RESEARCH ARTICLE

Characterization of astrocytes and microglial cells in the hippocampal CA1 region after transient focal cerebral ischemia in rats treated with Ilexonin A

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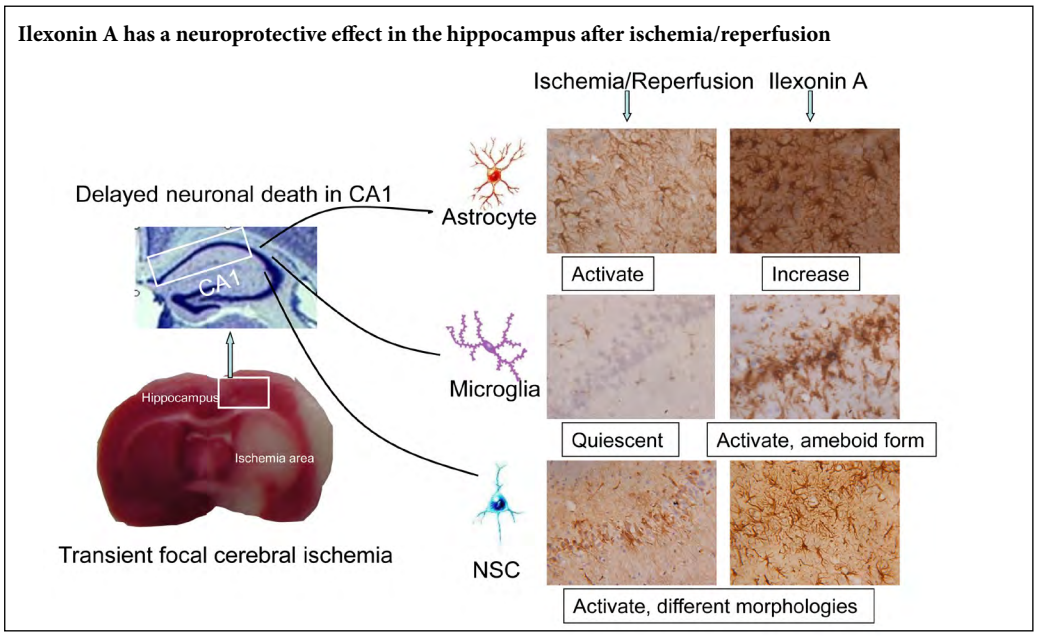
Graphical Abstract



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Abstract

Ilexonin A is a compound isolated from the root of Ilex pubescens, a traditional Chinese medicine. Ilexonin A has been shown to play a neuroprotective role by regulating the activation of astrocytes and microglia in the peri-infarct area after ischemia. However, the effects of ilexonin A on astrocytes and microglia in the infarct-free region of the hippocampal CA1 region remain unclear. Focal cerebral ischemia models were established by 2-hour occlusion of the middle cerebral artery in rats. Ilexonin A (20, 40 or 80 mg/kg) was administered immediately after ischemia/reperfusion. The astrocyte marker glial fibrillary acidic protein, microglia marker Iba-1, neural stem cell marker nestin and inflammation markers were detected by immunohistochemistry and western blot assay. Expression levels of tumor necrosis factor-α and interleukin 1β were determined by enzyme linked immunosorbent assay in the hippocampal CA1 tissue. Astrocytes were activated immediately in progressively increasing numbers from 1, 3, to 7 days post-ischemia/reperfusion. The number of activated astrocytes further increased in the hippocampal CA1 region after treatment with ilexonin A. Microglial cells remained quiescent after ischemia/reperfusion, but became activated after treatment with ilexonin A. Ilexonin A enhanced nestin expression and reduced the expression of tumor necrosis factor-α and interleukin 1β in the hippocampus post-ischemia/reperfusion. The results of the present study suggest that ilexonin A has a neuroprotective effect in the hippocampus after ischemia/reperfusion, probably through regulating astrocytes and microglia activation, promoting neuronal stem cell proliferation and reducing the levels of pro-inflammatory factors. This study was approved by the Animal Ethics Committee of the Fujian Medical University Union Hospital, China.

Key Words: *astrocytes; hippocampal CA1 region; ilexonin A; microglia; middle cerebral artery occlusion; neural stem cell; neuroprotection; transient focal cerebral ischemia*

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Introduction

The hippocampus is one of the most vulnerable brain areas post-ischemia, especially its CA1 region. Pyramidal neurons in the hippocampal CA1 region were selectively lost 2 to 3 days after global cerebral ischemia, whereas other neurons in the same area were unaffected (Duan et al., 2011; Yan et al., 2012; Sun et al., 2018). In recent findings, neuronal damage was also observed in the hippocampal CA1 region after middle cerebral artery occlusion (MCAO), a condition that does not directly affect the hippocampus (Uchida et al., 2010; Jiao et al., 2011). The underlying mechanisms remain poorly understood. Previous studies, including our own, suggested that after transient focal cerebral ischemia astrocytes and microglia are swiftly activated and play complex roles in the infarct and peri-infarct regions, with both beneficial and detrimental effects (Jiao et al., 2011; Hu et al., 2012; Xu et al., 2016). This phenomenon has also been found in the hippocampal CA1 region after global ischemia (Lee et al., 2010; Rauš et al., 2013). However, it is not known how or when astrocytes and microglia change and what effect they exert in the hippocampal CA1 region after transient focal cerebral ischemia.

Under normal conditions, astrocytes and microglia are ubiquitously distributed, quiescent cell populations that reside in the central nervous system. One of the main characteristics of astrocytes and microglia is their swift activation in response to various pathologies, such as trauma, neurodegenerative diseases and ischemia. The hallmarks of their activation include proliferation, morphological changes and the release of cytokines and growth factors (Yuan et al., 2007; Uchida et al., 2010). Both cell types can be either neuroprotective or neurotoxic, depending on their morphology and their releasing factors (Chvátal et al., 2007; Wang et al., 2013).

