Original Article
Puerarin modulates autophagy to ameliorate cerebral ischemia/reperfusion injury through JNK signaling pathway

Yao Cheng[1*], Xiao-Lu Liu[1*], Zhi-Gang Mei[1], Jing-Jing Wang[1], Lu Wang[1], Jin-Feng Wang[1], San-Jin Cai[1], Hua-Jun Zhou[2], Shi-Zhong Zhang[1], Zhi-Tao Feng[1]

[1]*Third-Grade Pharmacological Laboratory on Chinese Medicine Approved by State Administration of Traditional Chinese Medicine, Medical College of China Three Gorges University, Yichang, Hubei 443002, China;* [2]*Institute of Neurology, The First College of Clinical Medical Sciences, China Three Gorges University, Yichang, Hubei 443003, China.* *Equal contributors.*

Abstract: Puerarin, a major isoflavone in radix puerariae, exerts remarkable pharmacological effects on ischemic stroke patients in the clinic. However, the underlying mechanisms of the protective effects of puerarin are not fully elucidated. In this study, the effects of puerarin were explored on autophagy induced by cerebral ischemia/reperfusion injury (CIRI) through the c-Jun N-terminal kinase (JNK) signaling pathway. Male Sprague-Dawley (SD) rats were subjected to middle cerebral artery occlusion (MCAO) under deep anesthesia for 1.5 hours with subsequent 24 hour reperfusion. Neurological function deficits and infarct volume of brain tissue were assessed, and the expression of autophagy-related proteins was determined by Western blot with the ischemic cerebral penumbra cortex. The results demonstrate that puerarin alleviates cerebral infarct volume and neurological deficits when compared with I/R group. In addition, pre-treatment with puerarin notably decreased p-JNK-1/JNK-1, p-JNK-2/JNK-2, LC3-2/LC3-1, and expression of Beclin1, and significantly increased expression of Bcl2 and p62 when compared with the I/R group. Compared to the group pretreated with puerarin (100 mg/kg), p-JNK-1/JNK-1, p-JNK-2/JNK-2, LC3-2/LC3-1, Beclin1, and p62 levels had no significant differences in group pretreated with puerarin and SP600125. Moreover, pre-treatment with puerarin and SP600125 exerted similar effects as pre-treatment with puerarin when compared with the I/R group. These studies show that puerarin can attenuate autophagy in a dose-dependent manner and that SP600125 shows similar effects to puerarin. The mechanism is likely that puerarin can decrease expression of JNK and p-JNK, and then increase Bcl2 and interfere with the functions of Beclin1 during the execution of autophagy.

Keywords: Puerarin, autophagy, JNK signaling pathway, CIRI

Introduction

Stroke is regarded as the second leading-cause of death, of which ischemic stroke accounts for approximately 85 percent and it is characterized by significant rates of disability and mortality worldwide, due to lack of valid treatment currently [1]. Rapid revascularization to restore perfusion remains one of the most crucial approaches to salvage cerebral ischemic injury. However, reperfusion might aggravate the injury initially caused by ischemia and result in cerebral ischemia/reperfusion injury (CIRI) [2]. It has been proposed that inflammation [3], oxidative stress [4], apoptosis [5], and hemorrhagic transformation (HT) [6] might take part in the process of CIRI. Accumulating evidence indicates that autophagy also plays an important role in the underlying mechanisms of CIRI [7, 8].

In different circumstances, autophagy could either prompt cell survival or enhance cell death [9]. Researchers had demonstrated that moderate autophagy might have neuroprotection by providing neuronal cells with free fatty acids and amino acids and removing damaged organelles [10, 11], whereas excessive autophagy can be a contributing factor for neuronal death
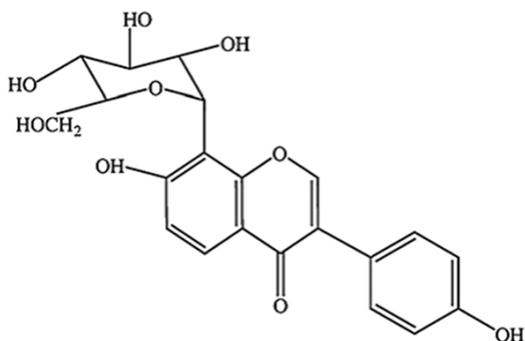
Figure 1. Chemical structure of puerarin.

