



# Hydroxysafflor yellow A from safflower (*Carthamus tinctorius* L.) prevents cerebral ischemia-reperfusion injury in rats

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## Abstract

In this study we extracted hydroxysafflor yellow A (HSYA) from safflower and investigate its preventive effect on cerebral ischemia-reperfusion injury (CIRI) in rats. HSYA with 89.78% purity was extracted from safflower. Ninety rats were divided into sham-operated, model, low-dose HSYA, high-dose HSYA and nimodipine groups. The CIRI model was established in latter three groups. After 30 min from ischemia beginning, the low-dose and high-dose HSYA groups were treated with 2 and 4 mg/kg HSYA via tail-vein injection, respectively. The nimodipine group was treated with 2 mg/kg nimodipine. Results showed that, at 24 h after ischemia, compared with model group, in high-dose HSYA and nimodipine groups the neurological symptom score was significantly decreased ( $P < 0.05$ ), the brain water content was significantly decreased ( $P < 0.05$ ), the brain infarction area percentage was significantly decreased ( $P < 0.05$ ), the serum IL-6 and IL-1 $\beta$  levels were significantly decreased ( $P < 0.05$ ), and the brain tissue phosphorylated p38 mitogen-activated protein kinase (p-p38 MAPK) and phosphorylated nuclear factor kappa B (p-NF- $\kappa$ B) p65 protein expression levels were significantly decreased ( $P < 0.05$ ). In conclusion, HSYA can inhibit the p38 MAPK/NF- $\kappa$ B pathway and reduce the inflammatory response, thus exerting the preventive effect on CIRI in rats.

**Keywords:** hydroxysafflor yellow A; cerebral ischemia-reperfusion injury; rats; p38 MAPK; NF- $\kappa$ B.

**Practical Application:** This study has provided a basis for obtaining hydroxysafflor yellow A from safflower and application of it to prevention of cerebral ischemia-reperfusion injury.

## 1 Introduction

Ischemic stroke has the high recurrence rate, disability rate and mortality rate, which seriously endangers the health and life of people (Skajaa et al., 2021). At present, the treatment principle for this disease is mainly to dissolve the thrombus and restore the blood supply. However, the reperfusion after cerebral ischemia can aggravate the pathological damage of ischemic brain tissues and worsen the cerebral injury, which is called the cerebral ischemia-reperfusion injury (CIRI) (Pan et al., 2007). Reducing CIRI has become an important link in the treatment of ischemic cerebrovascular diseases. The pathological mechanism of CIRI is complex. It involves the energy depletion, calcium overload, production of inflammatory mediators, production of free radicals, activation of apoptosis pathway, and so on (Cao & Phillis, 1995; Zhao et al., 2013; Zhang et al., 2016; Liu & Zhang, 2019). The inflammatory response plays a very important role in the physiological and pathological development of CIRI. Seeking drugs for preventing and treating CIRI through inhibiting the inflammatory response has become a research hotspot (Xian et al., 2019; Zheng et al., 2019). It is found that, p38 mitogen-activated protein kinase (p38 MAPK)/nuclear factor kappa B (NF- $\kappa$ B) is a classical inflammatory pathway, which can regulate the expression of a variety of inflammatory factors, thus regulating the inflammatory response (Li et al., 2012). Hydroxysafflor yellow A (HSYA) is the main active component of traditional Chinese medicine safflower (*Carthamus tinctorius* L.) (Bai et al., 2012).

Its molecular formula is  $C_{27}H_{32}O_{16}$ , with molecular weight of 612.53. Pharmacological and clinical studies have shown that HSYA has the anti-inflammatory (Chen et al., 2008), antioxidant (Chen et al., 2016), platelet aggregation-inhibitory (Zang et al., 2002) and other effects. It is applied to treating of ischemic heart disease (Ji et al., 2009), preventing hypoxia injury (Ye & Gao, 2008) and protecting against chronic liver fibrosis (Zhang et al., 2011). In view of this, this study intended to extract HSYA from safflower and investigate the preventive effect of HSYA on CIRI in rats and the related mechanisms.