Ilexonin A, extracted from the root of pubescent holly, Ilex pubescens, is a 3β-succinyl-18-dehydro-disodic ursolate (**Figure 1**), which has been clinically used as cardiovascular drug. Our group has done a lot of research on the neuroprotective effect of ilexonin A. It has been shown to reduce the infarction volume and to improve neurological deficits after focal brain ischemia in adult rats (Zheng et al., 2011; Xu et al., 2016). Ilexonin A acts as a neuroprotector by promoting neural regeneration, enhancing the secretion of neurotrophic factors and mitigating cerebral edema (Sheng et al., 2009; Zheng et al., 2011). Our previous research shows that ilexonin A plays a neuroprotective role by regulating the activation of astrocytes and microglial cells in the peri-infarct area post-ischemia (Xu et al., 2016), and by activating wnt and notch signaling pathways (Zhang et al., 2016; Han et al., 2018). A previous study has shown that curcumin, another herbal component, had similar effects to ilexonin A, improving GFAP and nestin protein levels in the hippocampal CA1 region after ischemia (Zhang et al., 2011). In this study, we established the role of ilexonin A in activating astrocytes and microglia in the hippocampal CA1 region of the ipsilateral brain and determined the expression of nestin, TNF-α and IL-1β after ischemia/reperfusion. This study tested doses of 20, 40, and 80 mg/kg ilexonin A to develop a therapeutic strategy.

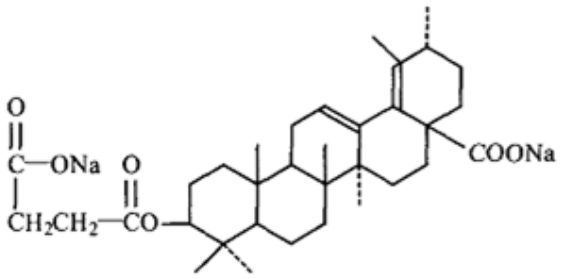

Figure 1 Chemical structure of ilexonin A.

Materials and Methods

Animals and drug administration

Male clean Sprague-Dawley rats aged 6–8 weeks old and weighing 250 ± 10 g were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd., China (license No. SCXK (Hu) 2007-0005). These rats were randomly divided into six groups: (1) control, (2) sham, (3) ischemia, and ischemia groups treated with ilexonin A at (4) 20 mg/kg, (5) 40 mg/kg, or (6) 80 mg/kg. Each group has four subgroups (n = 6/subgroup) for each of the time following MCAO (1, 3, 7, and 14 days). Two rats in each subgroup were intraperitoneally injected with either 20, 40, or 80 mg/kg ilexonin A (Guangdong Boro Pioneer Pharmaceutical Group Co., Ltd., Huizhou, Guangdong Province, China; Product No. Z44023366) after ischemia/reperfusion. Rats in the control, sham and ischemia groups were intraperitoneally injected with an equal volume (2 mL) of saline. Our study was approved by the Animal Ethics Committee of Union Hospital of Fujian Medical University, China.

Procedure of transient focal cerebral ischemia

Transient focal cerebral ischemia was induced by MCAO using the method developed by Longa et al. (1989). Rats were intraperitoneally anesthetized with 10% chloral hydrate (300 mg/kg). Blunt separation of the left common carotid artery, internal carotid artery and external carotid artery was performed through a ventral midline incision of the neck. The distal end of the external carotid artery branches was ligated and dissociated by electric coagulation. A nylon monofilament suture (diameter 0.26 mm) with a paraffin-rounded tip was inserted from the external carotid artery into the internal carotid artery for 18 ± 0.5 mm, to occlude the middle cerebral artery. The nylon suture was fixed and the incision was closed. After 2 hours of ischemia, the suture was withdrawn for reperfusion. Rats in the sham group underwent identical surgery, without the nylon suture that induced ischemia. Rats in the control group did not undergo surgery. The method of 2,3,5-triphenyltetrazolium chloride staining of MCAO was as published in our previous study (Xu et al., 2016).

Criteria for MCAO modeling

Following ischemia/reperfusion, neurological findings were evaluated at five levels of behavior based on Longa's method (Zhang et al., 2011), as follows: 4, no spontaneous walking

with a depressed level of consciousness; 3, falling down to the right; 2, circling to the right; 1, failure to extend right forelimb fully; 0, no deficit. MCAO was considered successful in rats scoring 1–3 points. Dead or non-compliant animals were removed from the experiment.

Immunohistochemistry

Rats were intraperitoneally anesthetized with 10% chloral hydrate (300 mg/kg) and lavaged with 100 mL of saline, followed by 200 mL of 0.1 M phosphate-buffered 4% paraformaldehyde, pH 7.4. Brains were isolated, fixed in the same fixative for 24 hours, and dehydrated in 15%, 20%, and 30% sucrose gradient in 0.1 M phosphate buffer, pH 7.4. These 8 μm-thick sections, cut by a cryomicrotome (MICROM HM525, Walldorf, Germany), were used for immunohistochemical examination. Immunohistochemistry was carried out according to the protocol provided by Elivision kit (1110219901, Fuzhou Maixin Biotechnology Development Co., Ltd., Fuzhou, China), with 1:5000 rabbit polyclonal anti-GFAP antibody (ab7260; Abcam, Cambridge, UK) to mark astrocytes, 1:100 goat polyclonal anti-Iba-1 antibody (ab5076; Abcam) to mark microglia, and 1:100 mouse monoclonal anti-rat nestin antibody (sc-33677; Santa Cruz Biotechnology, Dallas, TX, USA) to mark neural progenitor cells. After incubation overnight at 4°C, a negative control, 0.01 M phosphate-buffered saline was used in place of the primary antibody. The enzyme-labeled anti-mouse/rabbit polymers in the kit were incubated at room temperature for 30 minutes. A 3,3′-diaminobenzidine kit (Zhongshan Biotechnology, Beijing, China) was used to visualize the results. An optical microscope (CX40; Olympus, Tokyo, Japan) was used for image acquisition. Three sections of each brain of each group (n = 6) were viewed, and positive cells were evaluated by Imagine Pro Plus 5.0 (Media Cybernetics, Inc., Rockville, MD, USA) in 400× microscopic fields for each section in the middle part of the hippocampal CA1 region.