by excessive degradation of cellular contents in cerebral ischemia [12]. The infarct volume and the neurological outcome depend on a number of factors such as the duration and severity of ischemia [13]. Therefore, inhibition of excessive autophagy might reduce damage caused by CIRI. Emerging studies indicate that the Beclin1/Bcl2 complex [14], adenosine monophosphate-activated protein kinase (AMPK)-mammalian target of rapamycin (mTOR) [15, 16], and the c-Jun N-terminal kinase (JNK) signaling pathway [17] could modulate activation of autophagy in neuronal death following cerebral ischemia and that selective inhibition of the JNK signaling pathway with JNK inhibitor SP600125 can produce protective effects against CIRI [18].

Thrombolysis, antiplatelet agents, and anticoagulants are considered the main rational therapies for ischemic stroke in a timely fashion and the tissue-type plasminogen activator (rt-PA) is appropriate for patients and has remained the mainstay of early treatment of acute ischemic stroke [19]. However, the use of antiplatelet agents and anticoagulants have been limited due to the increasing incidence of symptomatic intracranial hemorrhage [20] and rtPA has remained limited by a narrow time window [21] and the occurrence of hemorrhagic transformation (HT) [6, 22]. Therefore, great efforts on the development of potential neuroprotectants against ischemic stroke have to be made. Accumulating pharmacological studies exhibit that some traditional Chinese medicine (TCM) could be used for protecting neurons against CIRI by dilating the cardio-cerebral vessels [23], suppressing intracellular oxidative stress [24], improving circulation, and enhancing the tolerance of ischemic tissue to hypoxia [25]. Furthermore, recent studies have focused on the

possible effects of TCM such as rhubarb root and rhizome [26], gardeniae fructus [27] and radix puerariae [28].

Puerarin is a major isoflavone derived from the TCM, radix puerariae, which has been used for the treatment of fever, diabetes, and cardiovascular diseases for more than 2000 years [29]. Puerarin injection has been reported to exhibit anti-platelet aggregation [30], anti-oxidant [31] and anti-inflammatory effects [32]. Furthermore, puerarin exerts significant pharmacological effects on cardio-cerebrovascular and microcirculation disturbance diseases as a Chinese patent drug in clinic [33, 34]. In this study, the effects of puerarin on CIRI were investigated and it was hypothesized that puerarin exerts protective effects on ischemic stroke by inhibiting CIRI-induced autophagy in a dose-dependent manner.

Materials and methods

The preparation of puerarin

The puerarin (relative molecular weight 416.38, **Figure 1**) (Batch Number: FALT150901) applied in this research was purchased from Yichang Hospital of Traditional Chinese Medicine, Hubei province, China, and was identified by Maohua Chen, a pharmacist who is the chief of the Pharmacy Department of Yichang Hospital of Traditional Chinese Medicine.

Animals

Male SD rats weighing 250-280 g, were obtained from the laboratory animal center of China Three Gorges University, and housed in a maintained ambient temperature at (22±3)°C with (60±5)% humidity and alternating 12 hour light/dark cycle. They were given free access to water and food. Rats were acclimatized for 1 week prior to experiments. Animal care in this study was in accordance with guidelines approved by China Three Gorges University.

Groups and drug administration

To evaluate the effects of puerarin on the autophagy, rats were randomly divided into 5 groups composing of 7 animals respectively, including group I (Sham), 2 mL/kg normal saline (NS) intraperitoneally per day; group II (I/R), 2 mL/kg NS intraperitoneally per day; group III, 50 mg/kg of puerarin intraperitoneally per day;

group IV, 100 mg/kg of puerarin intraperitoneally per day and group V (SP600125+puerarin), 100 mg/kg of puerarin intraperitoneally per day and 15 mg/kg of SP600125 (sc-200635, Santa Cruze Biotechnology, California, America). NS and puerarin were both injected for 7 days.

