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Abstract
Stroke is a leading cause of disability and death in the worldwide. Therefore, prevention of stroke is critical 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 (OVX) rats has been rarely evaluated. In this study, OVX 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 OVX 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 alleviated neuronal apoptosis induced by cerebral ischemia in OVX 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.

Keywords: genistein; PI3K-Akt-mTOR; apoptosis; cerebral ischemia; neuroprotection

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Abstract

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Keywords: genistein; PI3K-Akt-mTOR; apoptosis; cerebral ischemia; neuroprotection

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

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 [1, 2]. Nearly 80% of strokes are caused by ischemic attack (consequent of thrombosis, embolism and/or hypoperfusion) and the others are the result of hemorrhage [3]. 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 [4,5].

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 [6-9]. However, the underlying molecular mechanisms remains largely unknown.

As an important signal transduction pathway, PI3K-Akt-mTOR is involved in many cellular processes, including cell apoptosis, survival and proliferation [10]. Phosphatidylinositol 3 kinase (PI3K) is an intracellular phosphatidylinositol kinase [11]. 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 [12] and maintains normal functions [13]. And 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 [14]. A number of researches have demonstrated the critical role of PI3K-Akt-mTOR signal pathway in alleviating cerebral ischemic injury [15,16]. 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 OVX rats, which would supply a new molecular mechanism for genistein neuroprotection.

Materials and Methods:

Animals

One hundred and forty adult female Sprague–Dawley (SD) 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) based on whether they underwent ovariectomy (OVX) surgeries, genistein treatment or MCAO-R injury: 1) sham group was sham operation group, 2) control group (Con) consisted of female rats with intact ovaries who received MCAO-R, 3) the rats in OVX group received ovariectomy surgeries, vehicle (sesame oil) treatment and MCAO-R injury, 4) the rats in Genistein (Gen) group 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±1°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.

OVX and Drug treatment

OVX was performed through dorsolateral incisions as previously described [17] (n=35). The animals in the sham group were subjected to the same operation; however, their ovaries were kept intact. Following OVX, vaginal smears were performed for 5 days to confirm the success of the OVX 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 intraperitoneally at a dose of 10 mg/kg once daily for two weeks, which was based on the dosage used in a previous study [8].

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 [18] (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 table 1. 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. And the rats in Con, OVX and Genistein 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 [19], as shown in the following table (n=12). There are six scoring items in Garcia Test. And each scoring item include 0 point, 1 point, 2 points and 3 points. Each rat was scored by all these six items. The higher the scores, the better the neurological function of the rat.

Garcia Test

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)	Left side: no movement	Left side: slight movement	Left side: moves slowly	Both sides:move symmetrically
Symmetry of forelimbs(out	Left side: no movement, no	Left side: slight movement to	Left side: moves and outreaches less than	Symmetrically outreach

stretching while held by tail)	outreaching	outreach	right	
Climbing wall of wire cage	...	Fails to climb	Left side is weak	Normal climbing
Reaction to touch on either side of trunk	...	No response on left side	Weak response on left side	Symmetrical response
Response to vibrissae touch	...	No response on left side	Weak response on left side	Symmetrical response

Assessment of infarct volume

After neurological scoring, infarct volume was assessed via 2,3,5-triphenyltetrazolium chloride (TTC) staining as previously described [18] (n=12). Briefly, after the rats were anesthetized with 10% chloral hydrate (0.3 ml/100g weight, i.p), their brains were rapidly removed and cooled in ice-cold saline for 10 mins. 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 were taken out. Then the region of the ischemic penumbra were microdissected according to established protocols in rodent models of unilateral proximal MCAO [20]. Then 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 specifc 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 place into 20% and 30% sucrose solutions at 4°C. After the brains were equilibrated with the sucrose, 12-μm-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 were counted in 5 different fields of view for each section by an observer blinded to the treatment group manner via light microscopy at ×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) H2O2 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 were counted in 5 different fields of view for each section by an observer blinded to the treatment group manner via light microscopy at ×400 magnification (BX51; Olympus, Tokyo, Japan).

