Effect of Cerebral Ischemia/Reperfusion Injury on Hydroxysafflor Yellow A Penetrating Across the Blood-Brain Barrier

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Abstract

The blood-brain barrier (BBB) is a formidable obstacle for the delivery of therapeutic agents into the central nervous system. Hydroxysafflor yellow A (HSYA) has been shown to be effective in protecting cerebral ischemia. However, whether HSYA could cross the BBB has not been elucidated. In this work, the effect of cerebral ischemia/reperfusion injury on the BBB penetration for HSYA was investigated in normal, sham-operated and cerebral ischemia/reperfusion rats. The concentration of HSYA in homogenate of brain tissue was determined by reversed phase high performance liquid chromatography and the protein was assayed by Bradford assay. The results showed lower concentration of HSYA could be detected in normal rats at 10 to 60 min after HSYA administration. The HSYA concentration in ischemic hemisphere significantly increased, while there is no change in non-ischemic hemisphere. These results suggest that HSYA can penetrate the BBB with weak ability in physiologic condition, but cerebral ischemia/reperfusion injury increases HSYA penetration across BBB markedly.

Keywords

Hydroxysafflor yellow A • Cerebral ischemia/reperfusion • Blood-brain barrier • HPLC
Introduction

The flower of the safflower plant, *Carthamus tinctorius* L., has been used extensively in traditional Chinese medicine for treatment of cerebrovascular and cardiovascular diseases. The compounds present in *C. tinctorius* contain yellow and red pigments including hydroxysafflor yellow A (HSYA), safflor yellow B, safflomin A, safflomin C, as well as other less prevalent pigments [1]. HSYA has been demonstrated to antagonize platelet activating factor receptor binding and has inhibitory effects on both thrombosis formation and platelet aggregation [2]. Wei and colleagues [3] indicated that HSYA might exert neuroprotection against cerebral ischemia/reperfusion injury (CIRI) and the mechanism is related to the antioxidant action of HSYA. It is also reported that HSYA showed a neuroprotective action on the cortex mitochondrial injuries induced by cerebral ischemia and glutamate-mediated neuron injury [4, 5].

The blood-brain barrier (BBB) is a structural and functional barrier between cerebral capillaries and brain parenchyma [6]. It is well known that the BBB restricts the transport of most substances from the systemic circulation to the central nervous system (CNS) in order to maintain homeostasis. However, the BBB is also the main obstacle for the delivery of therapeutic substances into the brain. In order to exert their therapeutic effect, the compounds for the treatment of stroke should adequately cross the BBB and reach effective concentration in the brain. The State Food and Drug Administration of China recently approved HSYA for clinical trials of treating patients with cerebral ischemia as HSYA has showed protective action on cerebral ischemia in preclinical studies. However, it remains unclear whether HSYA can penetrate across the BBB and the effects of cerebral ischemia/reperfusion injury on the penetration of HSYA in BBB. In this study, the ability of the penetration of HSYA in BBB and the effect of CIRI on the penetration of HSYA in BBB was investigated.

Experimental

Chemicals

HSYA standard (purity: 98.49%) was supplied by the Shandong Engineering Research Center for Natural Drugs. The internal standard (rutin purity > 98%, batch number 0080-9705) was purchased from National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). Acetonitrile, methanol and dichloromethane (HPLC-grade) were obtained from Tianjin Kermel Chemical Reagents Development Center. Phosphoric acid (analytical grade) was purchased from Tianjin Chemical and Empirical Company (China). Distilled water filtered by 0.45 μm filter membrane was used throughout the study.

The model of middle cerebral artery ischemia (MCAO)

Transient focal cerebral ischemia was produced by the MCAO procedure as described by Zea Longa [7]. Briefly, rats were anesthetized with chloral hydrate (350 mg/kg, i.p.). The left common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were exposed. A nylon suture (18 mm) was carefully introduced into the ECA lumen and advanced into the ICA to block the origin of the middle cerebral artery. Occlusion was done for a period of 2 h. Reperfusion was accomplished by withdrawing the nylon filament. The subarachnoid haemorrhage was identified during sacrifice from the presence of blood
clot near the circulus Willisil. The sham operated rats received all surgical procedures but without the suture inserted. The normal control rats had no any treatment.

**Animals and study design**

Adult male Sprague–Dawley rats weighing (200–250g) were obtained from the Experimental Animal Center of Shandong Luye Pharmaceutical Company (Certificate No 20030020). Animals were adapted to handling for an adaptation period of 1 week before being used for experiments. Animals were housed in a room at a temperature of (22±2°C) and a relative humidity of (50±5%) with 12 h light/12 h dark cycle and free access to food and water. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 80-23, revised 1996).