Western blot assay

Rats were lavaged with 100–200 mL saline after anesthesia with 10% chloral hydrate (300 mg/kg, intraperitoneally) and their brains were isolated. The hippocampal CA1 regions were disassociated in 10 μg/mL radioimmunoprecipitation assay lysis buffer containing phenylmethyl sulfonylfluoride (final concentration 0.01 M). The supernatant was collected after sonication and centrifugation at 14,000 × g at 4°C for 5 minutes. A 100 μg sample of total protein, as quantified by bicinchoninic acid protein quantitation, was diluted with lysis buffer, boiled for 5 minutes, and resolved on sodium dodecyl sulfate polyacrylamide gels with various percentages, depending on the molecular weight of the target protein. Following electrophoresis, proteins were transferred to nitrocellulose membranes at a constant current of 250–300 mA for 2 hours. The membranes were blocked in 5% non-fat milk in Tris-buffered saline/Tween-20 (pH 7.5, Tris-HCl 0.1 M, 0.05% Tween-20 and 0.9% NaCl) at room temperature for 1 hour and then incubated with the primary antibodies: rabbit polyclonal anti-GFAP (ab7260, 1:20,000; Abcam), goat polyclonal anti-Iba-1 (ab5076, 1:200; Abcam), mouse monoclonal anti-nestin (sc-33677, 1:300; Santa Cruz Biotechnology), or mouse monoclonal anti-β-actin (sc-47778, 1:2000; Santa Cruz Biotechnology) at 4°C overnight. After Tris-buffered saline/Tween-20 washes, membranes were incubated with 1:6000 peroxidase-conjugated rabbit anti-goat IgG (ZB-2306; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) at room temperature for 2 hours. Enhanced chemiluminescence detection reagents (KPL, Gaithersburg, MD, USA) were used to visualize the result. The band absorbance was measured using ImageMaster® VDS gel imaging and analysis systems (alpha Innotech Corporation, San Leandro, USA), and normalized to the absorbance of the β-actin band, using grey levels.

Enzyme-linked immunosorbent assay

Levels of TNF-α and IL-1β in collected supernatants (see above) were determined by standard enzyme-linked immunosorbent assay (ELISA), as per the supplier protocol (Boster, Wuhan, China). All assays were carried out in duplicate.

Statistical analysis

All data, expressed as the mean ± SD, were analyzed by one-way analysis of variance using the SPSS 17.0 Software package (SPSS, Chicago, IL, USA). The differences between groups were analyzed using the least significant difference test for homogeneity of variance. A value of $P < 0.05$ was considered statistically significant.

Results

Astrocyte activation in the hippocampal CA1 region in an ischemia/reperfusion rat model with ilexonin A treatment

GFAP-positive cells in CA1 were scattered in the control and sham groups. Astrocytes were activated swiftly after ischemia/reperfusion and their morphology changed: the soma swelled, the cytoplasm stained darkly and cell processes grew and thickened. Following ilexonin A treatment, the number of GFAP-positive cells increased at 1, 3, and 7 days post-ischemia/reperfusion, peaked at 7 days in the subgroup treated with 80 mg/kg ilexonin A, and had significantly decreased at 14 days (**Figure 2A** and **B**). Western blot assay results confirmed the results of immunohistochemistry (**Figure 2C** and **D**). Compared with the ischemia group, GFAP expression was significantly increased in the ilexonin A 40 mg/kg and 80 mg/kg groups ($P < 0.05$). There were no significant differences in GFAP expression among the 20, 40 and 80 mg/kg ilexonin A groups. Changes in astrocyte morphology and protein expression were consistent with those observed in the peri-infarct region (data not shown).

Microglial activation in the hippocampal CA1 region in an ischemia/reperfusion rat model with ilexonin A administration

Microglial cells in the hippocampal CA1 region remain quiescent after ischemia/reperfusion. Following treatment with ilexonin A, Iba-1 positive cells became activated and distributed in a specific pattern in the pyramidal layer (**Figure 3A**

and **B**). Compared with the ischemia group, the number of Iba-1 positive cells in the ilexonin A 40 mg/kg and 80 mg/kg groups were increased at the time points of 3, 7 and 14 days ($P < 0.05$). This change was most obvious in the ilexonin A 80 mg/kg group at 7 days after ischemia/reperfusion compared with the ilexonin A 20 mg/kg or 40 mg/kg groups ($P < 0.01$). The morphology of reactive microglia in the ilexonin A-treated groups did not change into a macrophage-like shape, but rather into bushy shapes. Western blot analysis of Iba-1 protein is shown in **Figure 3C** and **D**.

Proliferation of neuronal stem cells in the hippocampal CA1 region in an ischemia/reperfusion rat model after ilexonin A treatment

No nestin-positive cells were observed in the hippocampal CA1 region of either the control or sham groups. The number of nestin-positive cells increased significantly after ischemia/reperfusion, increasingly more with increasing doses of ilexonin A (**Figure 4A** and **B**). Compared with the ischemia group, the number of nestin-positive cells was significantly different in the ilexonin A 80 mg/kg group at each time point ($P < 0.05$). The number of nestin-positive cells in the ilexonin A 80 mg/kg group was also significantly greater than those in the ilexonin A 20 mg/kg or 40 mg/kg groups ($P < 0.05$). Two types of nestin-positive cells were detected, characterized by different morphology, different distribution and different activation (**Figure 5A**). The first type stained lighter and had hypertrophic soma and slender cellular processes. They were observed mostly in the ischemia group and with only a few in the ilexonin A 20 mg/kg group at 3 days after ischemia/reperfusion. They were specifically distributed in the pyramidal cell layer of the hippocampal CA1 region but also appeared in different parts of the CA1 region at different times after ischemia/reperfusion. Nestin-positive cells appeared increasingly closer to the center line of the brain from 3 to 14 days, while the total number of cells decreased over the same period of time (**Figure 5B**). The second type of cells stained darker and had smaller soma and thicker cellular processes, similar to astrocytes. They were non-specifically distributed around the CA1 region. After treatment with ilexonin A, the number of the second type of nestin-positive cells was significantly increased in the hippocampal CA1 region, especially in the ilexonin A 80 mg/kg group at 3 and 7 days post-ischemia/reperfusion. The two types of nestin-positive cells did not co-localize spatially and temporally, but rather had appeared sequentially in the reperfused tissue. The first type peaked at 3 days, whereas the second peaked at 7 days after treatment with ilexonin A. Changes in nestin protein levels are shown in **Figure 4C** and **D**.

TNF-α and IL-1β concentrations in the hippocampal tissue in an ischemia/reperfusion rat model after ilexonin A administration

The concentrations of TNF-α and IL-1β in the hippocampal tissue increased continuously with time after reperfusion. TNF-α and IL-1β concentrations were significantly inhibited by ilexonin A, especially after administration at 80 mg/kg dose, compared with the ischemia group ($P < 0.01$; **Figure 6**). The effect in the ilexonin A 80 mg/kg group was significantly greater than that in the ilexonin A 20 mg/kg and 40 mg/kg groups at 14 days ($P < 0.05$).