Statistical Analyses

Data was presented as mean ± 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 were performed using Student's *t*-tests. $p<0.05$ 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 hours 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. And 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% ± 4.9%.

The OVX surgery significantly increased infarct volume to 29.7% ± 5.1% ($^{**}p<0.01$ vs. the Con group), and the genistein treatment significantly decreased the infarct volume to 24.3% ± 4.1% ($^{\#}p<0.05$ vs. the OVX group). There was no significant difference between the Con group 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 hours after MCAO. As shown in Figure 3, the injured neurons showed shrunken cell bodies accompanied by shrunken and pyknotic nuclei. The ratio of intact neurons in the Con group was 71.2% ± 7.0%, the OVX treatment significantly decreased the ratio of intact neurons to 59.6% ± 6.4% ($^{**}p<0.01$ vs. the Con group). And genistein treatment significantly increased the the ratio of intact neurons to 65.4% ± 6.9% ($^{\#}p<0.05$ vs. the OVX group). There was no significant difference between the Con group and Gen group.

As shown in Figure 4, the proportion of TUNEL-positive cells in the Con group was 27.4% ± 4.40%, and the OVX group exhibited a significant increase in this proportion to 38.1% ± 4.8% ($^{**}p<0.01$ vs. the Con group), while genistein treatment significantly decreased the proportion to 30.9% ± 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.

Then, we used Western blot to detect the activity of the PI3K-Akt-mTOR signal pathway in the ischemic penumbra 72 hours 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 OVX 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

234 with the Con group.

235

236 **Discussion**

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

241 Accumulating researches demonstrate that estrogen replacement treatment (ERT)
242 could significantly induce cerebral ischemia tolerance in OVX rats and mice [21,22];
243 thus, ERT has been regarded as an potential method to prevent stroke, probably in the
244 postmenopausal women, who possess a significantly increased stroke incidence rate
245 compared with non-menopausal women and also suffer much worse outcomes
246 compared with the men in the same age [23]. However, long-term ERT also
247 significantly increased the occurrence of tumors in female reproduction organs such
248 as ovary cancer and breast cancer; therefore, alternative treatments which exert
249 neuroprotective effects but little adverse effects of estrogen have been largely
250 explored.

251 Recently, diet and dietary components have been regarded as important strategies
252 to prevent the stroke. Several researches have found the promising effect of genistein
253 in the alleviation of stroke injuries [6-9]. In this study, we explored the effect of
254 genistein treatment on ischemic stroke in OVX rats and found that 10 mg/kg genistein
255 treatment significantly improved the neurological outcomes and decreased the infarct
256 volume in OVX rats subjected to MCAO and reperfusion injury, which was consistent
257 with several previous studies [6,7]. These results definitely verified the
258 neuroprotective effects of genistein treatment against stroke injury in OVX female
259 animals. Thus, genistein-related replacement treatment may become a new strategy
260 for stoke therapy. But the underlying molecular mechanism remains largely unknown.

261 Our study found that estrogen deprivation by OVX surgery significantly
262 increased the neuron damage and cell apoptosis in ischemic penumbra compared to
263 rats with intact ovarian; and genistein treatment to the OVX 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 [24]. Lots of studies have demonstrated that inhibition of the apoptosis pathway exerts significant neuroprotective effects against cerebral ischemia injuries [21,25]. Apoptosis is the result of a series of cascade activation of nucleases and proteases that involve caspases [26]. A recent previous study found that genistein treatment significantly decreased the cleaved-Caspase-3 expression via promoting Nrf-2 expression and inhibiting ROS production [6]. And *Wang et al*. found that ERK1/2 activation may be involved in the anti-apoptotic neuroprotective action of genistein[7]. 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 [15,16]. Some researchers have found that Akt signaling, which is activated after transient cerebral ischemia, inhibits delayed neuronal apoptosis and promotes cell survival [12, 27]. 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 [28]. Activation of mTOR pathway is necessary for preventing apoptotic neuronal cell death and aggravation of oxidative stress response during cerebral ischemia [29,30]. Thus, mTOR has been considered as one promising target to develop therapeutic strategies for stroke and other neurodegenerative disorders [29]. 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 were 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 [27, 31]. And the activity of PI3k-Akt-mTOR signal pathway were further significantly decreased in the OVX 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 [32], provide growth factor neuronal cell protection during apoptosis [33], and block apoptosis during oxidative stress exposure [34].

