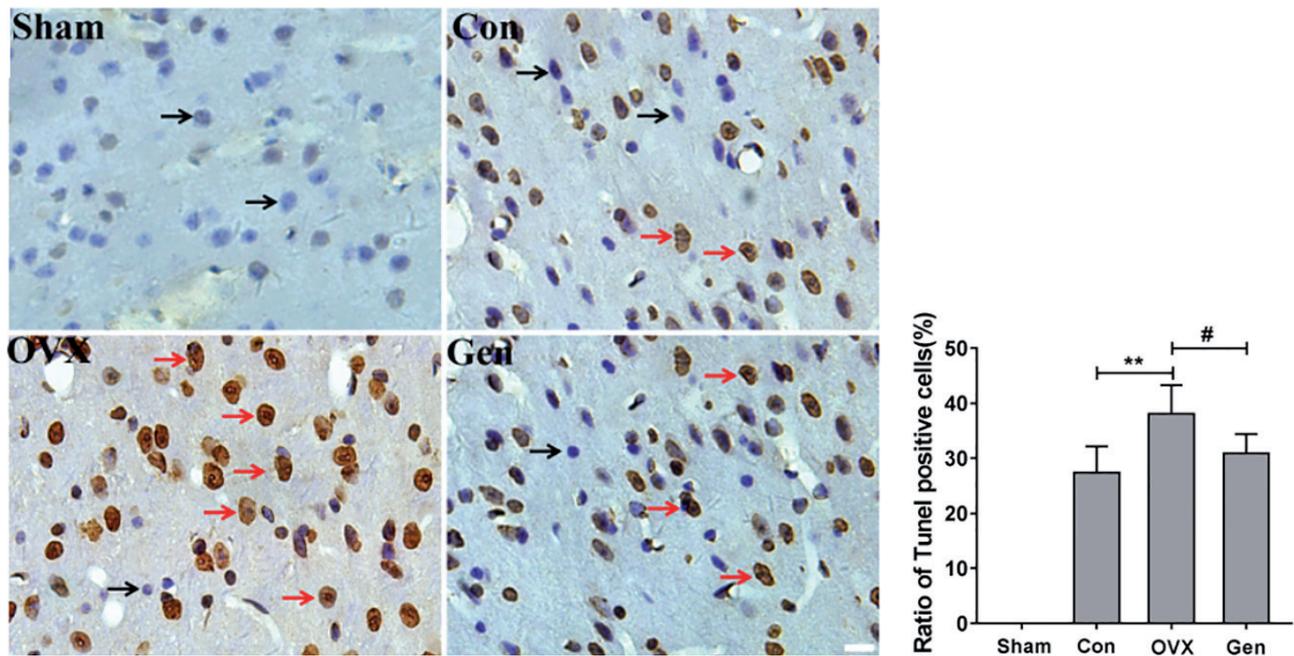


Figure 4. TUNEL staining indicating cell apoptosis in ischemic penumbras following ischemia–reperfusion. **A.** Representative photographs showing cell apoptosis in ischemic penumbras. The black arrow indicates living cells, with nuclei stained blue, and the red arrow indicates TUNEL-positive cells, which showed a pyknotic and sepia nuclei. Bar = 10 μ m. **B.** Graph of the extent of apoptosis in the ischemic penumbras of the different groups. (** $p < 0.01$, # $p < 0.05$; $n = 6$). For abbreviations, see Fig. 1. (See online version for color figure.)

As shown in Figure 4, the proportion of TUNEL-positive cells in the Con group was $27.4 \pm 4.4\%$, and the OVX group exhibited a significant increase in this proportion to $38.1 \pm 4.8\%$ (** $p < 0.01$ vs. the Con group), while genistein treatment significantly decreased the proportion to $30.9 \pm 3.2\%$ (# $p < 0.05$ vs. the OVX group). No significant difference in TUNEL-positive cells was observed between the Con and Gen group.