Rats were randomly divided into five groups: control group, sham-operated 3 h group, sham-operated 24 h group, ischemia/reperfusion (IR) 3 h group, and IR 24 h group. The rats in each group were further divided into four subgroups (n=9) according to the time of HSYA administration. The rats in IR 3 h group and IR 24 h group were obtained by occluding left middle cerebral artery for 2 h at T1, T3 (Fig.1), followed by reperfusion at T2, T4 respectively. Then the homogenates for measuring the concentration of drugs in the brain tissues were made according to the methods of Alfinito and Germano [9, 10]. The rats in sham-operated groups operated paralleled with model groups. At 10, 30, 60, and 90 min before perfusion with cold saline through the left ventricle, rats were administered HSYA intravenously at a dose of 12 mg/kg respectively. Briefly, rats were anesthetized with chloral hydrate (350 mg/kg, i.p.), and were perfused with 50 mL cold normal saline through the left ventricle (T0). Following decapitation, the brains were carefully removed and hemispheres were separated, weighed, and homogenized in pre-cooled 0.9% saline (at a ratio of 1:2, g/mL) and stored at −20°C for assay.

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**HSYA Perfusion**

**Fig. 1.** Chronology of protocol indicating elapsed times at top, with arrows indicating cerebral ischemia/reperfusion, and relative times at bottom, with arrows indicating drug administration and perfusion (The rats in IR 3 h groups and IR 24 h groups were obtained by occluding left middle cerebral artery for 2 h at T1, T3, followed by reperfusion at T2, T4 respectively, rats in each group were administered HSYA intravenously at T5, and then rats were perfused with cold normal saline through the left ventricle at T0)
**Chromatographic system**
The HPLC system (Agilent 1100 series) consisted of following: a binary pump, a vacuum degasser, a thermostatted column compartment and a UV detector set at 400 nm. Samples were analyzed on a Discovery C18 Column (250 mm×4.6 mm i.d., 5 μm particle size, Supelco, Bellefonte, PA, USA), equipped by a guard column (10 mm×4.6 mm i.d.) of the same material. The chromatographic mobile phase was acetonitrile: 2mM phosphoric acid (16:84, V/V), at a flow-rate of 1.0 mL/min performed at 30°C. Under these conditions, the runtime analysis was set at 15 min.

**Preparation of standard solution**
The stock solution of HSYA were prepared in 50 % methanol at a concentration of approximately 384 μg/mL, and the solution of internal standard was prepared at a final concentration of 15 μg/mL in methanol. All solution were stored at 4 and brought to room temperature before used.

**Sample pre-treatment**
The homogenized brain tissue solution 500 μL was diluted with 110 μL 50% methanol, 50 μL internal solution and 1 mL acetonitrile by vortex for 1 min, ultrasonic for 5 min at room temperature. The denatured protein precipitate was separated by centrifugation at 12000 rpm for 10 min. The supernatant was transferred to clear centrifuge tubes and aliquots of dichloromethane were added. These tubes were vortex-mixed for 3 min and centrifuged for 10 min at 3600 rpm. The supernatant was collected. 20 μL of the supernatant was injected into the HPLC system for analysis. The same sample handling process was used for standard curve preparation and precision, recovery determinations in brain tissue homogenate.

**Standard curve preparation**
A calibration curve was constructed by adding 110 μL seven different concentrations of HSYA and 50 μL internal standards to blank brain tissue homogenate. Seven spiked brain homogenate samples covering a concentration range from 16 to 640 ng/mL were assayed (16, 20, 40, 80, 160, 320, 640 ng/mL). The peak area ratios (HSYA: internal standard) to HSYA concentrations were used for the linear regression.

**Precision and stability**
The precision was evaluated by the intra-day and inter-day variability with relative standard deviation (RSD). The blank brain homogenate spiked with three different concentrations (20, 80, 640 ng/mL) of HSYA and 50 μL internal standard were assayed at 0, 1, 2, 3, 4, 6, 8 and 10 h, when the sample was not detected, it was stocked at 4°C, the changes in peak area ratios (HSYA: internal standard) were calculated as the intra-day RSD and stability. The inter-day variance was assayed over three consecutive days (stored at −20°C), RSD were calculated from these values.

**Recovery**
The biological samples spiked with three different concentrations (20, 80, 320 ng/mL) of HSYA were assayed and the recovery of HSYA was established by comparing peak area of standards in prepared samples with those of standards in 50% methanol.
**Determination of protein content in brain tissue**

The content of protein in brain tissue homogenate was determined by the Bradford protein assay kit. Brain tissue concentrations of HSYA were expressed as the ratio of the HSYA concentration to protein content in brain tissue.

**Statistical analysis**

Results were expressed as means±S.D. One-way analysis of variance (one-way ANOVA) and Student’s t-test was used to determine statistical significance. Values of P<0.05 were considered to be statistically significant in all cases.

**Results**

**Specificity and selectivity**

Under the conditions described above, the retention times of HSYA and rutin (internal standard) were about 5.2 and 12.7 min, respectively. No interfering peaks were observed within the time frame in which HSYA and rutin were detected. Chromatograms of 50% methanol containing HSYA, blank brain tissue homogenate, blank brain tissue homogenate spiked with HSYA and internal standard, and rat brain tissue homogenate sample are shown in Fig. 2.