Rat model of middle cerebral artery occlusion

The rat model of middle cerebral artery occlusion (MCAO) was employed in this research to fabricate ischemic stroke according to a previously described method [35]. In detail, rats were fasted for 12 hours with free access to water before surgery. Rats were anesthetized with chloral hydrate (0.35 mL/100 g, intraperitoneal injection). Then a short breach was made to expose the left common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA), subsequently separated adjacent nerves and tissues. CCA and ICA were temporarily clamped, in the meantime, ECA was ligated close behind the carotid bifurcation. A blunt-tip 5-0 nylon monofilament was inserted into the ICA through the ECA stump and gently impelled to the Circle of Willis to occlude the right middle cerebral artery (MCA). Then nylon monofilament (50 mm) was placed into the artery nearly 18-20 mm from bifurcation to MCA. The ECA stump and intraluminal filament were tightened to prevent bleeding, then bulldog clamps were removed. The filament was withdrawn gently after an ischemic period of 1.5 hours to induce reperfusion (24 hours).

Evaluation of neurological deficit

The neurological defcit scores were assessed prior to the sacrifice after 24 hours of reperfusion, and neurological defcit scoring system was described by Longa *et al*. (1989). The neurological findings were scored on a 4-point scale: 0 point, behave normally (normal); 1 point, fail to flex left forepaw fully (light); 2 point, turn around into a circle (moderate); 3 point, lean to the left (severe); 4 point, fail to walk autonomously and unconsciousness (critical). Higher scores indicate worse neurobehavioral dysfunction.

Determination of brain infarct volume

After 24 hours of reperfusion, rats were sacrificed by decapitation under deep anesthesia using 10% (v/v) chloral hydrate (350 mg/kg),

following which the whole brains were rapidly removed, cut into five coronal sections (2 mm thick) and stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C for 20 minutes, followed by overnight fixation in 4% paraformaldehyde. TTC-stained sections were photographed and lesion volume was quantitatively analyzed with image analysis software Image-Pro Plus 6.0 (Media Cybernetics, USA), the infarct volume was calculated by taking infarct area on both sides of the slice and multiplying it by the section thickness. The percentage of lesion volume (% HLV) was expressed as the percentage of infarct volume/brain slice volume (%).

Western blot analysis

At 24 hours after reperfusion in each group, the rats were sacrificed under deep anesthesia. The tissues from the ischemic penumbra cortex and the corresponding area of sham-operated rats were dissected rapidly. Subsequently, the tissues were homogenized with lysis buffer for 30 minutes, and the lysates were extracted by centrifugation at 12000 r/min for 10 minutes at 4°C. Then the resulting supernatant was collected and preserved at -20°C. Different samples with an equal amount of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane, which was then blocked with 5% nonfat milk in Tris buffered saline with 0.1% Tween 20 (TBST) for 2 hours at room temperature and incubated overnight at 4°C with following corresponding primary antibodies: anti-β-actin (Boster Biological Technology, Ltd, Wuhan, CN, 1:200), anti-microtubule-associated protein1 light chain3 (anti-LC3) (CST, MA, USA, 1:1000), anti-B-cell lymphoma 2 (anti-Bcl2) (Sanying, Wuhan, CN, 1:500), anti-JNK (Sanying, Wuhan, CN, 1:2000), anti-p-JNK (Abcam, Cambridge, MA, USA 1:800) and anti-Beclin1 (Abcam, Cambridge, MA, USA, 1:500) and anti-p62 (CST, Danvers, MA, USA 1:1000). Afterward, the membrane was completely washed with TBST six times (5 minutes per time) and incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit IgG (Boster Biological Technology, Ltd, Wuhan, CN, 1:5000) for 2 hours at 37°C. After washing, the protein bands were visualized by enhanced chemiluminescence (ECL), Western blot detection reagent
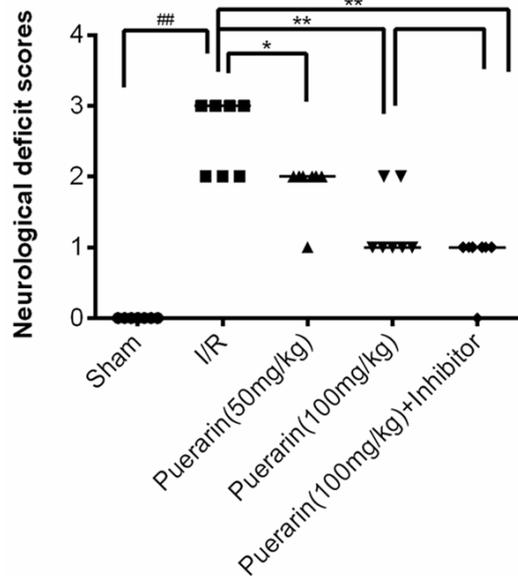
Figure 2. Effects of puerarin on neurological deficits 24 hours after CIRI in rats. Neurological function was assessed by Longa's score, with higher scores indicating more severe neurological deficits. Values are expressed as mean ± SD, n = 7 rats per group. $^{##}P<0.01$ vs. Sham group, $^{**}P<0.01$ vs. I/R group, $^{*}P<0.05$ vs. I/R group.