Genistein treatment increased the activity of the PI3K-Akt-mTOR signal pathway

Western blot was used to detect the activity of the PI3K-Akt-mTOR signal pathway in the ischemic penumbra 72 h after MCAO. As shown in Figure 5, compared with the Sham group, there was significant decrease in PI3K (0.64-fold, ** $p < 0.01$), phospho-Akt (0.56-fold, ** $p < 0.01$) and phospho-mTOR (0.55-fold, ** $p < 0.01$) in the Con group. And the ovariectomy surgery further significantly decreased the expression levels of PI3K (0.60-fold, ## $p < 0.01$), phospho-Akt (0.63-fold, # $p < 0.05$) and phospho-mTOR (0.62-fold, ## $p < 0.01$) compared with the Con group. And genistein treatment significantly increased the expression levels of PI3K (1.35-fold, & $p < 0.05$), phospho-Akt (1.38-fold, & $p < 0.01$) and phospho-mTOR (1.38-fold, & $p < 0.05$) compared with the Con group.

Discussion

Stroke is a leading cause of disability and death in the world-wide. By now, there is still no effective treatment for the stroke except rTPA (recombinant tissue-type plasminogen activator) thrombolytic therapy within a narrow treatment time window. Therefore, the prevention of stroke is of great importance.

Accumulating researches demonstrate that estrogen replacement treatment (ERT) could significantly induce cerebral ischemia tolerance in ovariectomy rats and mice (Ma et al. 2013, 2016); thus, ERT has been regarded as an potential method to prevent stroke, probably in the postmenopausal women, who possess a significantly increased stroke incidence rate compared with non-menopausal women and also suffer much worse outcomes compared with the men in the same age (Appelros et al. 2009). However, long-term ERT also significantly increased the occurrence of tumors in female reproduction organs such as ovary cancer and breast cancer; therefore, alternative treatments which exert neuroprotective effects but little adverse effects of estrogen have been largely explored.

Recently, diet and dietary components have been regarded as important strategies to prevent the stroke. Several researchers have found the promising effect of genistein in the alleviation of stroke injuries (Liang et al. 2008; Castelló-

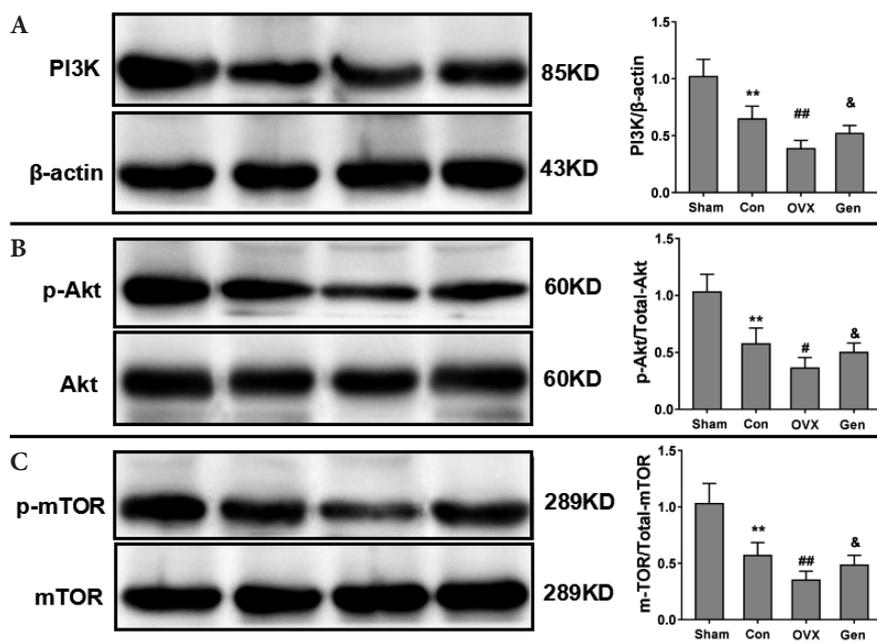


Figure 5. Results of the PI3K-Akt-mTOR signal pathway expression levels in ischemic penumbra. Cropped gels, blots and graph of PI3K (A), p-Akt (B) and p-mTOR (C) expression levels in ischemic penumbras of the different groups. (** $p < 0.01$ vs. Sham group; # $p < 0.05$ vs. Con group; ## $p < 0.01$ vs. Con group; & $p < 0.05$ vs. OVX group; $n = 6$). For abbreviations, see Fig. 1.

