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Research Article

The Effect of Angelica sinensis Polysaccharide on Neuronal Apoptosis in Cerebral Ischemia-Reperfusion Injury via PI3K/AKT Pathway

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In the present study, the protective effects and mechanism of Angelica sinensis polysaccharide (ASP) were investigated in rats with cerebral ischemia-reperfusion injury (CIRI). Rats were randomly divided into sham group, CIRI group, ASP treatment group, and ASP and LY294002 treatment group. H&E results confirmed the successful induction of CIRI in Sprague-Dawley rats. Compared with the sham group, the neurological function score, percentage of myocardial infarction area, neuronal apoptosis, oxidative stress, and inflammation in the CIRI group were significantly increased. Compared with the CIRI group, the ASP group's neurological function score, percentage of myocardial infarction area, neuronal apoptosis, oxidative stress, and inflammation were significantly reduced. However, compared with the ASP group, LY294002 inhibited the effect of ASP in CIRI rats. CIRI downregulated the PI3K/AKT pathway and upregulated the apoptosis level. And ASP activated the PI3K/AKT pathway and Bcl-2 protein expression, while it inhibited caspase-3 and Bax expression. LY294002 could significantly inhibit the protective effect of ASP on nerve injury and the expression and phosphorylation of PI3K and Akt protein in CIRI rats. ASP could effectively improve nerve function and nerve cell apoptosis of CIRI rats by activating the PI3K/AKT signaling pathway.

1. Introduction

Cerebral ischemia-reperfusion injury (CIRI) is a brain injury after ischemic stroke [1]. The most typical feature of CIRI is transient ischemia following reperfusion [2]. Neuronal apoptosis by CIRI causes damage to the hippocampus and cortical neurons [3]. Currently, thrombolytic therapy is the most common clinical treatment for CIRI [4]. However, perfusion increases the production of oxides from reactive proteins, causing intracellular DNA damage, oxidative stress-related injuries, protein oxidation, lipid peroxidation, and thus further worsening the blood-brain barrier and edema [5]. Hence, it is necessary to look for new treatment strategies for post-CIRI.

Traditional Chinese medicine is a rich source of biologically active substances and can be used to prevent or treat various human diseases. In the past few years, it has been proved that the extraction of polysaccharides from traditional Chinese medicine is beneficial to pharmacological activity with low toxicity [6]. *Angelica* has been used as Chinese herbal medicine and functional food in many Asian countries [7]. A water-soluble polysaccharide was isolated from the dried roots of *Angelica sinensis* and named *Angelica sinensis* polysaccharide (ASP). ASP is one of the active ingredients of *Angelica sinensis*. It is composed of xylose, galactose, glucose, arabinose, fructose, and glucuronic acid [8, 9]. ASP has been proven to have various pharmacological effects, with gastrointestinal protective effects, immunomodulatory effects, hematopoietic antitumor activities, antiaging, antidamage, antioxidation, and anti-inflammatory activities [10, 11]. Studies have shown that ASP has a protective effect on cerebral ischemic brain injury. However, there is still a lack of corresponding research on the impact and specific mechanisms of CIRI [12].

The phosphoinositide 3-kinase/AKT (PI3K/AKT) signaling pathway is one of the critical members involved in the

process of brain tissue damage [13]. The PI3K/AKT signaling pathway plays an important regulatory role in a variety of diseases, and it is involved in nerve cell apoptosis, oxidative stress, and inflammation [14]. AKT inhibits apoptosis by regulating apoptosis-related protein cleaved-caspase-3 and apoptosis regulator Bcl-2/Bax after activation. LY294002 is a specific inhibitor of PI3K. The purpose of this paper is to study the effect of ASP on neuronal apoptosis induced by CIRI in rats and to explore the mechanism of the PI3K/AKT pathway in the protective effect of ASP.