(Thermo, Waltham, MA, USA) and exposure to X-ray film. Quantification of band intensity was carried out on scanned Western blot images using Bandscan.

Statistical analysis

All data are presented as mean ± standard deviation (SD), and statistical analysis was performed by the SPSS software 18.0. Analysis of variance (ANOVA) was used for multiple comparison of groups. Least significant difference (LSD) analysis was used to compare the means between each of the two groups. A result was considered significant when $P<0.05$.

Results

Puerarin improved neurological deficits induced by CIRI

At 24 hours after cerebral I/R, rats in the sham group did not display any signs of neurological dysfunction (Longa's score 0), while rats in the I/R group showed significant neurological dysfunction when compared to the sham group ($P<0.01$). Pre-treatment with puerarin (50 mg/kg) made an improvement in neurological function when compared to the I/R group ($P<0.05$).

Pre-treatment with puerarin (100 mg/kg) made a prominent improvement in neurological function when compared to the I/R group ($P<0.01$). Furthermore, the puerarin+SP600125 treatment improved neurological function of rats when compared with the I/R group ($P<0.01$) but there were no significant differences when compared to pre-treatment with puerarin (100 mg/kg) ($P>0.05$) (**Figure 2**).

Puerarin reduced infarct volume after cerebral I/R

At 24 hours after cerebral I/R, the infarct volume was measured by TTC staining. Apparent cerebral infarct was detected in I/R group. As shown in **Figure 3**, pre-treatment with puerarin (100 mg/kg) reduced infarct volume of brains when compared to the I/R group ($P<0.05$). Pre-treatment with puerarin (50 mg/kg) reduced infarct volume of brains when compared to the I/R group but there were no significant differences. Furthermore, pre-treatment with puerarin+SP600125 decreased the infarct volume of brains when compared with I/R group ($P<0.05$), but there were no significant differences when compared to pre-treatment with puerarin (100 mg/kg) ($P>0.05$) (**Figure 3**).