Discussion

Unlike global cerebral ischemia or forebrain ischemia, transient focal cerebral ischemia induced by MCAO does not primarily affect the hippocampus. However, delayed neuronal death occurred in the pyramidal layer of the hippocampal CA1 region (Uchida et al., 2010; Jiao et al., 2011). Previous studies have suggested that neuroglial cells undergo dynamic changes: microglia were immediately activated and specifically distributed in the pyramidal layer, while astrocytes were activated later and distributed around the pyramidal layer. These changes were strongly associated with the delayed neuronal death that occurred following global cerebral ischemia (Duan et al., 2011; Yan et al., 2012; Lee et al., 2019). In our study, gliocytes that responded to delayed neuronal death after transient focal cerebral ischemia, showed different characteristics from those mentioned above. This may be due to the microenvironment created by the combination of blood reperfusion and ilexonin A.

Results from our study showed that astrocytes were activated rapidly around the pyramidal layer after ischemia/reperfusion, which is consistent with previous studies (Uchida et al., 2010; Jiao et al., 2011). However, the intensity of astrocyte activation in the hippocampus is weaker than in other peri-infarct regions. Ilexonin A plays a dual role, by either enhancing the activation of astrocytes in the early stages (1, 3, 7 days post-MCAO) or reducing the formation of glial scars at a later stage (14 days post-MCAO). It is accepted that reactive astrocytes can play a protective role in brain ischemia (Jeong et al., 2013). Some astrocytes acquire stem cell properties after injury and hence may differentiate to cell types needed to initiate repairs (Shin et al., 2013). They release neurotrophic factors such as glia-derived neurotrophic factor, neurotrophin receptors such as TrkB (a receptor for brain-derived neurotrophic factor), antioxidants and extracellular matrix proteins to promote functional repair (Lin et al., 2006; Tonchev et al., 2008; Ding et al., 2009). They were also shown to be involved in the neurovascular remodeling after ischemia/reperfusion (Hayakawa et al., 2010; Jing et al., 2013). The infarct volume expanded 2–3 fold when the activation of astrocytes was rescinded in deficient mutant mice (Li et al., 2008). However, reactive astrocytes proliferating in later stages played an key role in the formation of the glial scar, which promoted morphological repair of the central nervous system, but inhibited functional recovery (Davies et al., 1999). Therefore, the dual effects of ilexonin A provide a favorable condition for neuronal restoration.

Microglia are activated and proliferate swiftly after ischemia/reperfusion in the peri-infarction region and their morphology changes to rod-like or to amoeba-like cells, which are difficult to distinguish from blood-derived macrophages. After treatment with ilexonin A, the number of

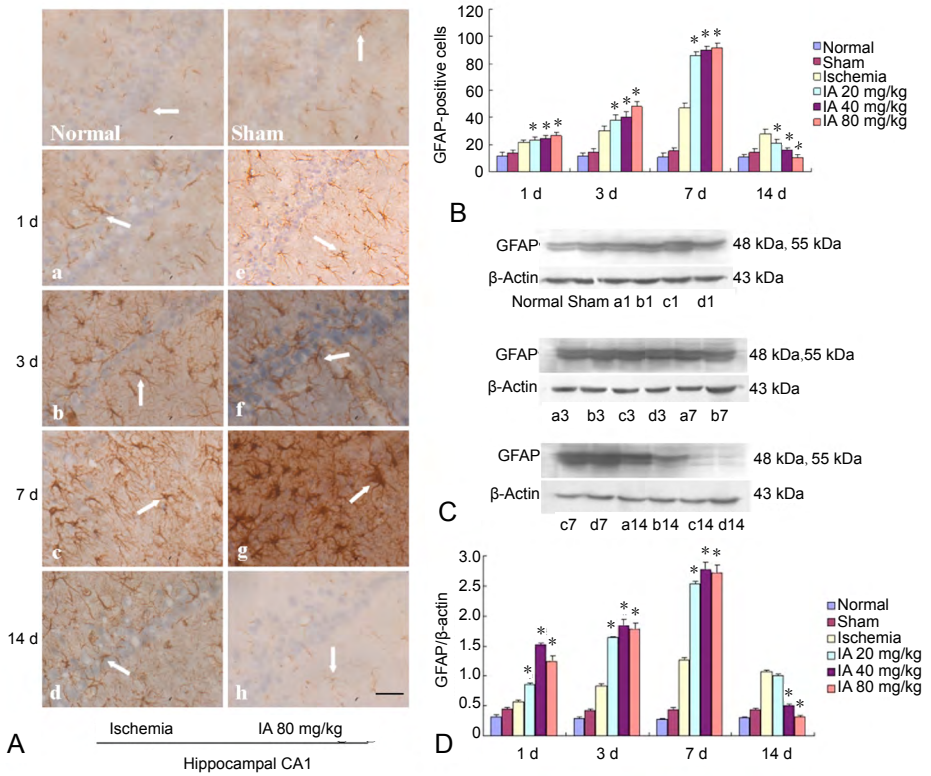

Figure 2 Astrocyte activation in the hippocampal CA1 region after ischemia/reperfusion and ilexonin A treatment.

(A, B) GFAP immunostaining (arrows) in the hippocampal CA1 region at different time points after ischemia/reperfusion and treatment with 80 mg/kg ilexonin A (IA). (a–d) Ischemia group at 1, 3, 7 and 14 days post-reperfusion; (e–h) Ilexonin A 80 mg/kg group at 1, 3, 7 and 14 days (d) post-reperfusion (400×, scale bar: 50 μm). (C, D) The GFAP protein in the hippocampus was detected by western blot analysis (C) and analyzed in (D) using β-actin protein as an internal reference. a1, 3, 7, 14: Ischemia group at 1, 3, 7 and 14 days post-reperfusion; b1, 3, 7, 14: Ilexonin A 20 mg/kg group at 1, 3, 7 and 14 days post-reperfusion; c1, 3, 7, 14: Ilexonin A 40 mg/kg group at 1, 3, 7 and 14 days post-reperfusion; d1, 3, 7, 14: Ilexonin A 80 mg/kg group at 1, 3, 7 and 14 days post-reperfusion. Data are expressed as the mean ± SD. ∗$P < 0.05$, *vs.* ischemia group (one-way analysis of variance followed by the least significant difference test).