2. Methods

2.1. Reagents. Angelica sinensis was purchased from Yutiancheng Pharmacy and collected from Min County, Gansu Province, and the collection time was mid-October 2019. LY294002 was purchased from Thermo Fisher Scientific (California, USA). SOD, MDA, IL-6, and TNF-α detection kits were purchased from Nanjing Jiancheng Biotechnology Company (Nanjing, China). The primary antibodies caspase-3, Bcl-2, and Bax were purchased from Cell Signaling Technology (Massachusetts, USA), and the primary antibodies PI3K, AKT, p-AKT, and GAPDH antibodies were purchased from Abcam (Cambridge, UK).

2.2. Experimental Protocols

2.2.1. Separation and Purification of ASP. The materials of *Angelica sinensis* were pulverized and sieved; then, 100 g powder was added into 800 mL distilled water. After heating and refluxing for boiling for 30 minutes, the obtained decoction was filtered, and the filtrate was lyophilized. The average yield of lyophilized powder was about 19%. The crude ASP was extracted using repeated freezing and thawing to remove protein. The purified ASP was obtained by ultrafiltration, dialysis (MWCO = 3.5 kD), gel filtration chromatography (Sephadex G-50), and lyophilization. Then, the purified ASP was analyzed by high-performance liquid chromatography (HPLC). 20 μL ASP was injected into a Waters Ultrahydrogel™ Linear column (7.8 mm × 300 mm) (Massachusetts, USA). The detector was Waters 2410 refractive index detector. The elution was performed with 0.1 M $NaNO_3$ at a flow rate of 0.9 mL/min.

2.2.2. Preparation of Rat Model of Cerebral Ischemia Reperfusion (CIRI). Sprague-Dawley (SD) male healthy rats (280 ± 30 g body weight) were obtained from Zhejiang University of Traditional Chinese Medicine. Rats are given standard feeding conditions, and an adaptive feeding for one week. This experiment was approved by the Ethics Committee of Zhejiang Rehabilitation Medical Center. The rat CIRI model was established referring to the method of Li et al. [15]: (1) anesthesia: 3% sodium pentobarbital (40 mg/kg) for intraperitoneal injection; (2) preoperative preparation: fixed the rat in the supine position, and prepared skin for disinfection; (3) separate blood vessels and thread: exposed the right common carotid artery, neck external artery, and internal carotid artery; (4) ligation; (5) insert the slit; (6) sewing leather; and (7) postoperative: raised the head slightly in prone position in the suitable temperature and humidity. In the sham group, except that the nylon fishing line was not inserted to occlude the middle cerebral artery, the rest of the steps were the same as the model group.

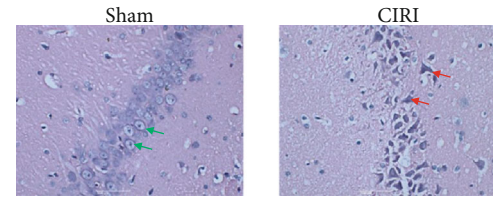

Figure 1: Histopathological changes in hippocampus tissues of rats in the sham and CIRI group were observed by H&E staining. Green arrows point to living cells; red arrows point to dead cells. Magnification ×200.

Hematoxylin-eosin (H&E) staining was used to observe histopathological changes. The neurons in the sham group were regular in shape and neatly arranged, with specific gaps and integrity; the pyramidal neurons in CIRI rats were scattered, the cell bodies were swollen and vacuolated, the nuclei were contracted, and the overall alignment was disordered, as shown in Figure 1. We further confirmed the success of the CIRI rat model.

2.2.3. Grouping and Administration. The rats were randomly divided into sham operation groups, CIRI group, ASP group (intraperitoneal injection of 10 mg/kg ASP solution in CIRI rats), and ASP+LY group (intraperitoneal injection of 10 mg/kg ASP and 0.3 mg/kg LY294002 in CIRI rats), with 10 rats in each group. It is administered once a day for two consecutive weeks.

2.2.4. Neurological Deficit Score. This neurological deficit assessment is a 5-point scale. 0 point: no nerve function damage; 1 point: left forelimb extension disorder; 2 points: circling leftward when walking; 3 points: left leaning when walking; 4 points: coma; and 5 points: death.