Effects of puerarin on Beclin1, Bcl2, JNK, p-JNK, LC3, and p62 expressions

As shown in **Figure 4**, expression of JNK, p-JNK, Bcl2, Beclin1, LC3, and p62 was detected in the ischemic rats brain 24 hours after cerebral I/R by Western blot. Puerarin (50 mg/kg) decreased p-JNK-1/JNK-1 ($P<0.05$), p-JNK-2/JNK-2 ($P<0.05$), Beclin1 ($P<0.05$) and LC3-2/LC3-1 ($P<0.01$) when compared to the I/R group, while expression of Bcl2 ($P<0.05$) and p62 ($P>0.05$) were up-regulated. Puerarin (100 mg/kg) decreased p-JNK-1/JNK-1 ($P<0.01$), p-JNK-2/JNK-2 ($P<0.05$), Beclin1 ($P<0.01$), and LC3-2/LC3-1 ($P<0.01$) when compared with the I/R group, while expression of Bcl2 ($P<0.01$) and p62 ($P<0.05$) were up-regulated. Puerarin (100 mg/kg)+SP600125 decreased p-JNK-1/JNK-1 ($P<0.01$), p-JNK-2/JNK-2 ($P<0.05$), Beclin1 ($P<0.01$), and LC3 ($P<0.01$) while increasing Bcl2 ($P<0.01$) and p62 when compared to the I/R group. Puerarin (100 mg/kg)+SP600125 increased the expression of Bcl2 and LC3-2/LC3-1 when compared to puerarin (100 mg/kg) ($P<0.05$), and decreased expression of Beclin1, p62, p-JNK-1/JNK-1, and p-JNK-2/JNK-2 while there were no significant differences.
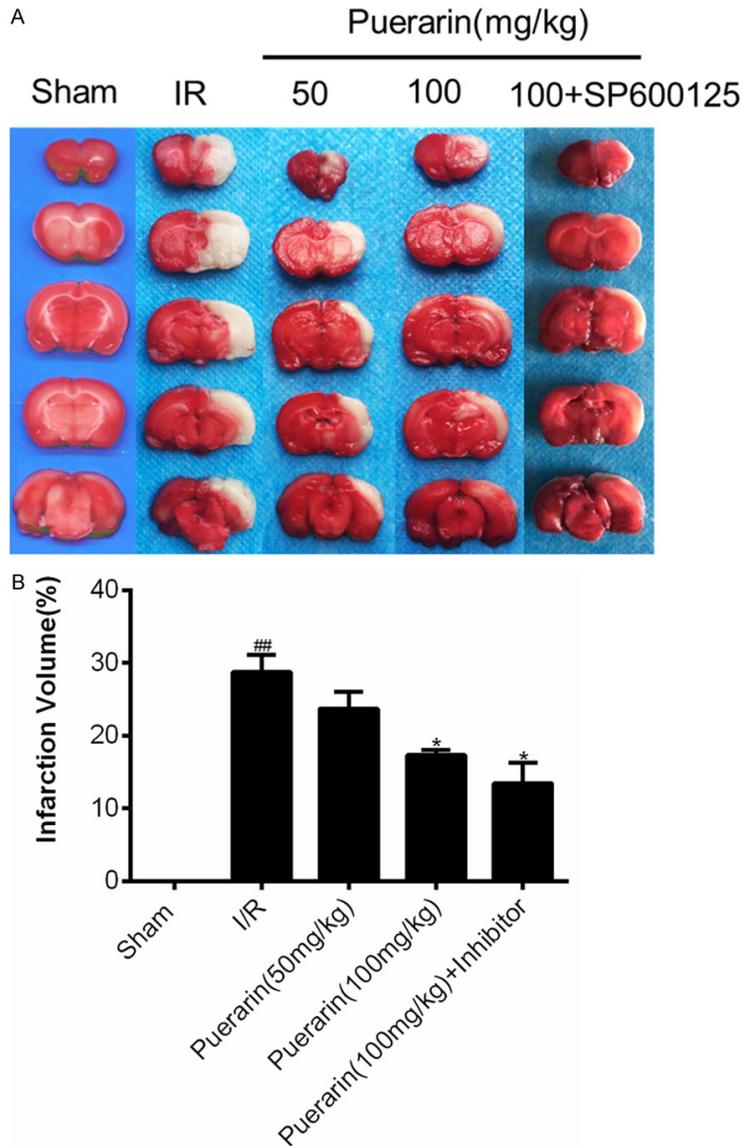
Figure 3. Effects of puerarin on infarct volume 24 hours after CIRI in rats. Infract volume was assessed with TTC staining. A. Infarct picture of TTC staining. B. Infarct volume n=3 rats per group, values are expressed as mean ± SD. ##$P<0.01$ vs. Sham group, *$P<0.05$ vs. I/R group.

arin could serve as a therapeutical drug against ischemic stroke.