## 2 Materials and methods

### 2.1 Extraction of HSYA

Safflower powder (500 g) was added into microwave digestion tank, followed by adding 10 L of 50% ethanol-water solution. After vortex shaking for 1 min, the microwave-assisted extraction was performed for 30 min. After cooling to room temperature, the mixture was filtered. The filtrate was concentrated by vacuum. The crude extract was obtained, and was dissolved in water. After standing at 4 °C over night, the mixture was filtered. The filtrate was loaded to X-5 macroporous resin column. The concentration of HSYA in load sample was adjusted to 1.0 mg/mL, and the load flow rate was 10 BV/h. Then, 70% ethanol-water solution was used for elution, and the elution flow rate was 5 BV/h. The effluent

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was collected, and concentrated by vacuum. The concentrated product was dissolved in water, followed by load on silica gel column. The elution was performed using ethyl acetate-95% ethanol (3: 1) solution. The effluent was collected, followed by vacuum concentration and freeze drying. Finally, the refined HSYA product was obtained. The high performance liquid chromatography showed that the purity of HSYA was 89.78%.

## 2.2 Establishment of CIRI model.

Ninety Sprague Dawley rats (280-300 g) were randomly divided into sham-operated, model, low-dose HSYA, high-dose HSYA and nimodipine groups, with 18 rats in each group. The CIRI model was established in latter four groups using the right middle cerebral artery embolization (MCAO) method. The rats were anesthetized with isoflurane. A median incision was made in the neck. The common carotid artery, external carotid artery and internal carotid artery were isolated. The internal carotid artery was clamped using the arterial clasper. An incision was made at the bifurcation of external carotid artery and internal carotid artery. A nylon thread was inserted into the internal carotid artery for about 22 mm distance until there was a slight sense of resistance. After 2 h of occlusion for ischemia, the nylon thread was removed for reperfusion. In sham-operated group, the surgical operations were the same as those in other groups, excepting insertion of nylon thread into internal carotid artery. The room temperature was kept at 23-25 °C during the surgery.

## 2.3 Treatment

After 30 min from the ischemia beginning, the rats in low-dose HSYA group and high-dose HSYA group were treated with 2 and 4 mg/kg HSYA via tail-vein injection, respectively. The rats in nimodipine group were treated with 2 mg/kg nimodipine via tail-vein injection. The rats in sham-operated group and model group were treated with the same volume of normal saline via tail-vein injection.

## 2.4 Neurological symptom scoring

After 3 h, 6 h 12 and 24 h from ischemia, the neurological symptoms of rats were evaluated according to the scoring system as follows: 0 point: no symptom of neurological deficit, normal activity; 1 point: the rats could not fully extend the forepaws on hemiplegic side; 2 points: the rats turned around to the hemiplegic side; 3 points: the rats tilted to the hemiplegic side; 4 points: the rats could not spontaneously walk, with loss of consciousness; 5 points: the rats died.

## 2.5 Determination of serum inflammatory factors

After the last neurological symptom scoring, the rats were anaesthetized with 10% chloral hydrate. The blood was collected from the heart in supine position. The blood samples were centrifuged at 1500 r/min for 20 min, the serum was taken and stored at -20 °C for testing. The serum interleukin 6 (IL-6) interleukin 1 $\beta$  (IL- $\beta$ ) levels were determined using enzyme-linked immunosorbent assay. The operations were according to the instructions of kits

## 2.6 Measurement of brain water content

Six rats in each group were randomly taken. The rats were sacrificed, and the brain was immediately taken. The brain was rinsed with normal saline. The liquid on the surface was sucked dry using filter paper. The brain was weighed to obtain the wet mass. Then, the brain was dried in an oven at 100 °C to constant weight to obtain the dry mass. The brain water content was calculated as follows: brain water content (%) = [(wet mass - dry mass) / wet mass]  $\times$  100%.