2.2.5. Detection of Percentage of Cerebral Infarction Area. The rats in each group were quickly sacrificed, and their brains were removed and sectioned into 2 mm coronal slices. The brain slices were suspended in 1% 2,3,5-triphenyltetrazolium chloride (TTC) and incubated at 37°C in the dark for 30 minutes. Mitochondrial dehydrogenases oxidize TTC, making living tissues appear dark red and necrotic tissues appear pale. Use ImageJ software to calculate the percentage of cerebral infarction area as the following formula: total pale area/total brain area × 100%.

2.2.6. Apoptosis Detection. Annexin V-PI staining was used to detect neuronal apoptosis. The brain tissue of each group was ground into a cell suspension, centrifuged at 800 rpm at 4°C for 5 min, and the supernatant was discarded. Wash the cells twice with precooled PBS, and centrifuge at 800 rpm at 4°C for 5 minutes each time. Discard the supernatant, add 500 μL of Annexin V binding buffer, and mix by pipetting. Add 5 μL of Annexin V-FITC and 10 μL of propidium iodide (PI), mix gently, and incubate for 15-20 min in the dark at room temperature. Flow cytometry was used to detect and analyze the percentage of apoptosis of the cells.

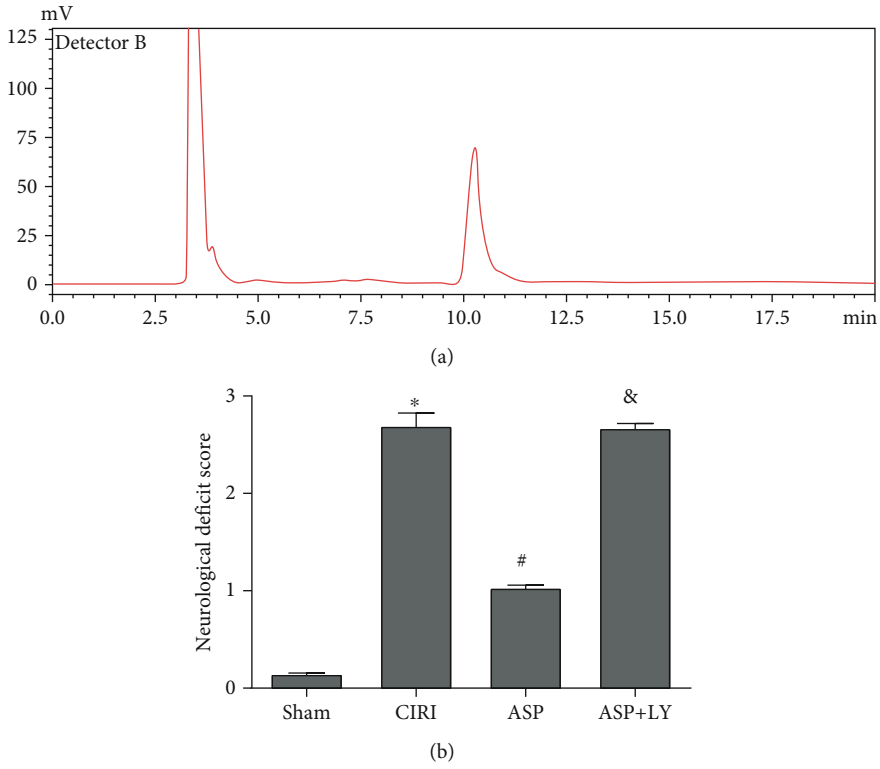

Figure 2: Effect of ASP treatment on rat neurological deficit after CIRI. (a) HPLC profile of ASP. (b) ASP significantly reduces the neurological deficit score of CIRI rats. Note: compared with the sham group, $^{*}P < 0.05$; compared with the CIRI group, $^{\#}P < 0.05$; compared with the ASP group, $^{\&}P < 0.05$. $N = 10$ per group.

