

Inhibition of cerebral ischemia/reperfusion injury-induced apoptosis: nicotiflorin and JAK2/STAT3 pathway

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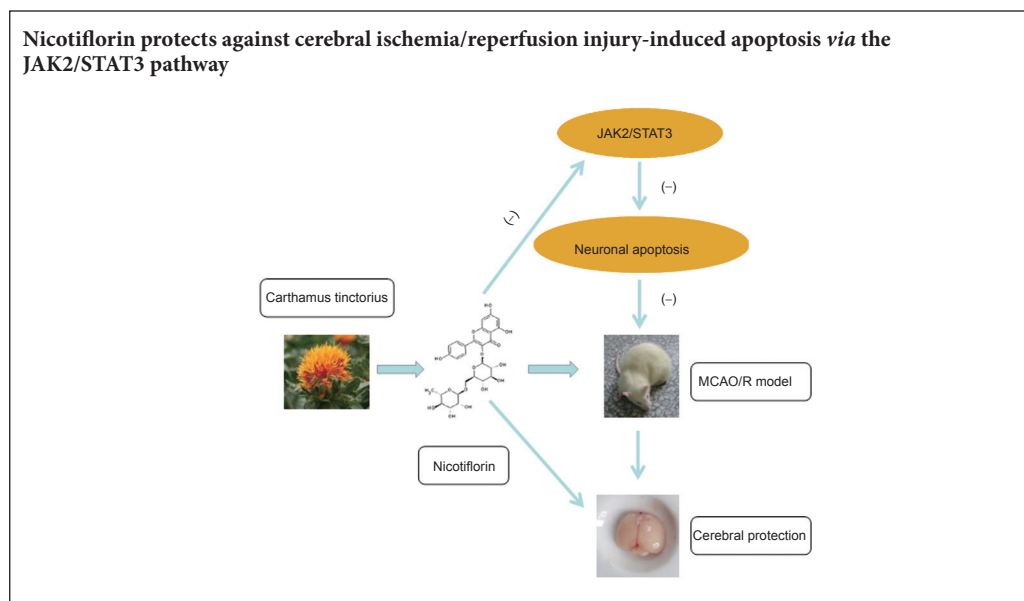
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How to cite this article: Hu GQ, Du X, Li YJ, Gao XQ, Chen BQ, Yu L (2017) Inhibition of cerebral ischemia/reperfusion injury-induced apoptosis: nicotiflorin and JAK2/STAT3 pathway. *Neural Regen Res* 12(1):96-102.

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Funding: This study was financially supported by the Natural Science Foundation of Education Department of Sichuan Province of China, No. 14ZB0152; the Joint Research Program of Luzhou and Southwest Medical University, in China, No. 14JC0120.

Graphical Abstract



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doi: 10.4103/1673-5374.198992

Accepted: 2016-10-22

Abstract

Nicotiflorin is a flavonoid extracted from *Carthamus tinctorius*. Previous studies have shown its cerebral protective effect, but the mechanism is undefined. In this study, we aimed to determine whether nicotiflorin protects against cerebral ischemia/reperfusion injury-induced apoptosis through the JAK2/STAT3 pathway. The cerebral ischemia/reperfusion injury model was established by middle cerebral artery occlusion/reperfusion. Nicotiflorin (10 mg/kg) was administered by tail vein injection. Cell apoptosis in the ischemic cerebral cortex was examined by hematoxylin-eosin staining and terminal deoxynucleotidyl transferase dUTP nick end labeling assay. Bcl-2 and Bax expression levels in ischemic cerebral cortex were examined by immunohistochemical staining. Additionally, p-JAK2, p-STAT3, Bcl-2, Bax, and caspase-3 levels in ischemic cerebral cortex were examined by western blot assay. Nicotiflorin altered the shape and structure of injured neurons, decreased the number of apoptotic cells, down-regulates expression of p-JAK2, p-STAT3, caspase-3, and Bax, decreased Bax immunoreactivity, and increased Bcl-2 protein expression and immunoreactivity. These results suggest that nicotiflorin protects against cerebral ischemia/reperfusion injury-induced apoptosis *via* the JAK2/STAT3 pathway.