## 2.7 Determination of brain infarction area

Six rats in each group were randomly taken. The rats were sacrificed, and the brain was immediately taken. The brain tissue slices with 2 mm thickness were prepared. The slices were stained in 1% 2,3,5-triphenyltetrazolium chloride solution at 37 °C for 20 min, followed by fixation with 10% paraformaldehyde. The normal brain tissue area presented rose red, and the infarction area presented white. The sections were photographed and analyzed using the ImageJ software. The percentage of brain infarction area was calculated.

## 2.8 Western blotting

The remaining six rats in each group were sacrificed, and the brain was immediately taken. The brain tissues were homogenized with RIPA lysate. After centrifugation, the supernatant was extracted and the protein content was determined by bicinchoninic acid method. A 20  $\mu$ g of crude protein sample was loaded on sodium dodecyl sulfonate-polyacrylamide gel electrophoresis. The separated proteins were transferred to the polyvinylidene fluoride membranes. After blocking using 1% bovine serum albumin, the membranes were incubated with primary antibody (anti-p-p38 MAPK, 1: 1000; anti-p-NF- $\kappa$ B p65, 1: 2000; anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 1: 5000) at 4 °C overnight, respectively. After rinsing with Tris buffered saline Tween (TBST) for three times, the membranes were incubated with the second antibody horseradish peroxidase-labeled anti-IgG (1: 2000) at 37 °C for 1.5 h. After rinsing with TBST for three times, the membranes were visualized using the enhanced chemiluminescence reagent. The absorbance of strips was measured by Image J software. Using GAPDH as internal reference, the expression level of the target protein is expressed according to the absorbance ratio of the target protein strip to the GAPDH strip.

## 2.9 Statistical analysis

The analysis was performed using SPSS 20.0 statistical software. All data were represented as mean $\pm$ standard deviation. The data were analyzed using one-way analysis of variance followed by Bonferroni test to determine the difference among groups. Significant differences were accepted for  $P < 0.05$ .

## 3 Results

### 3.1 Comparison of neurological symptom score among five groups

Table 1 showed that, at 3 h, 6 h 12 and 24 h after ischemia, the neurological symptom score in model, low-dose HSYA, high-dose HSYA and nimodipine groups was obviously higher

**Table 1.** Comparison of neurological symptom score among five groups (n = 18).

Group	Neurological symptom score (points)			
	3 h	6 h	12 h	24 h
Sham-operated	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Model	2.83 ± 0.23 <sup>#</sup>	3.51 ± 0.48 <sup>#</sup>	3.97 ± 0.63 <sup>#</sup>	4.32 ± 0.81 <sup>#</sup>
Low-dose HSYA	2.80 ± 0.47 <sup>#</sup>	3.12 ± 0.31 <sup>#S</sup>	3.56 ± 0.52 <sup>#</sup>	3.47 ± 0.77 <sup>#S</sup>
High-dose HSYA	2.59 ± 0.42 <sup>#S</sup>	2.75 ± 0.69 <sup>#S%</sup>	3.27 ± 0.49 <sup>#S</sup>	2.82 ± 0.59 <sup>#S%</sup>
Nimodipine	2.53 ± 0.36 <sup>#S%</sup>	2.61 ± 0.47 <sup>#S%</sup>	2.88 ± 0.58 <sup>#S%<sup>^</sup></sup>	2.62 ± 0.43 <sup>#S%</sup>

Data were represented as mean ± standard deviation. <sup>#</sup>P < 0.05 compared with sham-operated group; <sup>S</sup>P < 0.05 compared with model group; <sup>%</sup>P < 0.05 compared with low-dose HSYA group; <sup>^</sup>P < 0.05 compared with high-dose HSYA group. HSYA, hydroxysafflor yellow A.

than that in sham-operated group, respectively (P < 0.05). Compared with model group, the neurological symptom score in high-dose HSYA and nimodipine groups at 3 h and 12 h and in low-dose HSYA, high-dose HSYA and nimodipine groups at 6 h and 24 h were significantly decreased, respectively (P < 0.05).