2.2.7. ELISA Detection of SOD, MDA, IL-8, and TNF-α Level. The rat brain homogenate was centrifuged at 3500 rpm for 10 min to make a supernatant. The SOD, MDA, IL-8, and TNF-α level was determined by ELISA according to the manufacturer's instructions. The absorbance value of the sample was converted to the concentration according to the standard curve.

2.2.8. Western Blot Analysis. The rat brain was made into homogenate and added with cell lysate to extract total protein. The protein concentration was determined using a BCA kit (Beyotime, Shanghai, China). The quantified protein was loaded, and the protein was separated by SDS-PAGE, and the membrane was electrotransferred to the PVDF membrane. The protein was sealed by 5% skim milk at room temperature for two hours. The membrane was washed and incubated with the primary antibody overnight at 4°C. The primary antibodies used in this study were caspase-3 (1 : 1500), Bcl-2 (1 : 1000), Bax (1 : 1000), PI3K (1 : 1500), Akt (1 : 2000), p-Akt (1 : 2000), and GAPDH (1 : 800). On the next day, the membrane was washed and incubated with the secondary antibodies at room temperature for 2 hours. After washing the membrane, the membrane was developed, imaged, and quantitatively analyzed by Image Lab 3.0 software. GAPDH was used as an internal reference.

2.2.9. Statistical Analysis. SPSS 17.0 software was used to analyze data, and the results were expressed as mean ± standard deviation. The statistical significance among groups was analyzed using one-way ANOVA. The Student t-test was used to compare the difference between the 2 groups. A significance was considered at $P < 0.05$.

3. Results

3.1. The Effects of ASP on Neuronal Functions in CIRI Rats. After the ASP was purified, it was analyzed by HPLC. And a single peak was detected, suggesting that purified ASP was a homogeneous polysaccharide (Figure 2(a)). The neurological deficit score was detected in each rat. As shown in Figure 2(b), compared to the sham group, the scores of neurological deficit in the CIRI group increased significantly ($P < 0.05$). The scores of neurological deficit in the ASP group were significantly lower than those in the CIRI group ($P < 0.05$). Compared to the ASP group, the neurological deficit scores of rats in the ASP+LY group were upregulated significantly ($P < 0.05$).

3.2. Effect of ASP on Percentage of Cerebral Infarction Area in CIRI Rats. TTC staining was used to analyze the cerebral infarct of the rats in each group. The results are shown in Figure 3. Compared to the sham group, the cerebral infarction area percentage in the CIRI group increased significantly ($P < 0.05$). Compared to the CIRI group, the cerebral infarct decreased significantly in the ASP group ($P < 0.05$). Compared to the ASP group, the cerebral infarct in the ASP+LY group increased ($P < 0.05$).

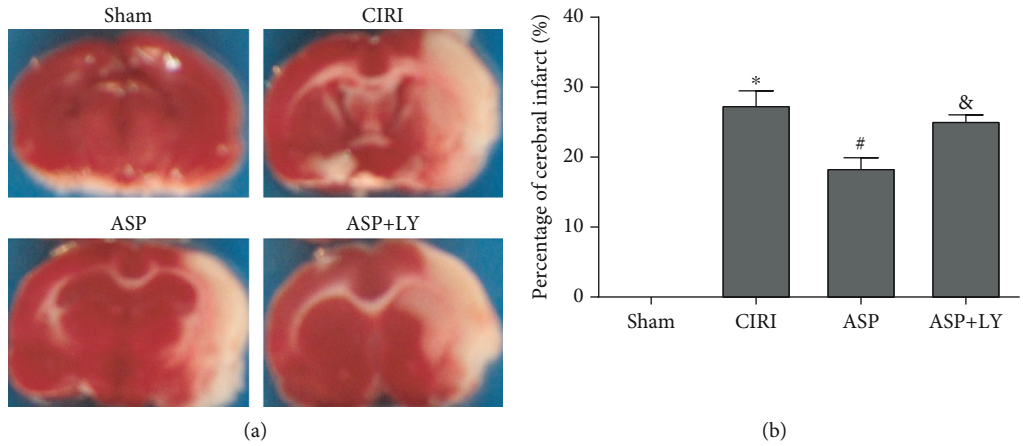

Figure 3: Effect of ASP on cerebral infarct of CIRI rats. (a) Cerebral infarction in SD rats and the represented image of TTC staining. (b) The percentage of cerebral infarction area was quantified and analyzed. Note: compared with the sham group, $^{*}P < 0.05$; compared with the CIRI group, $^{\#}P < 0.05$; compared with the ASP group, $^{\&}P < 0.05$. $N = 10$ per group.