Key Words: nerve regeneration; brain injury; nicotiflorin; ischemic stroke; cerebral ischemia/reperfusion injury; treatment; cell apoptosis; terminal deoxynucleotidyl transferase dUTP nick end labeling; JAK2/STAT3 pathway; Bcl-2; Bax; caspase-3; neural regeneration

Introduction

Stroke is a serious leading cause of death that causes financial burden, especially in low-income and middle-income countries (Feigin et al., 2014; Levine et al., 2015; Banerjee and Das, 2016). Ischemic stroke accounts for 75% of all stroke patients (Zevallos et al., 2015). Within a certain time window, thrombolysis is thought to be the most effective treatment method, but many people cannot arrive at hospital within 4.5–6.0 hours, therefore systemic recombinant tissue plasminogen activator is limited (Zaidat et al., 2012; Akbik et al., 2016). Due to developments in pathophysiological stroke research, different mechanisms provide varied treatment opportunities (Lo et al., 2003; Hachinski et al., 2010; Liu et al., 2014).

In recent decades, studies have shown that significant blood flow reductions within the ischemic core accompany irreversible nerve cell necrosis, while programmed cell death (namely apoptosis) appears within the ischemic penumbra, and is reversible until a few hours after cerebral ischemia (Xu and Zhang, 2011; Ghosh et al., 2012; Kalogeris et al., 2012). Hence, saving apoptotic cells is an important strategy in stroke treatment.

Cerebral ischemia/reperfusion (I/R) injury triggers multiple cell apoptotic pathways (Li et al., 1997; Polster and Fiskum, 2004; Wang et al., 2013; Feng et al., 2016). Indeed, there is overwhelming evidence that ischemia-induced oxidative stress and inflammation are principally responsible for subsequent cell death by necrotic or apoptotic mechanisms (Nita et al., 2001; Jin et al., 2013). Reactive oxygen species regulate cell survival/death by activating various cell signaling pathways, such as p38, c-Jun N-terminal kinases, nuclear factor-kappa B, and Janus kinase/signal transducers and activators of transcription JAK/STAT (Nakka et al., 2008; Wang et al., 2014; Hou et al., 2016). Studies show that JAK2/STAT3 activation contributes to cell apoptosis following transient focal cerebral ischemia (Satriotomo et al., 2006; Xie et al., 2007).

Traditional Chinese medicines are effective and have less clinical side-effects for cerebral ischemia patients, but their mechanisms and targets need further investigation (Murphy, 2003; Sun et al., 2015). Nicotiflorin, kaempferol-3-O-rutinoside, a flavonoid glycoside extracted from *Carthamus tinctorius*, has shown protective effects against brain injury in a multi-infarct dementia model (Xie et al., 2007). Similarly, other studies have shown that nicotiflorin improves ischemic brain damage after transient focal cerebral ischemia (Li et al., 2006; Huang et al., 2007). Nicotiflorin is neuroprotective against hypoxia-, glutamate- or oxidative stress-induced retinal ganglion cell death (Nakayama et al., 2011). In addition, we have previously shown protective effects of nicotiflorin in brain injury and neuroinflammation by inhibiting STAT3 activation (Yu et al., 2013). As already noted, JAK2/STAT3 activation contributes to cell apoptosis following transient focal cerebral ischemia. However, it remains poorly understood whether nicotiflorin protects against cerebral I/R-induced cell apoptosis through the JAK2/STAT3 pathway. Accordingly, this is the focus of our present study, and indeed

has not previously been investigated. Thus, we examined the anti-apoptotic effect and underlying nicotiflorin signaling pathway in rats following transient ischemia induced by I/R.