Stroke, which includes ischemic stroke and hemorrhagic stroke, has been regarded as the second leading-cause of death and the third leading-cause of disability-adjusted life years worldwide [36]. Ischemic stroke which accounts for approximately 85% of stroke cases, is the result of cerebrovascular occlusion and neurologic changes [37]. Timely restoration of blood flow of the occluded vessel in acute ischemic stroke patients was associated with favorable clinical outcome [21]. It has been reported that intravenous recombinant tissue plasminogen activator (rt-PA) applied to patients with acute ischemic stroke could achieve reperfusion [38]. Moreover, according to the guidelines from American Stroke Association, rapid administration of rt-PA to eligible patients remained the mainstay of early treatment of ischemic stroke [39]. However, the usage of rtPA has been limited to approximately 4.5 hours therapeutic time window after ischemia stroke onset [21] and associated with the occurrence of hemorrhagic transformation (HT) [6, 22, 40]. The mechanisms of CIRI included inflammation [3], oxidative stress [4], and apoptosis [5]. Furthermore, accumulating evidence indicates that autophagy plays a crucial role in CIRI [7, 8] and reperfusion treatment could trigger autophagy which inversely results in subsequent neuronal injury [41]. Therefore, it is necessary to explore some new alternative medicine with the characteristics of high safety, high efficiency, and synthetic therapeutic effects for the treatment of ischemic stroke.

Autophagy is a highly regulated process that involves the degradation of excessive cellular

Discussion

The present study confirms the neuroprotective potential of puerarin, which could ameliorate the abnormalities in infarct volume and neurological deficits, decrease expression of LC3-2/LC3-1, p-JNK/JNK, and Beclin1, and increase expression of Bcl2 and p62 in a dose-dependent manner through JNK signaling pathway following CIRI. The results indicate that puerarin can attenuate cerebral I/R induced autophagy and brain injury, which implies that puer-
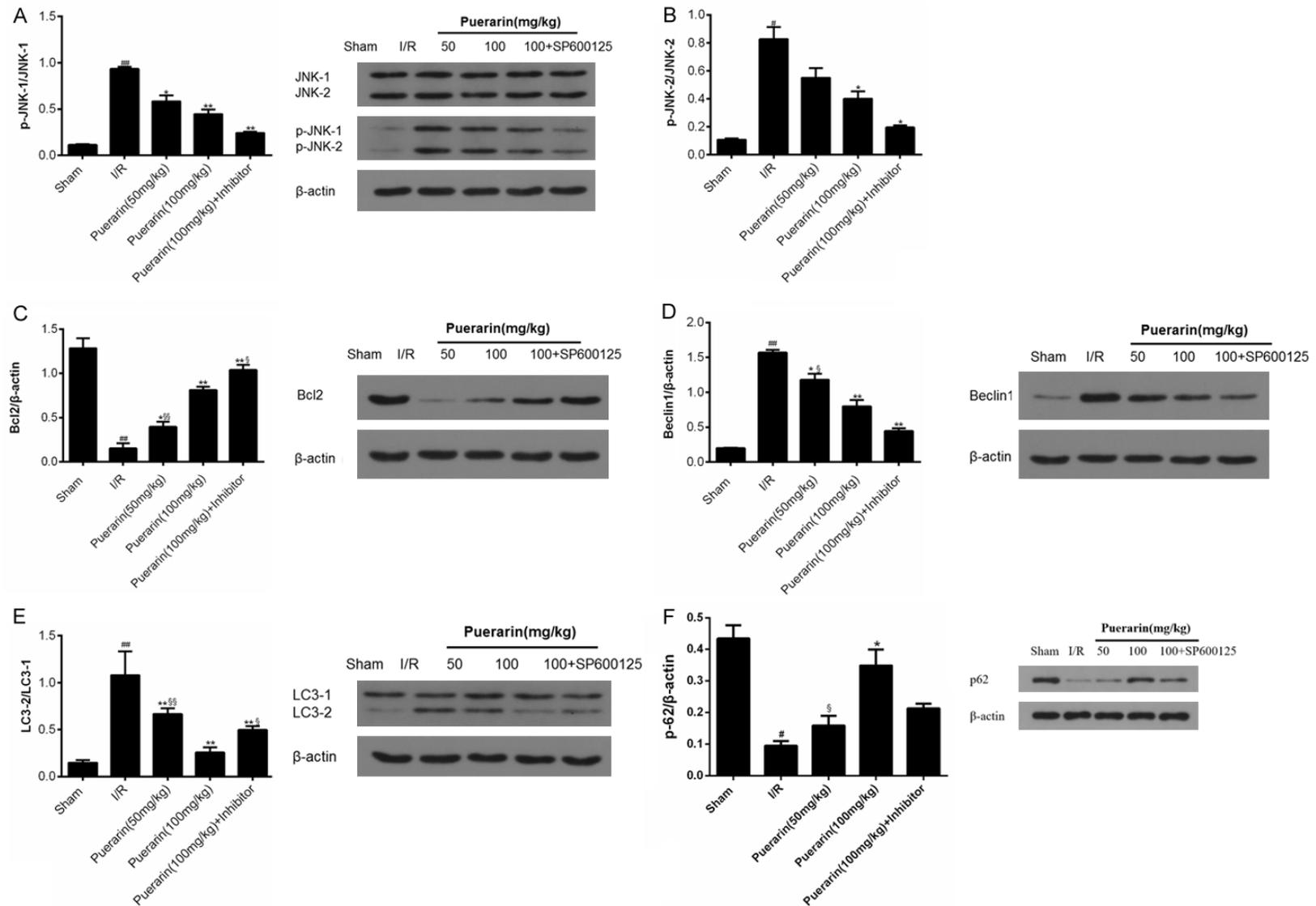
Figure 4. Effects of puerarin on p-JNK1/JNK1, p-JNK2/JNK2, Bcl2, Beclin1, p62, and LC3-2/LC3-1 in the ischemic penumbra cortex 24 hours after CIRI. A. B. Density analysis of protein p-JNK1/JNK1 and p-JNK2/JNK2 protein expressions. C. Density analysis of Bcl2 protein expressions. D. Density analysis of Beclin1 protein expression. E. Density analysis of LC3-2/LC3-1 protein expression. F. Density analysis of p62 protein expression. Actin was used as a loading control. n=3 rats per group. Values are expressed as mean ± SD. ##$P<0.01$ vs. Sham group, **$P<0.01$ vs. I/R group, *$P<0.05$ vs. I/R group, §$P<0.05$ vs. puerarin (100 mg/kg), §§$P<0.01$ vs. puerarin (100 mg/kg).