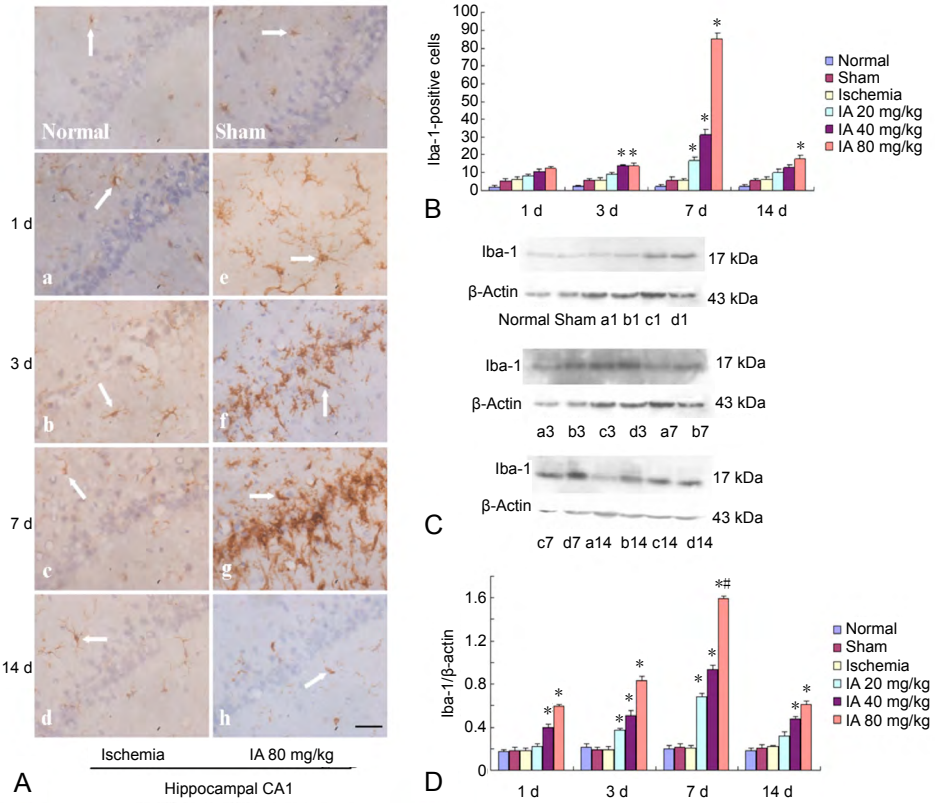

Figure 3 Microglial activation in the hippocampal CA1 region after ischemia/reperfusion and ilexonin A treatment.

(A, B) Iba-1 immunostaining (arrows) in the hippocampal CA1 region at different time points after ischemia/reperfusion and treatment with 80 mg/kg ilexonin A (IA). (a–d) Ischemia group at 1, 3, 7 and 14 days post-reperfusion; (e–h) Ilexonin A 80 mg/kg group at 1, 3, 7 and 14 days post-reperfusion (400×, scale bar: 50 μm). The Iba-1 protein in the hippocampus was detected by western blot assay (C) and analyzed in (D) using β-actin protein as an internal reference. a1, 3, 7, 14: Ischemia group at 1, 3, 7 and 14 days (d) post-reperfusion; b1, 3, 7, 14: Ilexonin A 20 mg/kg group at 1, 3, 7 and 14 days post-reperfusion; c1, 3, 7, 14: Ilexonin A .40 mg/kg group at 1, 3, 7 and 14 days post-reperfusion; d1, 3, 7, 14: Ilexonin A 80 mg/kg group at 1, 3, 7 and 14 days post-reperfusion. Data are expressed as the mean ± SD. ∗$P < 0.05$, *vs.* ischemia group; #$P < 0.01$, *vs.* IA 20 and IA 40 mg/kg groups (one-way analysis of variance followed by the least significant difference test).

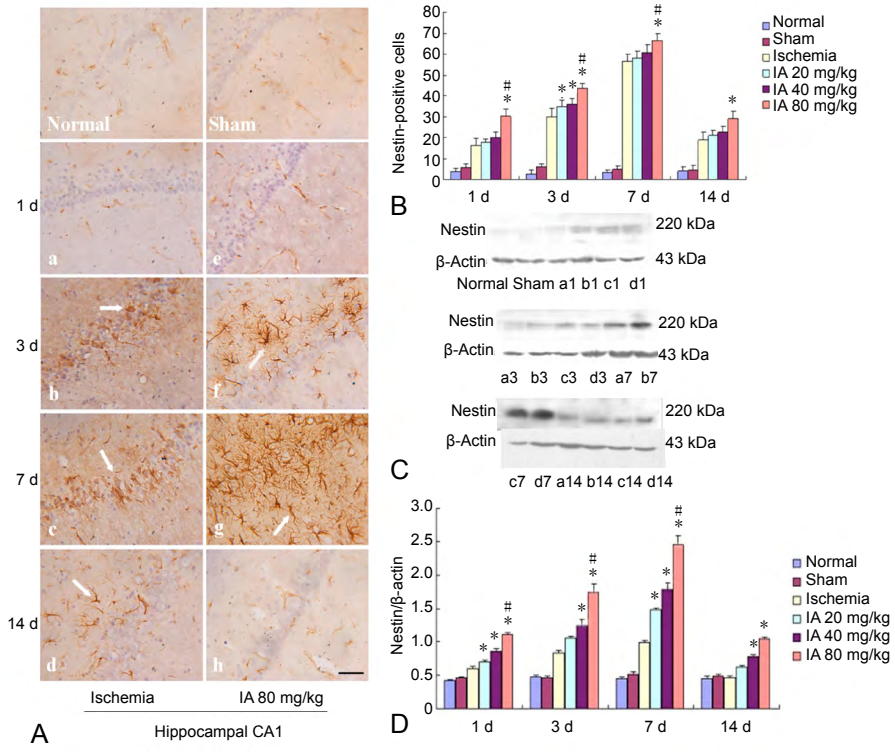

Figure 4 Neuronal stem cell proliferation in the hippocampal CA1 region after ischemia/reperfusion and administration of ilexonin A.

(A, B) Nestin immunostaining (arrows) in the hippocampal CA1 region at different time points after ischemia/reperfusion and administration of ilexonin A (IA). (a–d) Ischemia group at 1, 3, 7 and 14 days post-reperfusion; (e–h) Ilexonin A 80 mg/kg group at 1, 3, 7 and 14 days post-reperfusion (scale bar: 50 μm). The nestin protein in the hippocampus was detected by western blot analysis (C) and analyzed in (D) using β-actin protein as an internal reference. a1, 3, 7, 14: Ischemia group at 1, 3, 7 and 14 days post-reperfusion; b1, 3, 7, 14: Ilexonin A 20 mg/kg group at 1, 3, 7 and 14 days post-reperfusion; c1, 3, 7, 14: Ilexonin A 40 mg/kg group at 1, 3, 7 and 14 days post-reperfusion; d1, 3, 7, 14: Ilexonin A 80 mg/kg group at 1, 3, 7 and 14 days post-reperfusion. Data are expressed as the mean ± SD. $*P < 0.05$, *vs.* ischemia group; $\#P < 0.05$, *vs.* IA 20 and IA 40 mg/kg groups (one-way analysis of variance followed by the least significant difference test).