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 [35]. It should be pointed out that although OVX 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 [36]. But as there are many difficulties in constructing MCAO model in elderly animals, we used OVX 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 OVX 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.

Competing financial interests

The authors declare no competing financial interests.

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Figure legends

Figure 1. Regional cerebral blood flow during MCAO surgery and neurological scores after ischemia-reperfusion in the different groups.

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 ± 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). Con: Control; OVX: ovariectomy; Gen: genistein.

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 μm.

B. Graph of the number of intact neurons in the ischemic penumbras of the different groups. ($^{**}p<0.01$, $^{\#}p<0.05$, n=6). Con: Control; OVX: ovariectomy; Gen: genistein.

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 μm.

B. Graph of the extent of apoptosis in the ischemic penumbras of the different groups. ($^{**}p<0.01$, $^{\#}p<0.05$, n=6). Con: Control; OVX: ovariectomy; Gen: genistein.

Figure 5. Results of the PI3K-Akt-mTOR signal pathway expression levels in ischemic penumbra.

A. Cropped gels and blots and graph of PI3K expression levels in ischemic penumbras of the different groups. ($^{**}p<0.01$ vs. Sham group; $^{\#\#}p<0.01$ vs. Con group; $^{\&}p<0.05$ vs. OVX group, n=6).

B. Cropped gels and blots and graph of PI3K expression levels in ischemic penumbras of the different groups. ($^{**}p<0.01$ vs. Sham group; $^{\#}p<0.05$ vs. Con group; $^{\&}p<0.05$ vs. OVX group, n=6).

C. Cropped gels and blots and graph of PI3K expression levels in ischemic penumbras of the different groups. ($^{**}p<0.01$ vs. Sham group; $^{\#\#}p<0.01$ vs. Con group; $^{\&}p<0.05$ vs. OVX group, n=6). Con: Control; OVX: ovariectomy; Gen: genistein.

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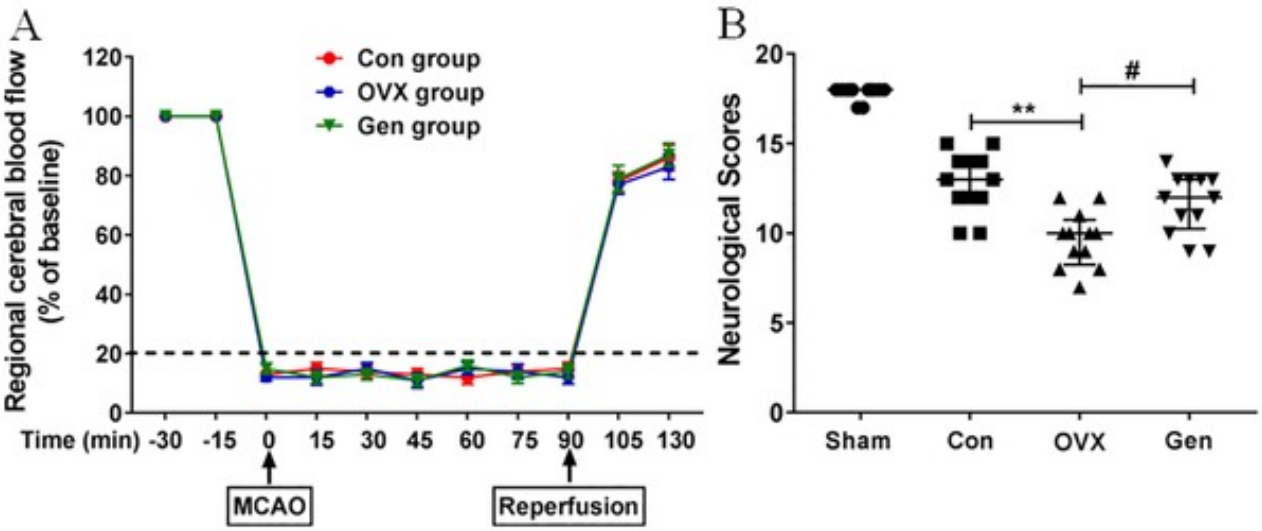