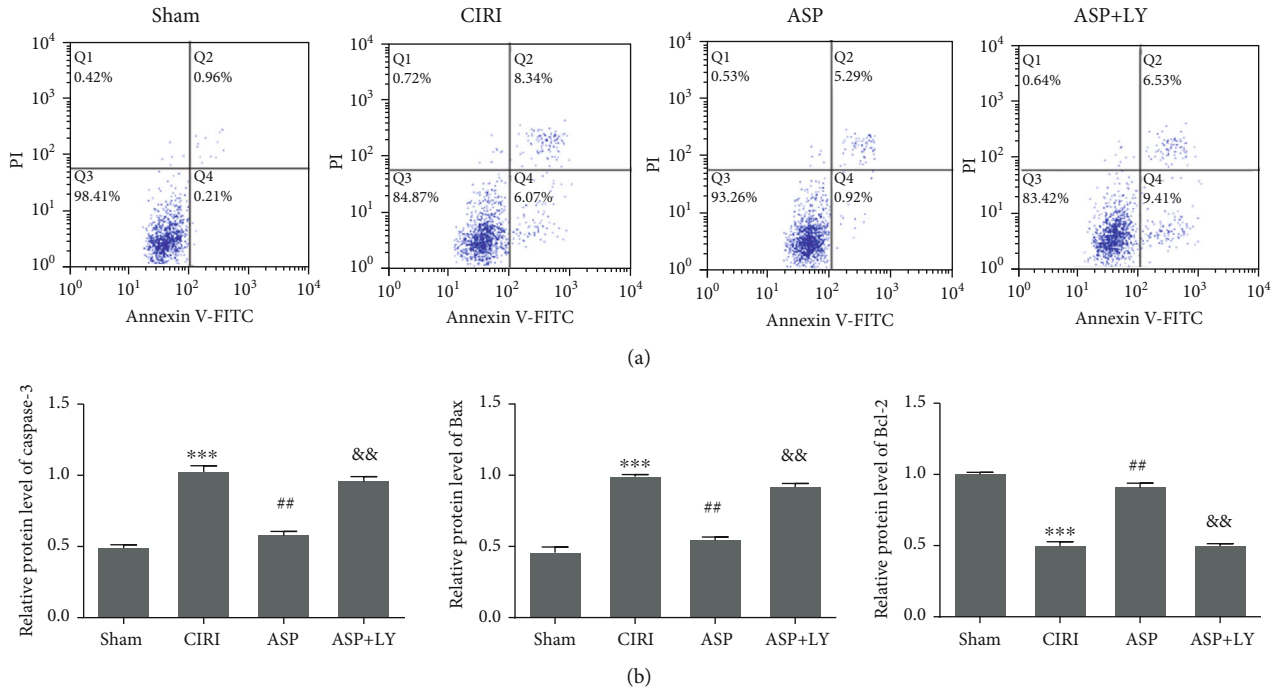

Figure 4: The effect of ASP on cell apoptosis of CIRI rats. (a) Flow cytometry analysis of the apoptosis of nerve cells in the brain tissues of rats in different groups. (b) Western blotting assay for the apoptosis related-proteins expression of caspase-3, Bax, and Bcl-2. Note: compared with the sham group, $^{***}P < 0.001$; compared with the CIRI group, $^{\#\#}P < 0.01$; compared with the ASP group, $^{\&\&}P < 0.01$. $N = 10$ per group.