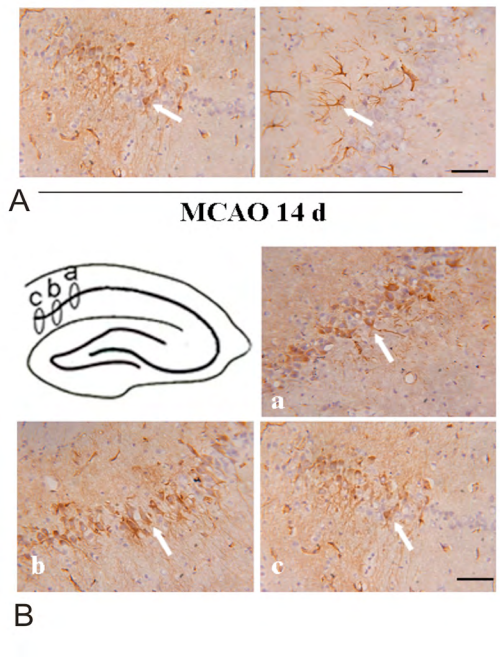

Figure 5 Characteristics of nestin-positive cells in the hippocampal CA1 region.

(A) Different morphologies of nestin-positive cells (arrows) in the ischemia group at 14 days. (B) Nestin-positive cells (arrows) appeared in different parts of the CA1 region at different time points after ischemia/reperfusion. (a–c) 3, 7, 14 days after MCAO. Original magnification: 400×; scale bars: 50 μm. IA: Ilexonin A. MCAO: middle cerebral artery occlusion.

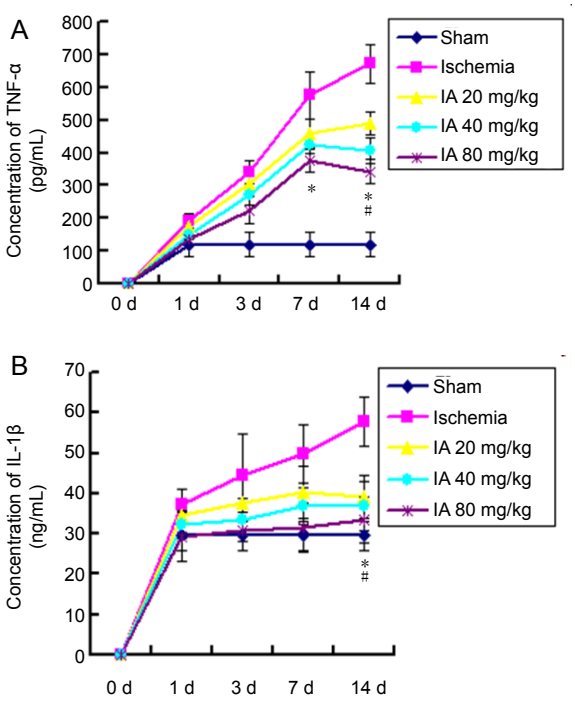

Figure 6 Concentration of TNF-α and IL-1β in the hippocampal tissue after ischemia/reperfusion and IA administration as determined by enzyme linked immunosorbent assay.

TNF-α (A) and IL-1β (B) concentrations were measured at 1, 3, 7 and 14 days after ischemia/reperfusion. Data are expressed as the mean ± SD. $*P < 0.01$, *vs.* ischemia group (one-way analysis of variance followed by the least significant difference test). $\#P < 0.05$, *vs.* IA 20 and IA 40 mg/kg groups. IA: Ilexonin A; IL-1β: interleukin 1β; TNF-α: tumor necrosis factor-α.

amoeba-like cells remarkably decreased, but the number of rod-like cells increased (Xu et al., 2016). Unlike microglia in the peri-infarction region, those in the hippocampal CA1 region were not activated after ischemia/reperfusion, but were activated after subsequent administration of ilexonin A. Previous studies indicated that different brain injuries led to microglia showing different phenotypes and performing different functions (Hu et al., 2012). It has been suggested that their functions (neurotoxic or neuroprotective) could be configured by the equilibrium among various microglial factors (Ekdahl et al., 2009; Wang et al., 2013). Recent *in vivo* and *in vitro* studies have suggested that activation and proliferation of resident microglia were essential for neuronal survival. Selective ablation of proliferating microglia or attenuated acute activation of microglia exacerbated the ischemic injury. Possibly linked to a reduction in the secretion of neurotrophic factors and neuronal plasticity proteins, such as glial cell-derived neurotrophic factor and insulin-like growth factor-1 (Montero et al., 2009; Arroba et al., 2011; Wang et al., 2013). These activated rod-like microglial cells may play a protective role against cerebral ischemia/reperfusion injury, however, further research is required to establish this role. Iba-1 labelling of microglia does not clearly differentiate between M1 and M2 microglial cell types; therefore, we plan to use CD86 and CD206 as markers to investigate the microglial cell type in a future study.

Neural stem cells are static in the intact brain. After brain injury, they begin to proliferate, undergo targeted migration, and differentiate into neurons or gliocytes necessary for neural regeneration and neurologic recovery (Liu et al., 2009). Previous findings suggested that new neurons were observed in the dentate subgranular zone, rostra subventricular zone and, recently, in the cerebral cortex after ischemia/reperfusion (Kim et al., 2009; Yao et al., 2009). Furthermore, ilexonin A enhanced the cortical differentiation of neural stem cells following transient focal cerebral ischemia (Zheng et al., 2011).

In this study, we found two types of nestin-positive neurons with different morphology distribution, and temporal expression after ischemia/reperfusion. Cerebral ischemic injury has induced mature neurons in the cerebral cortex to return to a naive state and re-express embryonic phase proteins (Schmidt-Kastner et al., 1997; Shen et al., 2008; Matsuda et al., 2013). Therefore, the first type of nestin-positive cells, found only in the ischemic model groups, may be mature neurons that start expressing nestin protein. This indicates that neurons may have a self-defense mechanism against ischemic injury. The spatio-temporal distribution of the second type of nestin-positive cells in the ischemic hippocampus mirrored that of astrocytes after ischemia/reperfusion and treatment with ilexonin A (Tao et al., 2014). Previous studies have indicated that reactive astrocytes assisted with neural stem cell proliferation and differentiation and even acquired the capacity to generate neurons (Kronenberg et al., 2010; Shin et al., 2013). Axonal growth cones grew only in the presence of healthy astrocytes but not in areas of injured astrocytes (Takano et al., 2009; Jing et al., 2013). This further confirms the neuroprotective effect of reactive astrocytes immediately after ischemia/reperfusion and indicates that ilexonin A may promote neuronal damage repair in the hippocampal CA1 region by inducing the proliferation of neural stem cells. However, it is unclear why the two types of nestin-positive cells do not co-localize.