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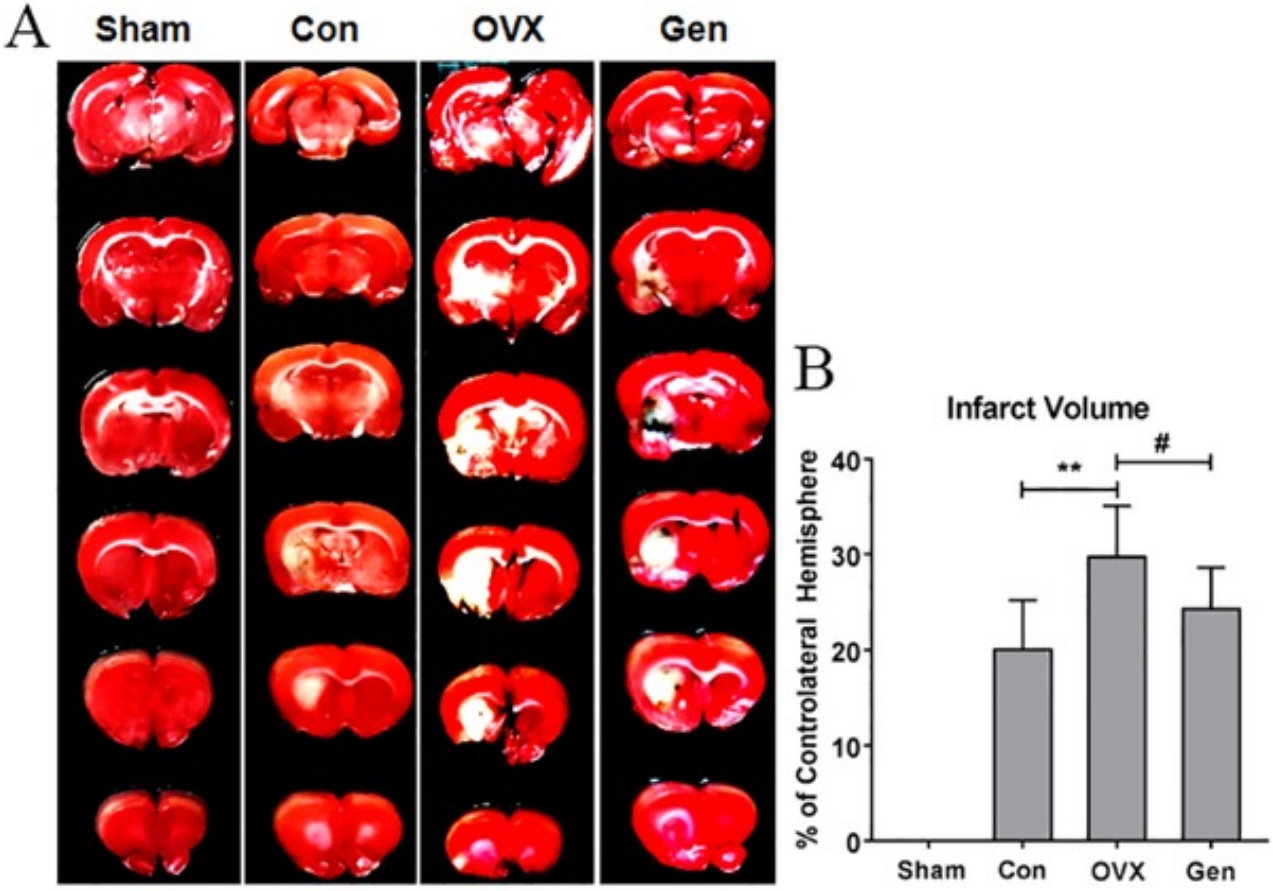