Ruiz et al. 2011; Wang et al. 2014; Miao et al. 2018). In this study, we explored the effect of genistein treatment on ischemic stroke in ovariectomized rats and found that 10 mg/kg genistein treatment significantly improved the neurological outcomes and decreased the infarct volume in ovariectomized rats subjected to MCAO and reperfusion injury, which was consistent with several previous studies (Wang et al. 2014; Miao et al. 2018). These results definitely verified the neuroprotective effects of genistein treatment against stroke injury in ovariectomized female animals. Thus, genistein-related replacement treatment may become a new strategy for stroke therapy. But the underlying molecular mechanism remains largely unknown.

Our study found that estrogen deprivation by ovariectomy surgery significantly increased the neuron damage and cell apoptosis in ischemic penumbra compared to rats with intact ovarian; and genistein treatment to the ovariectomized rats markedly reduced the neuron damage and cell apoptosis in ischemic penumbra. Apoptosis is reported to be responsible for a significant proportion of the ischemia-induced neuronal loss (Hossain et al. 2008).

Lots of studies have demonstrated that inhibition of the apoptosis pathway exerts significant neuroprotective effects against cerebral ischemia injuries (Ma et al. 2013; Liu et al. 2014). Apoptosis is the result of a series of cascade activation of nucleases and proteases that involve caspases (Troy et al. 2011). A recent previous study found that genistein treatment significantly decreased the cleaved-Caspase-3 expression *via* promoting Nrf-2 expression and inhibiting ROS production (Miao et al. 2018). Wang et al. (2014)

found that ERK1/2 activation may be involved in the anti-apoptotic neuroprotective action of genistein. These results suggest that genistein protects neurons from mitigating cell apoptosis. However, the specific molecular pathway underlying the genistein's anti-apoptotic neuroprotection remains largely unknown.

Many studies have shown that PI3K-Akt-mTOR signaling plays a major role in cerebral ischemic stroke injury (Kamada et al. 2007; Pastor et al. 2009). Some researchers have found that Akt signaling, which is activated after transient cerebral ischemia, inhibits delayed neuronal apoptosis and promotes cell survival (Ouyang et al. 1999; Noshita et al. 2001). mTOR, which is a critical downstream substrate of PI3K-Akt signal pathway, governs the programmed cell death pathways of apoptosis that can determine neuronal cell development, cell differentiation, cell senescence, cell survival, and ultimate cell fate (Maiese 2016). Activation of mTOR pathway is necessary for preventing apoptotic neuronal cell death and aggravation of oxidative stress response during cerebral ischemia (Shi et al. 2011; Chong et al. 2012). Thus, mTOR has been considered as one promising target to develop therapeutic strategies for stroke and other neurodegenerative disorders (Chong et al. 2012). However, the role of PI3k-Akt-mTOR signal pathway in genistein's neuroprotection against cerebral ischemia has not been reported before. In this study, we found the activity of PI3k-Akt-mTOR signal pathway in ischemia penumbra was significantly decreased in the Con group compared with the Sham group. Some other researchers have found similar results indicating that cerebral ischemia induced the robust inhibition of PI3K-Akt-mTOR

pathway activity (Noshita et al. 2001; Nakajima et al. 2004). The activity of PI3k-Akt-mTOR signal pathway were further significantly decreased in the ovariectomized rats; genistein treatment markedly promoted the activity of PI3k-Akt-mTOR signal pathway compared to the OVX group. These results indicated that genistein alleviated the neuronal cell apoptosis induced by cerebral ischemia *via* promoting the activity of PI3k-Akt-mTOR signal pathway. In our future study, we will focus on the molecular mechanism underlying this promotion. For example, p70S6K, a component of the mTOR pathway, could block cortical ischemic injury (Koh 2013), provide growth factor neuronal cell protection during apoptosis (Chong et al. 2012), and block apoptosis during oxidative stress exposure (Zhou 2015).