Materials and Methods

Animals

Twenty-four male specific-pathogen-free Sprague-Dawley rats weighing 260–310 g and aged 13–15 weeks were provided by the Experimental Animal Center, Southwest Medical University, China (license No. SCXK(Chuan)2013-17). The rats were equally and randomly allocated to three groups: sham, I/R, and nicotiflorin. Rats were maintained under standardized temperature and humidity with a 12-hour light/dark cycle, and free access to food and water. All procedures were performed in accordance with China Animal Welfare Legislation. Protocols were approved by the Ethics Committee of Southwest Medical University, China.

Surgical operation

Transient middle cerebral artery occlusion (MCAO) was performed on the right side using a nylon filament, as previously described (Longa et al., 1989). Briefly, rats were anesthetized with 10% chloral hydrate (350 mg/kg, intraperitoneally). The internal carotid artery and external carotid artery were carefully detached and a prepared segment of 4-0 monofilament fiber was inserted from the external carotid artery into the internal carotid artery to block the origin of the middle cerebral artery. The sham operation involved a similar surgical procedure except for MCAO. After 2 hours of ischemia, the fiber was gently removed to enable reperfusion of the middle cerebral artery. Body temperature was maintained at 37°C throughout the operation. Rats with the following symptoms were assumed to be successful models: failure to fully extend left forepaw, circling to the left, or falling to the left. After 24 hours of reperfusion, rats were executed.

Drug administration

Nicotiflorin (Shanghai Winherb Medical Technology Co., Ltd., Shanghai, China) was dissolved in 25% polyethylene glycol 400. Nicotiflorin (10.0 mg/kg) was administered by tail vein injection to the nicotiflorin group at the beginning of reperfusion. Vehicle was administered to the I/R and sham groups in the same manner. Rats were executed after 24 hours of nicotiflorin treatment.

Brain tissue extraction

Brain tissue was removed after reperfusion for 24 hours, then fixed, embedded in paraffin blocks, and cut into 5 μ m-thick coronal sections (0.26–0.51 mm anterior to bregma) for conventional hematoxylin-eosin staining, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, and immunohistochemical staining.

Hematoxylin-eosin staining

Briefly, slices were deparaffinized, hydrated in water, then stained with hematoxylin for 15 minutes, and washed in

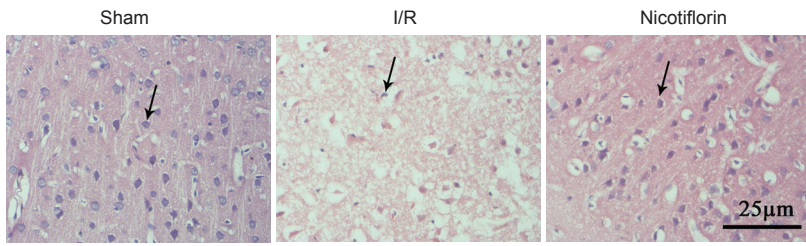


Figure 1 Representative hematoxylin-eosin (HE) stained micrographs of the cortical ischemic penumbra.

In the sham group, normal neurons show round and blue nuclei (arrow). In the I/R group, apoptotic or dead neurons exhibit pyknotic nuclei or side scatter (arrow). Nicotiflorin treatment significantly decreased pathological injury. Scale bar: 25 µm. I/R: Ischemia/reperfusion