3.3. Effects of ASP on Neuron Apoptosis Induced by CIRI in Rats. Annexin V-PI flow cytometry was used to detect neuronal apoptosis in the rat brain. As shown in Figure 4(a), compared with the sham group, the apoptosis of neurons in the CIRI group increased significantly ($P < 0.05$). Compared to the CIRI group, neuronal apoptosis decreased significantly in the ASP group ($P < 0.05$). Compared to the ASP group, the apoptosis of nerve cells in the ASP+LY group increased ($P < 0.05$).

Further, apoptosis-related protein expression was detected by western blotting. As shown in Figures 4(b), compared with the sham group, the caspase-3 and Bax expression levels of the rat brain in the CIRI group increased significantly, and Bcl-2 protein expression decreased significantly ($P < 0.001$). Compared to the CIRI group, the caspase-3 and Bax expression levels of brain tissue in the ASP group decreased significantly, while Bcl-2 protein expression significantly increased ($P < 0.01$). Compared to the ASP group, the caspase-3 and Bax expression levels of brain tissue in ASP+LY groups were increased, and Bcl-2 protein expression was decreased ($P < 0.01$). It was suggested that ASP might protect nerve cell from damage and inhibit nerve cell apoptosis.

3.4. Effects of ASP on Oxidative Stress and Inflammatory Factors in CIRI Rats. Cerebral ischemia usually leads to oxidative stress and inflammation. Next, we analyze SOD, MDA, IL-6, and TNF-α levels. As shown in Figure 5,

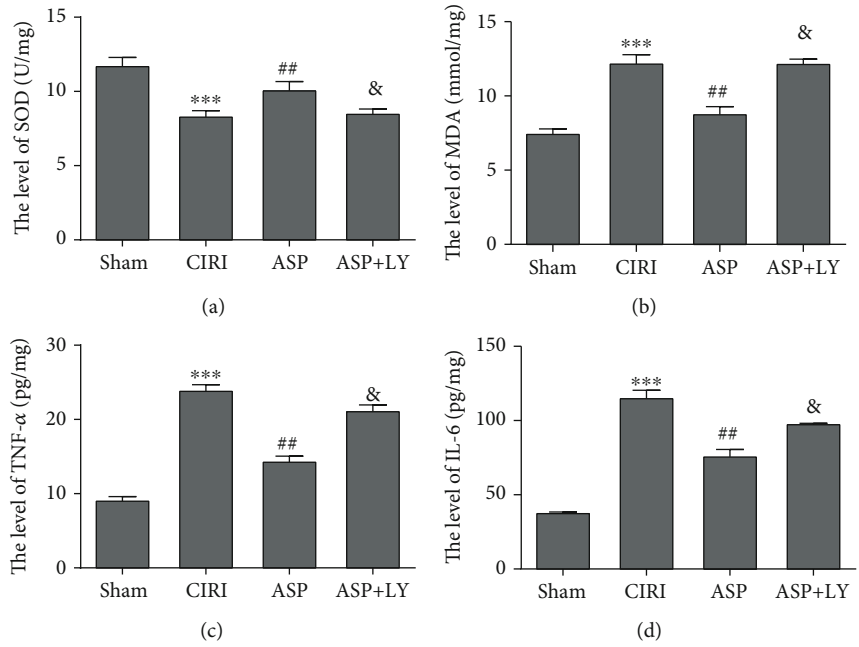

Figure 5: The effect of ASP on the levels of oxidative stress and inflammatory cytokines in rats with cerebral ischemia reperfusion. (a) The levels of SOD. (b) The levels of MDA. (c) The levels of TNF-α. (d) The level of IL-6. Note: compared with the sham group, $^{***}P < 0.001$; compared with the CIRI group, $^{\#\#}P < 0.01$; compared with the ASP group, $^{\&}P < 0.05$. $N = 10$ per group.