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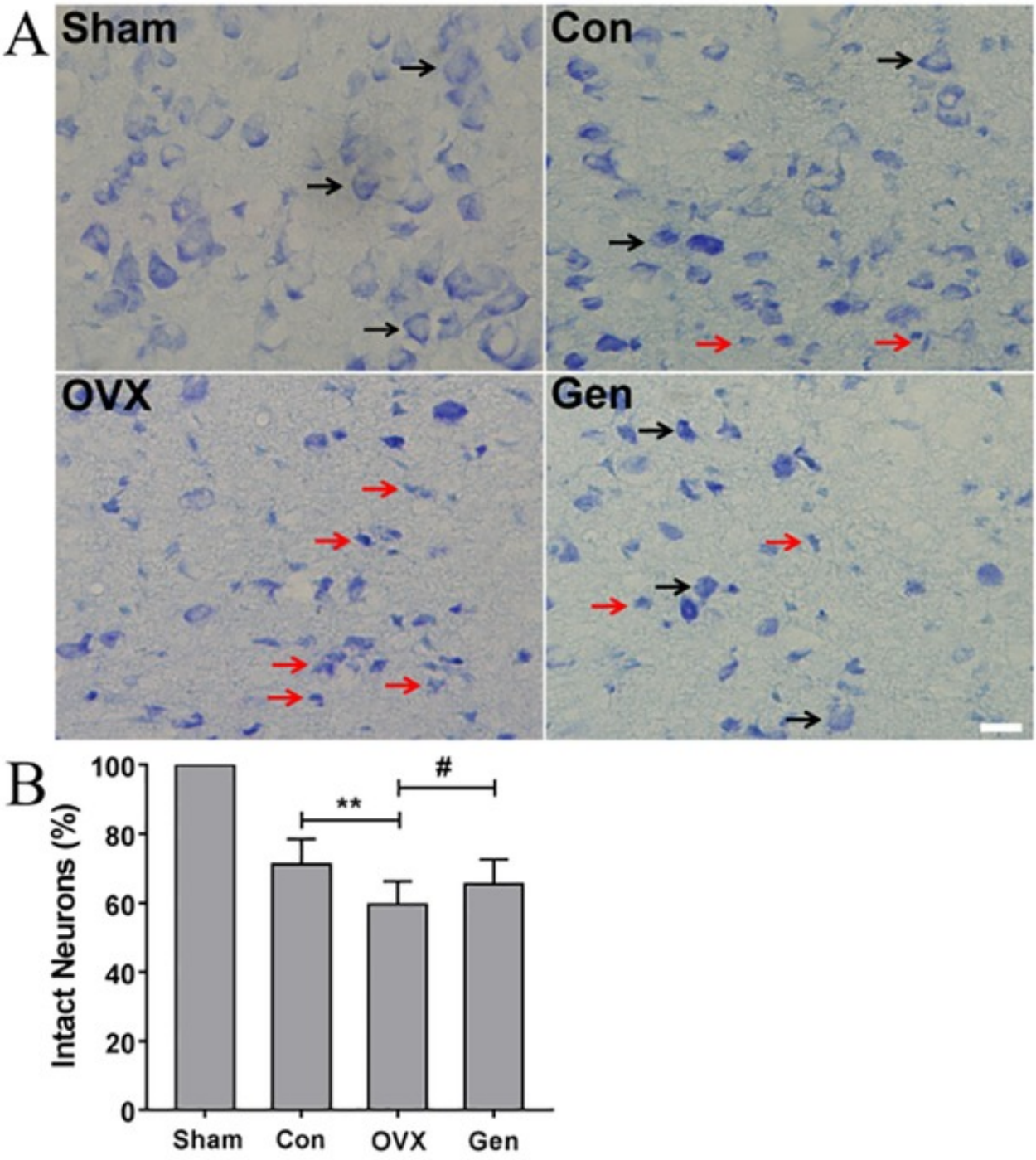