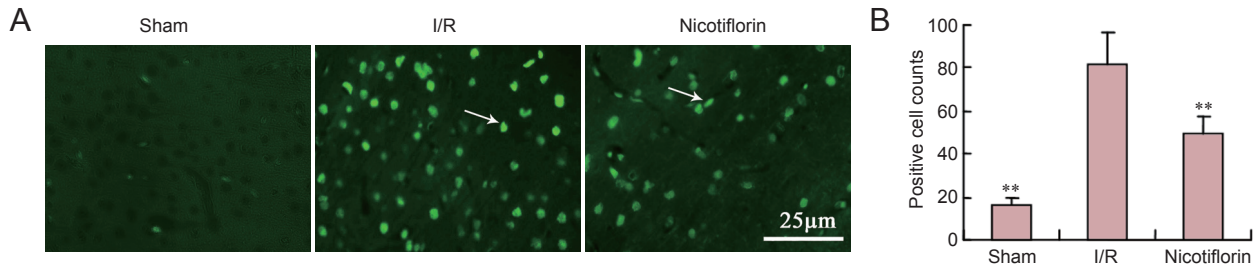


Figure 2 Effect of nicotiflorin on cell apoptosis in the I/R model.

(A) TUNEL staining in cortical cells under fluorescence microscopy (40×). Apoptotic cells are marked by arrows. Scale bar: 25 µm. (B) TUNEL-positive cell number was counted under fluorescent microscopy. $**P < 0.01$, vs. I/R group (mean ± SEM, $n = 3$, one-way analysis of variance followed by *post hoc* Tukey test). TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling; I/R: ischemia/reperfusion.

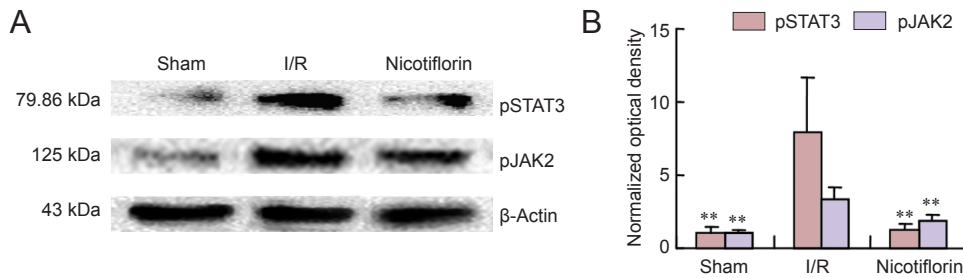


Figure 3 Nicotiflorin effect on expression of JAK2 and STAT3 phosphorylation in the ischemic cerebral cortex of I/R rats.

(A) Western blot images of pJAK2 and pSTAT3. (B) The resulting histogram shows increased pJAK2 and pSTAT3 expression in brain tissue from I/R rats. Nicotiflorin significantly reduced JAK2 and STAT3 phosphorylation. $**P < 0.01$, vs. I/R group (mean ± SEM, $n = 3$, one-way analysis of variance followed by *post hoc* Tukey test). I/R: Ischemia/reperfusion.