TNF-α and IL-1β are factors secreted by activated gliocytes and monocytes/macrophages, which migrated from the peripheral blood due to increased permeability of the blood-brain barrier after ischemia/reperfusion. TNF-α and IL-1β promote and aggravate post-ischemic inflammation, via direct damage or indirectly by inducing the expression of other inflammation mediators. Blockers against both TNF-α and IL-1β remarkably reduced the volume of infarction after ischemia/reperfusion (Intiso et al., 2004; Caso et al., 2007). Our study also indicated that inflammation may play an important role in the delayed neuronal death occurring in the hippocampal CA1 region and that this inflammation is inhibited by ilexonin A. This result further confirms that reactive astrocytes and microglia exert neuroprotective effects after focal cerebral ischemia/reperfusion.

Our previous studies found that ilexonin A exerted the strongest neuroprotective effect in the peri-infarct region after focal cerebral ischemia/reperfusion at the dose of 40 mg/kg (Zheng et al., 2011; Xu et al., 2016). However, in this study, ilexonin A produced the best results at 80 mg/kg. This suggests that cells around the ischemic core may be more sensitive than those of the hippocampus, after the ischemia has occurred.

In conclusion, activation of astrocytes and microglia is associated with neuronal damage in the hippocampal CA1 region and may play a neuroprotective role after transient focal cerebral ischemia. Ilexonin A has a neuroprotective effect, probably through regulation of astrocyte and microglial activation, and thus promotes neural stem cell proliferation and reduces secretion of pro-inflammatory factors.

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References

Arroba AI, Alvarez-Lindo N, van Rooijen N, de la Rosa EJ (2011) Microglia-mediated IGF-I neuroprotection in the rd10 mouse model of retinitis pigmentosa. Invest Ophthalmol Vis Sci 52:9124-9130.

Caso JR, Moro MA, Lorenzo P, Lizasoain I, Leza JC (2007) Involvement of IL-1beta in acute stress-induced worsening of cerebral ischaemia in rats. Eur Neuropsychopharmacol 17:600-607.

Chvátal A, Anderová M, Hock M, Prajerová I, Neprasová H, Chvátal V, Kirchhoff F, Syková E (2007) Three-dimensional confocal morphometry reveals structural changes in astrocyte morphology in situ. J Neurosci Res 85:260-271.

Davies SJ, Goucher DR, Doller C, Silver J (1999) Robust regeneration of adult sensory axons in degenerating white matter of the adult rat spinal cord. J Neurosci 19:5810-5822.

Ding J, Yu JZ, Li QY, Wang X, Lu CZ, Xiao BG (2009) Rho kinase inhibitor Fasudil induces neuroprotection and neurogenesis partially through astrocyte-derived G-CSF. Brain Behav Immun 23:1083-1088.

Duan YL, Wang SY, Zeng QW, Su DS, Li W, Wang XR, Zhao Z (2011) Astroglial reaction to delta opioid peptide [D-Ala2, D-Leu5] enkephalin confers neuroprotection against global ischemia in the adult rat hippocampus. Neuroscience 192:81-90.

Ekdahl CT, Kokaia Z, Lindvall O (2009) Brain inflammation and adult neurogenesis: the dual role of microglia. Neuroscience 158:1021-1029.

Hayakawa K, Nakano T, Irie K, Higuchi S, Fujioka M, Orito K, Iwasaki K, Jin G, Lo EH, Mishima K, Fujiwara M (2010) Inhibition of reactive astrocytes with fluorocitrate retards neurovascular remodeling and recovery after focal cerebral ischemia in mice. J Cereb Blood Flow Metab 30:871-882.

Han Y, Zheng GY, Zhang BQ, Chen XD, Jiang Q (2018) Effect of ilexonin A on the Notch signaling pathway following cerebral ischemia-reperfusion in rats. Biomed Res 29:39-45.

Hu X, Li P, Guo Y, Wang H, Leak RK, Chen S, Gao Y, Chen J (2012) Microglia/macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia. Stroke 43:3063-3070.

Intiso D, Zarrelli MM, Lagioia G, Di Rienzo F, Checchia De Ambrosio C, Simone P, Tonali P, Cioffi Dagger RP (2004) Tumor necrosis factor alpha serum levels and inflammatory response in acute ischemic stroke patients. Neurol Sci 24:390-396.

Jeong HK, Jou I, Joe EH (2013) Absence of delayed neuronal death in ATP-injected brain: possible roles of astrogliosis. Exp Neurobiol 22:308-314.

Jiao L, Zhang J, Li Z, Liu H, Chen Y, Xu S (2011) Edaravone alleviates delayed neuronal death and long-dated cognitive dysfunction of hippocampus after transient focal ischemia in Wistar rat brains. Neuroscience 182:177-183.

Jing L, Mai L, Zhang JZ, Wang JG, Chang Y, Dong JD, Guo FY, Li PA (2013) Diabetes inhibits cerebral ischemia-induced astrocyte activation - an observation in the cingulate cortex. Int J Biol Sci 9:980-988.

Kim HJ, Leeds P, Chuang DM (2009) The HDAC inhibitor, sodium butyrate, stimulates neurogenesis in the ischemic brain. J Neurochem 110:1226-1240.

Kronenberg G, Gertz K, Cheung G, Buffo A, Kettenmann H, Götz M, Endres M (2010) Modulation of fate determinants Olig2 and Pax6 in resident glia evokes spiking neuroblasts in a model of mild brain ischemia. Stroke 41:2944-2949.

Lee CH, Yoo KY, Hwang IK, Choi JH, Park OK, Li H, Kang IJ, Kwon YG, Kim YM, Won MH (2010) Hypothyroid state does not protect but delays neuronal death in the hippocampal CA1 region following transient cerebral ischemia: focus on oxidative stress and gliosis. J Neurosci Res 88:2661-2668.

Lee TK, Kim H, Song M, Lee JC, Park JH, Ahn JH, Yang GE, Kim H, Ohk TG, Shin MC, Cho JH, Won MH (2019) Time-course pattern of neuronal loss and gliosis in gerbil hippocampi following mild, severe, or lethal transient global cerebral ischemia. Neural Regen Res 14:1394-1403.