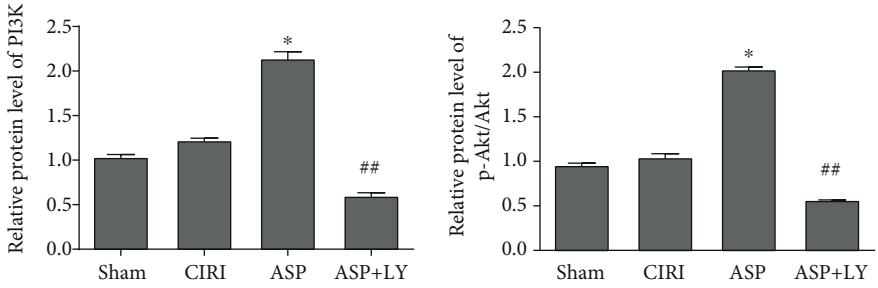

Figure 6: ASP activated PI3K/AKT signaling pathways. PI3K, AKT, and p-AKT protein levels in CIRI rats measured by western blot analysis. Note: compared with the CIRI group, $^{*}P < 0.05$; compared with the ASP group, $^{\#\#}P < 0.01$. $N = 10$ per group.

compared to the sham group, SOD activity decreased significantly in the CIRI group ($P < 0.001$), while MDA, IL-6, and TNF-α levels increased considerably ($P < 0.001$). Compared to the CIRI group, SOD activity had a significant increase in the ASP group ($P < 0.01$), while there was a significant decrease of MDA, IL-6, and TNF-α levels in the ASP group ($P < 0.01$). Compared to the ASP group, SOD activity decreased in the ASP+LY group ($P < 0.05$), whereas MDA, IL-6, and TNF-α levels increased ($P < 0.05$). Therefore, oxidative stress and inflammation occurred after CIRI. ASP had the function of resisting oxidative stress and reducing the expression of inflammatory cytokines.

3.5. ASP Activated the PI3K/AKT Signaling Pathway in CIRI Rats. By western blotting assay, the expression of critical proteins in the PI3K/AKT signaling pathway was detected. As shown in Figure 6, the expression level of PI3K and the ratio of p-AKT/AKT in the ASP group increased significantly in CIRI rats ($P < 0.05$) compared to the CIRI group. Compared to the ASP group, PI3K level and the ratio of p-AKT/AKT expression levels decreased ($P < 0.01$) in the ASP+LY group. The results showed that LY294002 intervention significantly inhibited the effect of ASP on the expression and phosphorylation of PI3K and AKT protein in rats after CIRI. ASP may affect neuronal cells in CIRI rats by activating the PI3K/AKT signaling pathway.

4. Discussion

Stroke is one of the major diseases with the highest mortality and disability rates [16]. It is classified as ischemic stroke and hemorrhagic stroke. Ischemic stroke is caused by a sudden occlusion of cerebral vessels, accounting for about 70% of all strokes [17]. Early and timely reperfusion is the most effective method to limit infarction and improve the clinical prognosis. However, it can also cause harmful effects such as secondary contamination and neuronal loss. Secondary neuronal loss is one of the most critical factors affecting neural function. Neural cells are considered to be the basis of the central nervous system, and the loss of nerve cells is one of

the golden clinical predictors of long-term prognosis. Neuronal loss caused by CIRI is a complex pathological process [18] such as energy failure, neuroinflammation, neuronal apoptosis, oxidative stress, and calcium overload. In this study, it was shown that CIRI rats suffered the pathological symptoms and characteristics, including deteriorated neuronal deficit and cerebral infarction, induced nerve cell apoptosis, inflammation, and oxidative stress. After ASP treatment, it was found that ASP has a protective effect on the nerve damage in CIRI rats and improves the nerve function and cerebral infarct of CIRI rats. The results were partly similar to the previous reports, which confirmed the disease protection of ASP. It has been reported that ASP has a protective effect on acute liver injury in mice induced by concanavalin A or acetaminophen [19]. ASP also has a certain protective effect in colitis [20] and myocardial cells [21]. This study confirmed the protective effect of ASP from CIRI and expanded the pharmacological action of ASP.