### 3.2 Comparison of brain water content among five groups

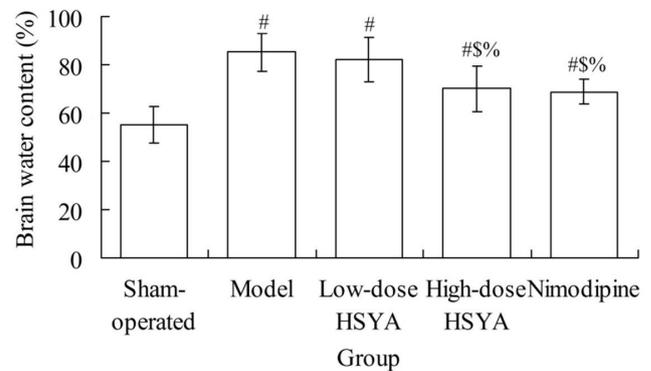
At 24 h after ischemia, the brain water content in sham-operated, model, low-dose HSYA, high-dose HSYA and nimodipine groups was (55.18±7.69)%, (85.20±7.80)%, (82.20±9.13)%, (70.12±9.59)% and (68.90±5.29)%, respectively. Compared with sham-operated group, the brain water content in model, low-dose HSYA, high-dose HSYA and nimodipine groups was obviously increased, respectively (P < 0.05). Compared with model group, the brain water content in high-dose HSYA and nimodipine groups was significantly decreased, respectively (P < 0.05) (Figure 1).

### 3.3 Comparison of percentage of brain infarction area among five groups

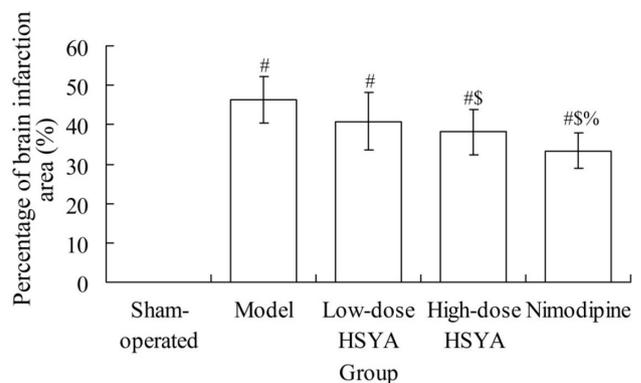
At 24 h after ischemia, percentage of brain infarction area in sham-operated, model, low-dose HSYA, high-dose HSYA and nimodipine groups was (0.00±0.00)%, (46.34±5.87)%, (40.76±7.32)%, (38.18±5.76)% and (33.39±4.45)%, respectively. There was no brain infarction area in sham-operated group, with obvious brain infarction area in model, low-dose HSYA, high-dose HSYA and nimodipine groups. Compared with model group, the percentage of brain infarction area in high-dose HSYA and nimodipine groups was significantly decreased, respectively (P < 0.05) (Figure 2).

### 3.4 Comparison of serum IL-6 and IL-1β levels among five groups

At 24 h after ischemia, compared with sham-operated group, the serum IL-6 and IL-1β levels in model, low-dose HSYA, high-dose HSYA and nimodipine groups was obviously increased, respectively (P < 0.05). Compared with model group, the serum IL-6 and IL-1β levels in low-dose HSYA, high-dose HSYA and nimodipine groups were significantly decreased, respectively (P < 0.05). In addition, the serum IL-1β level in high-dose HSYA group was significantly lower than that in nimodipine group (P < 0.05) (Table 2).



**Figure 1.** Comparison of brain water content among five groups (n = 6). Data were represented as mean ± standard deviation. <sup>#</sup>P < 0.05 compared with sham-operated group; <sup>S</sup>P < 0.05 compared with model group; <sup>%</sup>P < 0.05 compared with low-dose HSYA group. HSYA, hydroxysafflor yellow A.