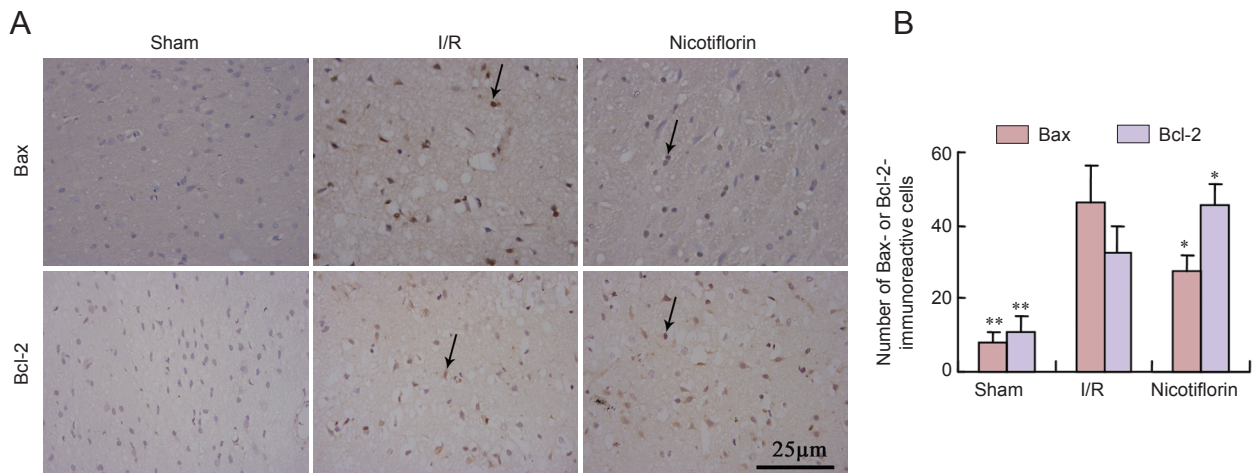


Figure 4 Nicotiflorin effect on Bcl-2 and Bax immunoreactivity in the ischemic cerebral cortex of I/R rats (immunohistochemical staining).

(A) High-magnification images of Bcl-2 and Bax immunohistochemical staining (arrows show immunoreactive cells). Scale bar: 25 µm. (B) For each antibody staining, immunoreactive cells in a 1 mm width were counted under optical microscopy. The average count number was used for quantitation and comparison between groups. Respective numbers of Bcl-2- and Bax-positive cells show that nicotiflorin decreases Bax immunoreactivity and increases Bcl-2 immunoreactivity. $*P < 0.05$, $**P < 0.01$, vs. I/R group (mean ± SEM, $n = 3$, one-way analysis of variance followed by *post hoc* Tukey test). I/R: Ischemia/reperfusion.

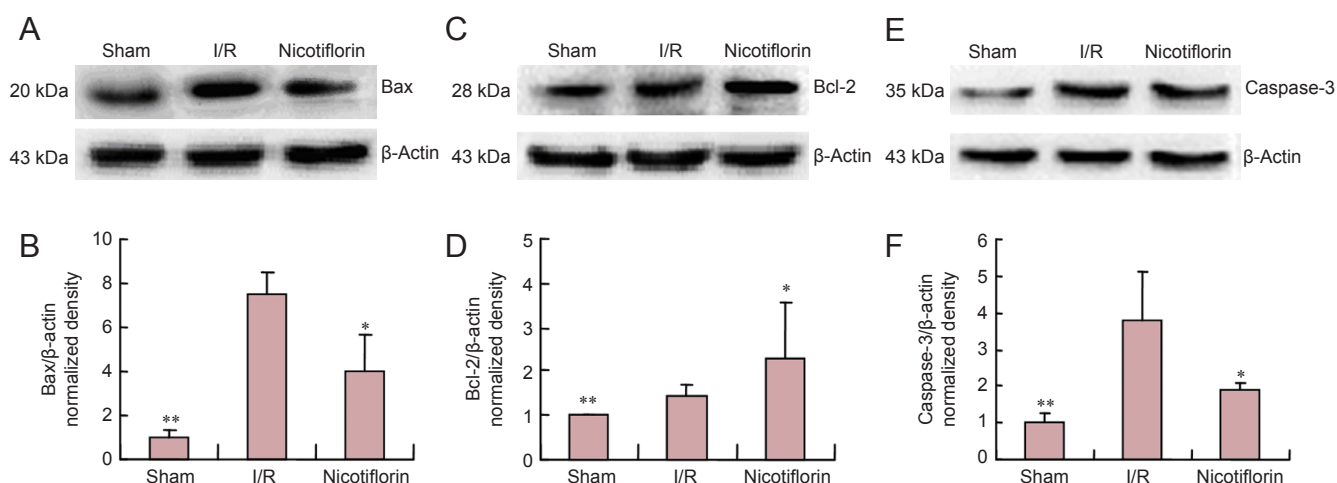


Figure 5 Nicotiflorin effect on Bcl-2, Bax, and caspase-3 expression in the ischemic cerebral cortex of I/R rats (western blot assay).