As is well known, age of animals in generally (as well as people) significantly affects the final neurological outcome after the stroke. And menopause most often occurs between the ages of 50 and 52, with 95% of women having final menstrual period between ages 44 and 56, while female rats enter menopause between ages 15 and 18 months (Durbin et al. 1966). It should be pointed out that although ovariectomy is a well-recognized animal model to mimic postmenopausal woman either by natural or by surgery, it could not completely represent the physiological and pathological changes of senile menopause (Rettberg et al. 2014). But as there are many difficulties in constructing MCAO model in elderly animals, we used ovariectomized models to present a preliminary study which could provide clues to the potential neuroprotective effects of genistein treatment for elderly postmenopausal animals or populations.

Above all, we concluded that estrogen deprivation by ovariectomized surgery inhibited the activity of PI3k-Akt-mTOR signal pathway after MCAO and reperfusion injury, which lead to excessive cell apoptosis and finally exacerbates cerebral ischemic injury; while, genistein treatment could significantly advance the activity of PI3k-Akt-mTOR signal pathway in ischemia penumbra after MCAO and reperfusion injury, then reduced neuronal cell apoptosis, and finally alleviated the cerebral ischemia injury. These results provide a new molecular pathway for exploring the neuroprotective effects of genistein against stroke, especially in postmenopausal women.

Conflict of interest. The authors declare no competing financial interests.

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Supplementary Material

A preliminary report: genistein attenuates cerebral ischemia injury in ovariectomized rats *via* regulation of the PI3K-Akt-mTOR pathwayLi-yan Lu¹, Yan Liu¹, Yu-feng Gong² and Xiu-ying Zheng³¹ *Personnel Department, The Second Affiliated Hospital of Mudanjiang Medical University, Heilongjiang, China*² *Department of Gynaecology and Obstetrics, The Second Affiliated Hospital of Mudanjiang Medical University, Heilongjiang, China*³ *The Second Affiliated Hospital of Mudanjiang Medical University, Heilongjiang, China*

Supplementary Table S1.

Group		MABP (mmHg)	Temp (°C)	Glu (dl/ml)	Hct (%)	pH	pO ₂ (mmHg)	pCO ₂ (mmHg)
Con	pre	73 ± 1	37 ± 0.2	195 ± 13	34 ± 3	7.4 ± 0.1	132 ± 4	46 ± 5
	during	70 ± 2	37 ± 0.2	178 ± 10	30 ± 4	7.4 ± 0.1	146 ± 5	60 ± 8
	post	68 ± 2	37 ± 0.2	170 ± 11	28 ± 2	7.4 ± 0.1	150 ± 7	55 ± 10
OVX	pre	71 ± 2	37 ± 0.2	193 ± 14	35 ± 4	7.4 ± 0.1	135 ± 7	48 ± 7
	during	68 ± 1	37 ± 0.3	172 ± 10	32 ± 3	7.4 ± 0.1	147 ± 4	62 ± 10
	post	65 ± 2	37 ± 0.3	165 ± 12	27 ± 4	7.4 ± 0.1	152 ± 7	58 ± 10
Gen	pre	71 ± 3	37 ± 0.3	192 ± 15	34 ± 5	7.4 ± 0.1	136 ± 8	47 ± 7
	during	67 ± 1	37 ± 0.3	171 ± 12	31 ± 3	7.4 ± 0.1	145 ± 5	63 ± 6
	post	65 ± 3	37 ± 0.3	166 ± 11	25 ± 4	7.4 ± 0.1	150 ± 6	53 ± 10

Data are means ± SD. MABP, mean arterial blood pressure; Temp, rectal temperature; Glu, glucose; Hct, hematocrit.