components or damaged organelles in mammalian cells through lysosomal system [42], which plays an important homeostatic role in cell survival, differentiation, and development [43]. It is involved in many pathological and physiological situations and is activated in response to many relevant factors e.g. starvation [44], ischemia [45, 46], and hypoxia [47, 48]. The roles of autophagy in CIRI are controversial. Research has suggested that moderate autophagy protects cells through mitochondrial clearance [49] and inhibition of P38 [50]. However, persistent or severe cerebral ischemia or excessive activation of autophagy could lead to the self-digestion of important intracellular components, contributing to the death of neurons [41, 51]. Inversely, autophagy deficiency could lead to accumulation of ubiquitinated proteins, endoplasmic reticulum stress, and cell death [52]. Therefore maintaining a balanced level of autophagy is critically important for neuronal survival and function in ischemic stroke and other serious brain injuries [53].

Autophagy is in the modulation of numerous different signaling pathways [54]. Beclin1/Bcl2 complex is well known to regulate autophagy: autophagy is induced when the Beclin1/Bcl2 complex is disrupted [55]. It has been reported that JNK facilitates phosphrylation of Bcl2, which triggers disruption of the Beclin1/Bcl2 complex, Beclin1 (a central regulator of autophagy) [56] release, and autophagosome formation [57]. Additionally, JNK promotes phosphorylation of c-Jun and further increases the mRNA expression of Beclin1 [58]. In this study, expression of JNK and Beclin1 in the group pretreated with puerarin significantly decreased when compared with the I/R group ($P<0.01$), which indicated that the autophagy level was down-regulated by puerarin. LC3 is a marker protein of autophagy [59] and the rate of LC3-2/LC3-1 is positively correlated with the activation of autophagy [60, 61]. Additionally, p62 is widely used as a predictor of autophagic flux [62, 63]. Activating autophagy reduces the expression of p62 [64]. Pharmacological and genetic inhibition of autophagy can increase the level of p62 in various cell lines [65]. Our results show that LC3-2/LC3-1 decreased ($P<0.01$) and p62 increased ($P<0.05$) in the group pretreated with puerarin (100 mg/kg) when compared to the I/R group, indicating that autophagy was inhibited. Puerarin also attenuated brain injury through decreasing expression of JNK, p-JNK,