(A, C, E) Western blot images of Bax, Bcl-2, and caspases-3 protein; (B, D, F) Western blot assay shows up-regulation of caspase-3 and Bax expression and down-regulation of Bcl-2 expression in the I/R group ($*P < 0.05$, $**P < 0.01$, vs. I/R group). Both Bax and caspase-3 expression were decreased and Bcl-2 expression increased in the nicotiflorin group. Data are expressed as the mean \pm SEM, $n = 3$, one-way analysis of variance followed by *post hoc* Tukey test. I/R: Ischemia/reperfusion.

running tap water for 20 minutes. Next, slices were counterstained with eosin for 2 minutes, dehydrated in 95% absolute alcohol until excess eosin was removed, permeabilized in xylene, and mounted. Pathological changes were observed under a light microscope.

TUNEL assay

TUNEL staining was used to detect fragmented nuclear DNA during apoptosis (ArunaDevi et al., 2010; Bahmani et al., 2011), according to standard protocols for the TUNEL assay kit (Boster, Wuhan, China). Briefly, slices were deparaffinized, rehydrated, and then incubated in proteinase K for 15 minutes to digest DNA. After washing in a Tris buffer, sections were incubated in labeling buffer mixed with TdT and digoxigenin (DIG)-d-UTP at 37°C for 2 hours. Blocking reagent was added at room temperature for 30 minutes. Next, sections were incubated in anti-DIG-biotin for 30 minutes, with Strept Avidin Biotin Complex (SABC)-FITC used for final detection. Stained cells were counted from three different views per section using a fluorescence microscope (AMG EVos FL Microscopy, Seattle, WA, USA). The average count number was used for quantification and comparison between groups.

Immunohistochemical staining

Immunohistochemistry of coronal sections was performed as described previously (Yu et al., 2013). Rabbit anti-B-cell lymphoma 2 (Bcl-2) associated X (Bax) polyclonal antibody (Boster; 1:200), and rabbit anti-Bcl-2 polyclonal antibody (Boster; 1:100) were used. Briefly, sections were dewaxed, rinsed with 3% H₂O₂ for 20 minutes, and incubated in 5% bovine serum albumin for 30 minutes to block nonspecific binding. Sections were then separately incubated in Bax and Bcl-2 antibodies overnight at 4°C. Next, ready-to-use goat

anti-rabbit IgG secondary antibody (Boster) was incubated at 37°C for 1 hour. Sections were then stained with 3,3'-diaminobenzidine and counterstained with hematoxylin. For each antibody staining, immunoreactive cells per 1 mm² of the cortex were counted using an optical microscope (Leica DM750, Solms, Germany) (Chu et al., 2006). The average count number was used for quantification and comparison between groups.

Western blot assay

After reperfusion for 24 hours, ischemic cortex was collected for total protein extraction ($n = 3$ each group), according to the manufacturer's instructions for the protein extraction kit (Boster). Equivalent amounts of protein were loaded onto 10% sodium dodecyl sulfate-polyacrylamide electrophoresis gels for standard electrophoresis. Afterwards, gels were transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% skimmed milk in Tris-buffered saline for 1 hour at room temperature, and then incubated overnight at 4°C with the following antibodies: monoclonal phospho-JAK2 (Cell Signaling Technology, Danvers, MA, USA; 1:1,000), monoclonal phospho-STAT3 (Cell Signaling Technology; 1:1,000), polyclonal Bax (Boster; 1:500), polyclonal caspase-3 (Boster; 1:500), polyclonal Bcl-2 (Boster; 1:500), and an internal control, monoclonal beta-actin antibody (Cell Signaling Technology; 1:1,000). Next, membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (1:1,000; Nantong, China) for 1 hour at room temperature. Protein bands were quantified by scanning of visualized bands using enhanced chemiluminescence (Millipore, Bedford, MA, USA) and gel imaging equipment (Bio-Rad, Hercules, CA, USA). Band optical density was measured using Quantity One 4.6.2 software (Bio-Rad).