Nerve cell apoptosis is the primary manifestation after CIRI. Many studies have shown that the process of apoptosis plays an essential role in CIRI [22]. It was detected that the expression of antiapoptotic proteins Bcl-2 and Bcl-xl was downregulated. In contrast, apoptotic proteins such as cleaved-caspase-3, Bax, and cytochrome c were upregulated after reperfusion [23–25]. The peak period of cell apoptosis is about 24-48 hours after transient ischemia [26]. There is also much evidence that blocking the process of cell apoptosis after cerebral ischemia-reperfusion has a neuroprotective effect. Overexpression of antiapoptotic proteins, such as Bcl-2 and Bcl-xl, or knocking out Bid, caspases, and other proteins with proapoptotic genes can lead to smaller infarctions [27]. Many potential targets or drugs for neuroprotection have been reported through their effects on the process of apoptosis [28]. In this study, it was found that ASP could effectively inhibit neuronal cell apoptosis after CIRI, inhibit the expression levels of caspase-3 and Bax, and increase the expression of Bcl-2 protein to protect neuronal cell damage. The antiapoptosis effect of ASP has also been demonstrated in other diseases. ASP inhibits the oxidative stress-induced chondrocyte apoptosis [29]. In acute liver injury, ASP suppresses the hepatic apoptosis *in vivo* and *in vitro* [30].

Ischemic stroke involves the interaction of many pathophysiological processes. Neuroinflammation and oxidative stress play an essential role in the pathological process [31, 32]. Neuroinflammation is an important marker of the pathology of ischemic stroke, which involves a series of inflammatory responses. Proinflammatory cytokines, TNF-α and IL-6, increased significantly during cerebral ischemia and stroke [33, 34]. In this study, the experimental results showed that after CIRI, the SOD activity decreased significantly. The MDA, IL-6, and TNF-α levels increased significantly, and oxidative stress and neuroinflammation occurred, while in the ASP group, the results showed a significant increase in SOD activity and a significant decrease in MDA, IL-6, and TNF-α levels, indicating that ASP effectively improved oxidative stress and inflammatory response after CIRI. Antioxidant effect is one of the main characteristics of ASP. Several studies have confirmed that ASP play the role of *in vivo* antioxidant effect in liver injury [35], diabetes [36], colitis [37], and urolithiasis [38]. ASP also play the role of antioxidant to chondrocytes*in vitro* [29]. In addition, the previous studies confirmed that ASP has an anti-inflammatory effect, which is similar to the results in this study. In liver injury, ASP pretreatment significantly reduces levels of proinflammatory cytokines (TNF-α, INF-γ, IL-2, and IL-6) [39]. In chronic kidney disease, ASP even inhibits the inflammatory NF-κB signaling pathway [40].

The PI3K/AKT pathway is one of the main signaling pathways affecting apoptosis. The PI3K/Akt pathway plays an important role in regulating cell proliferation, growth, survival, and angiogenesis [41]. Activation of PI3K/Akt pathways has been shown to reduce inflammatory genes and apoptotic protein [42]. In this present study, it was found that ASP activated the PI3K/AKT signaling pathway and exerted the protective effects on rats CIRI against neuronal damage, cerebral infarct, apoptosis oxidative stress, and inflammatory response. Then, the effect of ASP was intervened by LY294002, which was a specific inhibitor of PI3K. The results in this study showed that LY294002 inhibit the signaling pathway induced by ASP, such as PI3K and p-AKT protein expression, and destroyed the protective effect of ASP. This study revealed that ASP may have a protective effect on neuronal cells in CIRI rats by activating the PI3K/Akt signaling pathway.

It was found that ASP could improve the neurobiological function and cerebral infarct of CIRI rats. ASP increased SOD activity; decreased the levels of MDA, IL-6, and TNF-α; and significantly alleviated the apoptosis of nerve cells by activating the PI3K/AKT pathway. However, natural products may have multiple target therapeutic mechanisms and complex signal pathways. The finding of this study is not enough to explain the mechanism on CIRI by ASP in-depth, but the finding suggests that the reasonable combination of ASP and chemical drugs may provide a new thinking for the study of CIRI therapy. In summary, ASP may have a particular therapeutic effect on CIRI rats, providing a theoretical basis for clinical CIRI treatment.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Acknowledgments

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