**Figure 2.** Comparison of percentage of brain infarction area among five groups (n = 6). Data were represented as mean ± standard deviation. <sup>#</sup>P < 0.05 compared with sham-operated group; <sup>S</sup>P < 0.05 compared with model group; <sup>%</sup>P < 0.05 compared with low-dose HSYA group. HSYA, hydroxysafflor yellow A.

### 3.5 Comparison of brain tissue p-p38 MAPK and p-NF-κB p65 protein expression levels among five groups

Table 3 showed that, at 24 h after ischemia, the brain tissue p-p38 MAPK and p-NF-κB p65 protein expression levels in model, low-dose HSYA, high-dose HSYA and nimodipine groups were obviously higher than those in sham-operated

**Table 2.** Comparison of serum IL-6 and IL-1 $\beta$  levels among five groups (n = 18).

Group	IL-6 (pg/mL)	IL-1 $\beta$ (pg/mL)
Sham-operated	25.38 $\pm$ 3.56	7.34 $\pm$ 1.52
Model	55.76 $\pm$ 7.27 <sup>#</sup>	16.10 $\pm$ 2.39 <sup>#</sup>
Low-dose HSYA	50.29 $\pm$ 8.20 <sup>#S</sup>	13.67 $\pm$ 1.80 <sup>#S</sup>
High-dose HSYA	36.15 $\pm$ 4.19 <sup>#5%</sup>	10.09 $\pm$ 0.78 <sup>#5%</sup>
Nimodipine	33.26 $\pm$ 5.84 <sup>#5%</sup>	12.86 $\pm$ 1.14 <sup>#5^A</sup>

Data were represented as mean  $\pm$  standard deviation. <sup>#</sup>P < 0.05 compared with sham-operated group; <sup>S</sup>P < 0.05 compared with model group; <sup>%</sup>P < 0.05 compared with low-dose HSYA group; <sup>^</sup>P < 0.05 compared with high-dose HSYA group. HSYA, hydroxysafflor yellow A; IL-6, interleukin 6; IL- $\beta$ , interleukin 1 $\beta$ .

**Table 3.** Comparison of brain tissue p-p38 MAPK and p-NF- $\kappa$ B p65 protein expression levels among five groups (n = 6).

Group	p-p38 MAPK/GAPDH	p-NF- $\kappa$ B p65/GAPDH
Sham-operated	0.34 $\pm$ 0.06	0.45 $\pm$ 0.07
Model	0.88 $\pm$ 0.11 <sup>#</sup>	1.45 $\pm$ 0.12 <sup>#</sup>
Low-dose HSYA	0.74 $\pm$ 0.09 <sup>#S</sup>	1.23 $\pm$ 0.17 <sup>#S</sup>
High-dose HSYA	0.52 $\pm$ 0.05 <sup>#5%</sup>	0.82 $\pm$ 0.07 <sup>#5%</sup>
Nimodipine	0.46 $\pm$ 0.06 <sup>#5%</sup>	0.66 $\pm$ 0.05 <sup>#5%^A</sup>

Data were represented as mean  $\pm$  standard deviation. <sup>#</sup>P < 0.05 compared with sham-operated group; <sup>S</sup>P < 0.05 compared with model group; <sup>%</sup>P < 0.05 compared with low-dose HSYA group; <sup>^</sup>P < 0.05 compared with high-dose HSYA group. HSYA, hydroxysafflor yellow A; p-p38 MAPK, phosphorylated p38 mitogen-activated protein kinase; p-NF- $\kappa$ B p65, phosphorylated nuclear factor kappa B p65; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

group, respectively (P < 0.05). Compared with model group, the p-p38 MAPK and p-NF- $\kappa$ B p65 levels in low-dose HSYA, high-dose HSYA and nimodipine groups were significantly decreased, respectively (P < 0.05). In addition, the brain tissue p-NF- $\kappa$ B p65 level in nimodipine group was significantly lower than that in high-dose HSYA group (P < 0.05).