Statistical analysis

All data are presented as the mean \pm SEM, and were analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA). Differences among groups were analyzed by one-way analysis of variance followed by *post hoc* Tukey test. In all analyses, values of $P < 0.05$ were considered statistically significant.

Results

Nicotiflorin inhibited neuronal pathological changes

Hematoxylin-eosin staining showed that normal neurons in the sham group exhibited regulatory round and bright blue nuclei (Figure 1). While in the I/R group, neurons showed apparent disorder, and part of them presented apoptosis features: nuclear chromatin pyknosis, side scatter, or fracture. Nicotiflorin reduced pathological neuronal injury and the number of dead neurons (necrosis and apoptosis) induced by cerebral I/R.

Nicotiflorin decreased cerebral I/R-induced cell apoptosis

In the sham group, there were only a few TUNEL-positive cells (bright green fluorescence) in the cerebral cortex (Figure 2A). Further, in the I/R group, the TUNEL-positive cell number markedly increased after 2 hours of MCAO and 24 hours of reperfusion. In contrast, compared with the I/R group, TUNEL-positive cells significantly decreased after nicotiflorin treatment ($P < 0.01$; Figure 2B).

Nicotiflorin inhibited JAK2 and STAT3 phosphorylation

Phosphorylation of JAK2 and STAT3 is thought to play an important role in apoptosis regulation. Therefore, both proteins were assessed by western blot assay. Activated pJAK2 and pSTAT3 both increased after ischemic injury in the I/R group compared with the sham group ($P < 0.01$). However, compared with the I/R group, nicotiflorin inhibited pJAK2 and pSTAT3 activation (Figure 3A, B).

Nicotiflorin altered caspase-3, Bcl-2, and Bax immunoreactivity

Bcl-2 family members play important regulatory roles in cerebral I/R injury-induced apoptosis (Hardwick et al., 2012; Kalogeris et al., 2012; Kvensakul and Hinds, 2013; Troy and Jean, 2015). Therefore, we examined changes in Bcl-2 and Bax immunoreactivity. Immunohistochemical staining showed increased Bax immunoreactivity and lower Bcl-2 immunoreactivity in the cerebral cortex of the I/R group (Figure 4A). In contrast, nicotiflorin significantly reduced Bax immunoreactivity, but increased Bcl-2 immunoreactivity ($P < 0.05$ or $P < 0.01$; Figure 4B).

To further examine this anti-apoptotic effect of nicotiflorin, caspase-3, Bax, and Bcl-2 expression was examined by western blot assay in brain tissue after ischemia. Bax and Bcl-2 confirmed the changes observed by immunohistochemistry. Following transient MCAO and 24 hours of reperfusion in the I/R group, increased Bax and caspase-3 expression, and relatively less Bcl-2 expression was detected. However, in the nicotiflorin group, decreased Bax and caspase-3, and increased Bcl-2 expression was observed ($P < 0.05$ or $P < 0.01$,

$n = 3$; Figure 5).

Discussion

Neuronal damage includes both apoptosis and necrosis (Charriaut-Marlangue et al., 1996; Sugawara et al., 2004; Poon et al., 2010). Cerebral ischemia leads to irreversible neuronal injury in the ischemic core area within minutes of onset (Lavrik et al., 2005; Uyttenboogaart et al., 2009; Murray et al., 2010; Jiang et al., 2016). While in the infarct penumbra area, reversible apoptosis is the main cell death mechanism, and is closely related to the final infarct area (Olsen et al., 1983; Dirnagl et al., 1999; Lo, 2008; Popp et al., 2009; Deng et al., 2016). Promisingly, reversible apoptosis provides multiple opportunities for therapeutic intervention in ischemic stroke (Nakka et al., 2008; Broughton et al., 2009). Consequently, we examined the effect of nicotiflorin on neuronal apoptosis in I/R rats. TUNEL staining was chosen as an indicator of apoptotic neurons. Our results show significantly decreased apoptosis in the nicotiflorin group. In addition, hematoxylin-eosin staining showed that nicotiflorin reduces pathological neuronal injury and the number of dead neurons induced by cerebral I/R. This is the first time the effect of nicotiflorin on neuronal apoptosis has been studied.