Li L, Lundkvist A, Andersson D, Wilhelmsson U, Nagai N, Pardo AC, Nodin C, Ståhlberg A, Aprico K, Larsson K, Yabe T, Moons L, Fotheringham A, Davies I, Carmeliet P, Schwartz JP, Pekna M, Kubista M, Blomstrand F, Maragakis N, et al. (2008) Protective role of reactive astrocytes in brain ischemia. J Cereb Blood Flow Metab 28:468-481.

Lin CH, Cheng FC, Lu YZ, Chu LF, Wang CH, Hsueh CM (2006) Protection of ischemic brain cells is dependent on astrocyte-derived growth factors and their receptors. Exp Neurol 201:225-233.

Liu W, Song DL, Zhang GH, Chen JW, Xie RH, Li HJ, Du WJ (2009) Gene expression of Nestin in the ischemia/reperfusion injuried cerebral tissues in rats. Cuzhong yu Shenjing Jibing 16:34-36,40.

Longa EZ, Weinstein PR, Carlson S, Cummins R (1989) Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 20:84-91.

Matsuda Y, Suzuki G, Kusano T, Kawamoto Y, Yoshimura H, Fuse A, Yokota H, Naito Z, Ishiwata T (2013) Phosphorylation of Thr(1495) of nestin in a mouse model of cerebral ischemia and reperfusion damage. Pathol Int 63:448-456.

Montero M, González B, Zimmer J (2009) Immunotoxic depletion of microglia in mouse hippocampal slice cultures enhances ischemia-like neurodegeneration. Brain Res 1291:140-152.

Rauš S, Selaković V, Manojlović-Stojanoski M, Radenović L, Prolić Z, Janać B (2013) Response of hippocampal neurons and glial cells to alternating magnetic field in gerbils submitted to global cerebral ischemia. Neurotox Res 23:79-91.

Schmidt-Kastner R, Garcia I, Busto R, Ginsberg MD (1997) Nestin antibodies label reactive glial cells and some cortical neurons after global brain ischemia in adult rat. J Cerebral Blood Flow Metab 17 Suppl 1:S727.

Shen J, Xie L, Mao X, Zhou Y, Zhan R, Greenberg DA, Jin K (2008) Neurogenesis after primary intracerebral hemorrhage in adult human brain. J Cereb Blood Flow Metab 28:1460-1468.

Sheng HL, Dong XL, Jiang YF (2009) Protective effects of Radix Ilicis Pubescentis extract on focal cerebra l ischemia reperfusion injury in rats. Zhongguo Xinyao Zazhi 18:1020-1022.

Shin YJ, Kim HL, Park JM, Cho JM, Kim SY, Lee MY (2013) Characterization of nestin expression and vessel association in the ischemic core following focal cerebral ischemia in rats. Cell Tissue Res 351:383-395.

Sun JB, Li Y, Cai YF, Huang Y, Liu S, Yeung PK, Deng MZ, Sun GS, Zilundu PL, Hu QS, An RX, Zhou LH, Wang LX, Cheng X (2018) Scutellarin protects oxygen/glucose-deprived astrocytes and reduces focal cerebral ischemic injury. Neural Regen Res 13:1396-1407.

Takano T, Oberheim N, Cotrina ML, Nedergaard M (2009) Astrocytes and ischemic injury. Stroke 40:S8-12.

Tao J, Chen B, Gao Y, Yang S, Huang J, Jiang X, Wu Y, Peng J, Hong Z, Chen L (2014) Electroacupuncture enhances hippocampal NSCs proliferation in cerebral ischemia-reperfusion injured rats via activation of notch signaling pathway. Int J Neurosci 124:204-212.

Tonchev AB, Boneva NB, Kaplamadzhiev DB, Kikuchi M, Mori Y, Sahara S, Yamashima T (2008) Expression of neurotrophin receptors by proliferating glia in postischemic hippocampal CA1 sector of adult monkeys. J Neuroimmunol 205:20-24.

Uchida H, Fujita Y, Matsueda M, Umeda M, Matsuda S, Kato H, Kasahara J, Araki T (2010) Damage to neurons and oligodendrocytes in the hippocampal CA1 sector after transient focal ischemia in rats. Cell Mol Neurobiol 30:1125-1134.

Wang J, Yang Z, Liu C, Zhao Y, Chen Y (2013) Activated microglia provide a neuroprotective role by balancing glial cell-line derived neurotrophic factor and tumor necrosis factor-alpha secretion after subacute cerebral ischemia. Int J Mol Med 31:172-178.

Xu AL, Zheng GY, Wang ZJ, Chen XD, Jiang Q (2016) Neuroprotective effects of Ilexonin A following transient focal cerebral ischemia in rats. Mol Med Rep 13:2957-2966.

Yan BC, Park JH, Ahn JH, Choi JH, Yoo KY, Lee CH, Cho JH, Kim SK, Lee YL, Shin HC, Won MH (2012) Comparison of glial activation in the hippocampal CA1 region between the young and adult gerbils after transient cerebral ischemia. Cell Mol Neurobiol 32:1127-1138.

Yao RQ, Zhang L, Wang W, Li L (2009) Cornel iridoid glycoside promotes neurogenesis and angiogenesis and improves neurological function after focal cerebral ischemia in rats. Brain Res Bull 79:69-76.

Yuan QL, Yang CX, Xu P, Gao XQ, Deng L, Chen P, Sun ZL, Chen QY (2007) Neuroprotective effects of ginsenoside Rb1 on transient cerebral ischemia in rats. Brain Res 1167:1-12.

Zhang BQ, Zheng GY, Han Y, Chen XD, Jiang Q (2016) Ilexonin A promotes neuronal proliferation and regeneration via activation of the canonical Wnt signaling pathway after cerebral ischemia reperfusion in rats. Evid Based Complement Alternat Med 2016:9753189.

Zhang P, Yu TP, Zhang X, Li Y (2011) Repair effect of curcumin on injury of hippocampal CA1 area in rats with chronic cerebral ischemia and relevant mechanism. Zhongguo Shengwu Zhipin Xue Zazhi 24:1193-1196.

Zheng GY, Shi WQ, Chen XD, Zhu YG, Zhang J, Jiang Q (2011) Influence of ilexonin A on the expression of bFGF, GAP-43 and neurogenesis after cerebral ischemia-reperfusion in rats. Yao Xue Xue Bao 46:1065-1071.

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