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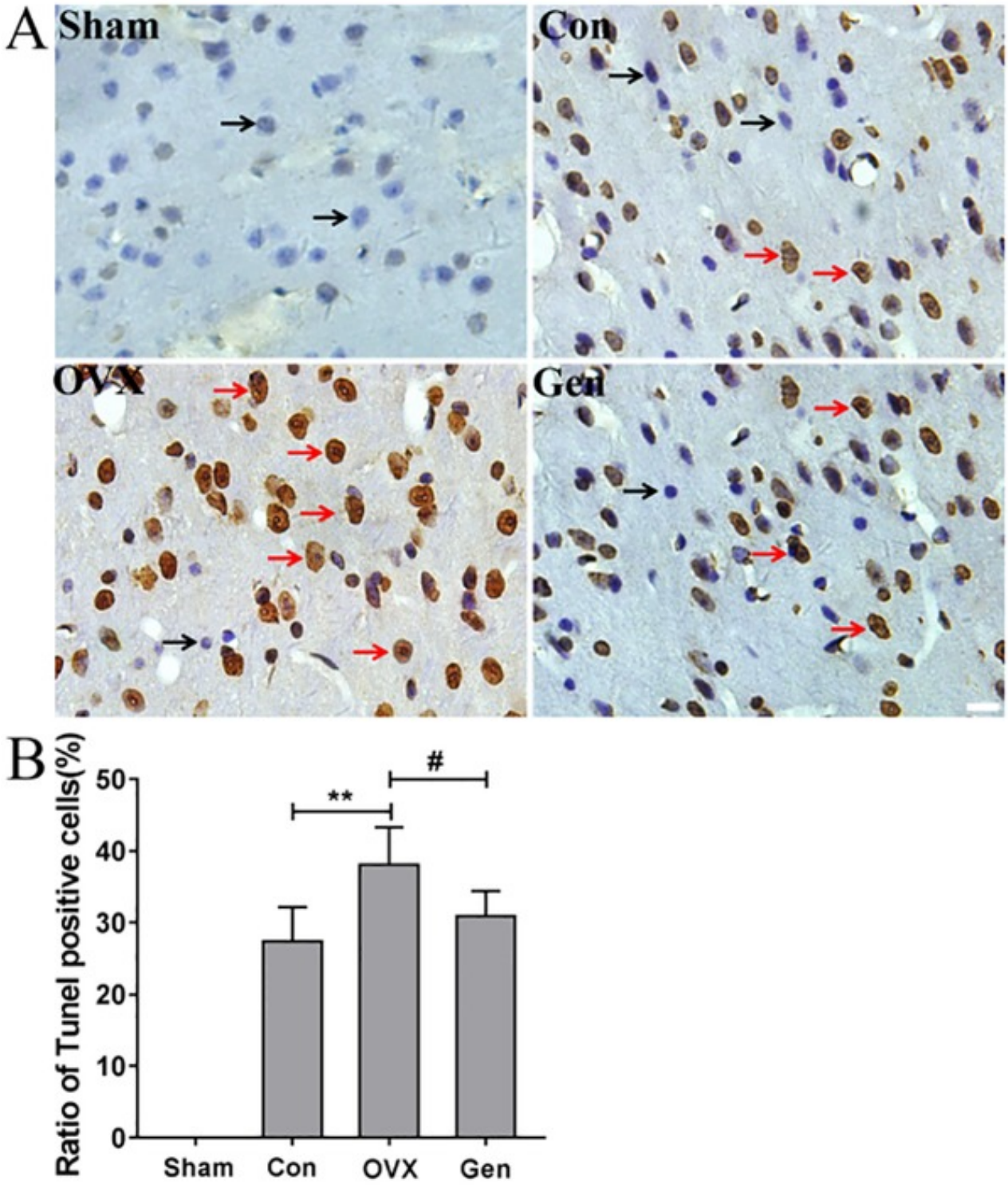

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