Apoptotic signal transduction mechanisms play a vital role (Chong et al., 2010; Wang et al., 2015). The JAK/STAT pathway is a critical human disease target (Ghoreschi et al., 2009; Babon et al., 2014; Tao et al., 2015; Chai et al., 2016). Within this family, JAK2/STAT3 is closely associated with ischemia-induced neuronal apoptosis and cancer cell apoptosis (Satriotomo et al., 2006; Xie et al., 2007; Du et al., 2012; Zhang et al., 2015). AG490, a potent JAK2 phosphorylation inhibitor, blocks post-ischemic JAK2 and STAT3 phosphorylation, and significantly decreases cerebral infarction, cell apoptosis, and neurological dysfunction (Satriotomo et al., 2006). We previously found that nicotiflorin is protective against brain injury and neuroinflammation by inhibiting STAT3 activation (Yu et al., 2013). Whether nicotiflorin prevents apoptosis and influences ischemic outcome *via* the JAK2/STAT3 pathway is not known. In the present study, we examined JAK2 and STAT3 phosphorylation in post-ischemic rat brain by western blot assay, in accordance with previous studies (Gorina et al., 2005; Satriotomo et al., 2006). Consistent with previous AG490 findings, we found nicotiflorin blocked post-ischemic JAK2 and STAT3 phosphorylation. Therefore, our results suggest that the protective effect of nicotiflorin against brain damage is related to the JAK2/STAT3 pathway.

To further understand the downstream anti-apoptotic mechanism of nicotiflorin in cerebral I/R injury, we also examined Bax, Bcl-2, and caspase-3 expression. Undoubtedly, both extrinsic and intrinsic pathways are responsible for activation of apoptosis (Gorina et al., 2005; Venderova and Park, 2012). Internally, cerebral ischemia increases intracellular calcium levels, activates calpains, and mediates cleavage of Bid to truncated Bid. Truncated Bid interacts with apoptotic proteins, such as Bax, which generally maintains balance with the anti-apoptotic protein, Bcl-2 (Hassan et al., 2014). Mitochondrial transition pores are

opened and release cytochrome c or apoptosis-inducing factor to activate caspase-9, and subsequently caspase-3, which leads to nuclear DNA damage and apoptosis (Mattson and Kroemer, 2003; Mishra and Kumar, 2005; Taylor et al., 2008; Broughton et al., 2009). Our results show significantly increased expression of the main pro-apoptotic proteins, Bax and caspase-3, in the ipsilateral cortical penumbra. Encouragingly, nicotiflorin strongly inhibits their expression. Alternatively, the anti-apoptotic protein, Bcl-2, was significantly increased in the nicotiflorin group compared with the I/R group. These findings provide improved understanding of the cerebral protective mechanism of nicotiflorin.

In conclusion, nicotiflorin ameliorates cortical neuronal shape and structure, and also decreases the number of apoptotic neurons. Further, this protective mechanism involves the JAK2/STAT3 signaling pathway. Our findings offer further theoretical evidence for nicotiflorin as a potential therapeutic drug for ischemic stroke. Of course, other possible mechanisms need to be further studied.

Acknowledgments: We would like to thank Drug Discovery Research Center of Southwest Medical University of China for providing technical platform.

Author contributions: LY designed the experiments and wrote the paper. GQH performed the experiments. XD and BQC were responsible for data analysis. YJL and XQG provided technical support. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Plagiarism check: This paper was screened twice using CrossCheck to verify originality before publication.

Peer review: This paper was double-blinded and stringently reviewed by international expert reviewers.

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Copyedited by James R, Hindle A, Wang J, Qiu Y, Li CH, Song LP, Zhao M