## 4 Discussion

In this study, HSYA was successfully extracted from safflower. The purity of HSYA product was 89.78%. This shows that the HSYA extraction technology in this study is feasible. CIRI is the secondary damage to brain tissue caused by cascade reaction due to blood flow reperfusion. It often leads to the neurological deficit, cerebral infarction and brain edema, and can cause the hemiplegia, aphasia and even death in severe cases (Wang et al., 2017a). This study investigated the preventive effect of HSYA on CIRI in rats. Results showed that, compared with model group, in HSYA groups the neurological symptom score, brain water content and percentage of brain infarction area were obviously decreased. It is suggested that HSYA can alleviate the CIRI in rats, thus exerting certain neuroprotective effect.

Inflammatory response plays an important role in CIRI (Zhong et al., 2019). Many inflammatory cells and mediators are involved in the inflammatory response. Inhibition of inflammatory response and reduction of brain tissue damage is one of the important means for treatment of stroke (Duris et al., 2018).

Interleukin is produced by a variety of cells and plays an important role in inflammatory response and humoral immunity. IL-6 can induce the leukocyte adhesion, and activate the complement, thus blocking the microcirculation and damaging the tissue cells (Lokau et al., 2017). IL-1 $\beta$  is an important medium to trigger the immune and inflammatory responses. Study has shown that IL-1 $\beta$  is in a biphasic release mode in the CIRI model, and its level is obviously increased in CIRI (Yang et al., 2016). In the present study, at 24 h after ischemia, compared with sham-operated group, the serum IL-6 and IL-1 $\beta$  levels in other groups was obviously increased. This confirms that the inflammatory response is involved in CIRI. Compared with model group, the serum IL-6 and IL-1 $\beta$  levels HSYA groups were significantly decreased. This suggests that, HSYA can reduce the inflammatory response, thus alleviating the CIRI in rats.

NF- $\kappa$ B is an important transcription activator, and its regulatory genes can encode the cytokines, immune regulatory molecules, etc.. It participates in the inflammatory response (Li et al., 2008). During cerebral ischemia, NF- $\kappa$ B is phosphorylated by inflammatory factors, cytokines, calcium overload and other factors. The p-NF- $\kappa$ B can induce the expression of cytokines, adhesion molecules, and inflammatory enzymes, forming a vicious circle of inflammatory response, which leads to brain tissue edema and nerve cell damage (Zhang et al., 2005; Ridder & Schwaninger, 2009). p38 MAPK, as a member of MAPK family, plays an important role in inflammatory response and its regulation (Ki et al., 2013). After stimulation, p38 MAPK is phosphorylated to form a specific substrate p-p38 MAPK. p-p38 MAPK is the upstream signal molecule of NF- $\kappa$ B. It can promote the phosphorylation of NF- $\kappa$ B to form p-NF- $\kappa$ B p65, thus regulating the transcription of inflammatory genes (Wang et al., 2017b). Results of our study showed that, the brain tissue p-p38 MAPK and p-NF- $\kappa$ B p65 protein expression levels in CIRI rats were obviously higher than sham-operated rats. This indicates that, the activation of p38 MAPK/NF- $\kappa$ B signal pathway is involved in the CIRI of rats. Compared with model group, the p-p38 MAPK and p-NF- $\kappa$ B p65 levels HSYA groups were significantly decreased. This indicates that, the HSYA can inhibit the p38 MAPK/NF- $\kappa$ B pathway to reduce the inflammatory response in CIRI rats.

## 5 Conclusion

In conclusion, HSYA product with 89.78% purity is successfully extracted from safflower. HSYA can inhibit the p38 MAPK/NF- $\kappa$ B pathway and reduce the inflammatory response, thus exerting the preventive effect on CIRI in rats. This study may provide a reference for further clarifying the action mechanism of HSYA in prevention of CIRI. This study still has some limitations. Firstly, the sample size of this study is relatively small. Secondly, other mechanisms related to the prevention of HSYA on CIRI have not been investigated. These issues should be solved in further studies.

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