and Beclin1 while increasing expression of Bcl2 and p62, and exerting similar effects of beta-asarone [66]. Details are as follows: in the group pretreated with puerarin (50 mg/kg and 100 mg/kg), Beclin1, p-JNK-1/JNK-1, p-JNK-2/JNK-2 decreased while the expression of Bcl2 and p62 was significantly increased when compared with the I/R group, indicating that autophagy was inhibited through JNK pathway. These biological parameters combined with the results of infarct volume and neurological deficits demonstrated the protective role of puerarin in CIRI rats which was similar to the results of our former researchers [28, 67, 68]. Therefore, we propose that CIRI caused the activation of JNK signaling pathway and up-regulated the level of autophagy.

Numerous Chinese medicinal herbs or effective constituents e.g. puerarin injection [69, 70], Buyang Huanwu decoction [25], Xuesetong injection [71], β-asarone [66], Weinaokang [72] and Shuan-Tong-Ling [73] have been widely used to treat ischemic stroke for a long time. Puerarin is the major bioactive ingredient isolated from the root of the pueraria lobate, which is well known as Gegen in traditional Chinese medicine. In the clinic, puerarin has been widely used in the treatment of cerebrovascular and cardiovascular diseases, cancer, Alzheimer's disease (AD), diabetes, and diabetic complications [33]. Furthermore, puerarin can improve neurological deficits of acute ischemic stroke patients, lower blood viscosity, and reduce fibrinogen production [74]. Gu et al. reported that puerarin suppressed hippocampal cell death by blockage of acid-sensing ion channels, indicating that puerarin protects the brain against ischemia [75]. Our previous research has shown that puerarin counteracts inflammatory responses after cerebral ischemia/reperfusion in rats through activating the cholinergic anti-inflammatory pathway [28]. In this study, rats in group III and group IV were pretreated with puerarin injection which decreased p-JNK-1/JNK-1, p-JNK-2/JNK-2, LC3-2/LC3-1 and the expression of Beclin1, increased the expression of Bcl2 and p62, and alleviated the cerebral infarct volume and neurological deficits when compared with group II. In the group pretreated with puerarin and SP600125 (group V), Bcl2 and LC3-2/LC3-1 both increased when compared to the group pretreated with puerarin (100 mg/kg) ($P<0.05$). Moreover, the pre-treat-

ment with puerarin and SP600125 exerted the similar effects as group III and group IV when compared with group II, indicating that autophagy was inhibited by puerarin and SP600125 and puerarin exerted similar effects as SP600125 through inhibiting JNK signaling pathway. In conclusion, our study recapitulates two important issues. First, puerarin could protect against brain injury by inhibiting the cerebral I/R induced autophagy in a dose-dependent manner. Second, puerarin protected against the CIRI possibly via the inhibition of JNK signaling pathway, which was similar to the effect of JNK inhibitor SP600125. The underlying mechanism might be that puerarin could suppress autophagy via modulating the expressions of Beclin1, Bcl2, LC3, JNK, and p-JNK. Therefore, puerarin could offer potential therapeutic benefit in the protection against cerebral ischemia in patients who are subjected to ischemic stroke.

Acknowledgements

Disclosure of conflict of interest

None.

Address correspondence to: Drs. Zhi-Gang Mei and Zhi-Tao Feng, Third-Grade Pharmacological Laboratory on Chinese Medicine Approved by State Administration of Traditional Chinese Medicine, Medical College of China Three Gorges University, Yichang, Hubei 443002, China. Tel: (+86)-717-6392568; E-mail: meizhigang@ctgu.edu.cn (ZGM); Tel: (+86)-717-6396558; E-mail: fengzhitao2008@126.com (ZTF)

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