![Chromatograms by HPLC](image)

**Fig. 2.** Chromatograms by HPLC (A, HSYA reference substance; B, blank brain homogenate; C, blank brain homogenate + HSYA + Rutin; D, brain homogenate after intravenous administration of HSYA; 1: HSYA; 2: Rutin)
Calibration curves
The calibration curve for HSYA was linear (r=0.9991) over the concentration range 16-640 ng/mL in rat brain tissue homogenate. A regression equation for the line was 
\[ y=0.0039x-0.0091 \] (where \( x \) expressed as the concentration of HSYA in the brain tissue homogenate and \( y \) expressed as the area HSYA/area internal standard).

Precision, stability, and recovery
The RSD of the intra- and inter-day precisions were 3.9%, 5.6%, 2.8% and 6.4%, 3.8%, 5.9% at concentrations of 20, 80, 640 ng/mL, respectively. The results suggested that HSYA was stable in rat brain tissue homogenate for at least 10 h when stored at 4°C. The method recovery of HSYA were 83.8±3.5%, 85.8±2.7%, 89.7±5.2% at concentrations of 20, 80, 320 ng/mL respectively (n=3). These validation results indicated that the method was suitable for the present study.

Determination of HSYA of brain in physiological condition
The HSYA concentrations of brain tissue in control group were shown in Fig. 3. HSYA could be detected during the period from 10 to 60 min and with highest concentration at 10 min after HSYA intravenous injection. At 90 min after HSYA injection, there was no HSYA detected in brain tissue. Furthermore, HSYA concentrations in left and right hemispheres were low with no significant difference from each other.

![Graph showing HSYA concentration over time and concentration across hemispheres.](image)

**Fig. 3.** The penetration of HSYA cross the BBB in physiological condition (Rats in control group were administered HSYA intravenously at 10, 30, 60, and 90 min before perfusion through the left ventricle. The HSYA concentration was expressed as the ratio of the HSYA concentration to protein content in homogenate of brain tissue. Data were presented as mean±S.D., n=9)
Effects of cerebral ischemia/reperfusion injury on the penetration of HSYA cross the BBB in non-ischemic hemisphere

There were no significant differences in the HSYA concentrations in right hemisphere between sham group and control group. Compared to sham group, the HSYA concentrations in non-ischemic hemisphere in IR 3 h and IR 24 h groups had no obvious changes (Fig.4).

Fig. 4. The effects of cerebral ischemia/reperfusion injury on the penetration of HSYA cross the BBB of non-ischemic hemisphere (Rats were administered HSYA intravenously at 10, 30, 60, and 90 min before perfusion through the left ventricle. The HSYA concentration was expressed as the ratio of the HSYA concentration to protein content in homogenate of brain tissue. Data were presented as mean±S.D., n=9)

Effects of cerebral ischemia/reperfusion injury on the penetration of HSYA cross the BBB in ischemic hemisphere

The HSYA concentrations of left (ischemic) brain tissue in IR groups were shown in Fig.5. Compared with control group, there were no marked changes in the HSYA concentrations of left brain tissue in sham-operated 3 h and sham-operated 24 h groups. At 10 min after intravenous injection, HSYA attained the highest concentration and from that time the HSYA concentration declined. Compared with sham-operated group, the HSYA concentrations of ischemic hemisphere in IR 3 h group increased significantly (p<0.05 or p<0.01). HSYA could be detected at 10 min and the concentration peaked at 30 min. The HSYA concentrations of ischemic hemisphere in IR 24 h group significantly increased (p<0.05 or p<0.01). The concentration kept on augmenting during the period from 10 to 90 min and attained highest concentration at 90 min after HSYA intravenous injection.
**Fig. 5.** The effects of cerebral ischemia/reperfusion injury on the penetration of HSYA cross the BBB of ischemic hemisphere (Rats were administered HSYA intravenously at 10, 30, 60, and 90 min before perfusion through the left ventricle, The HSYA concentration was expressed as the ratio of the HSYA concentration to protein content in homogenate of brain tissue. Data were presented as mean±S.D., n=9, *p<0.05 vs sham group, **p<0.01 vs sham group)

**Discussion**

In general, for CNS-active agents the extent of distribution to the target site is a prerequisite for their therapeutic effect and is determined by their physicochemical properties [11] as well as permeability of BBB [6]. The BBB is made of a complex vasculature lined by brain capillary endothelial cells (BCECs) and supported by astrocytic end-feet and extracellular matrix. The BBB is the specialized system of BCECs that were joined by continuous belts of tight junctions and are devoid of fenestrations. The critical zonularity of the tight junctions of the cerebral vasculature sets them apart from that of capillaries of other organs in the body and restricts the brain entry of most non-transported hydrophilic compounds. The brain microvasculature also contains a variety of enzymes that may either inactivate or activate compounds that traverse the brain capillary wall. In addition, high levels of ATP-dependent transporters are localized in the brain capillary wall like e.g., several nutrient carrier systems and P-glycoprotein that regulates the influx or efflux of a variety of compounds [12]. Consequently, transport across the BBB is strictly limited through both physical (tight junctions) and metabolic barriers (enzymes, diverse transport systems). As a result of restricted permeability, the BBB is a limiting factor for the delivery of therapeutic agents into the CNS. Due to its hydrophilic characteristic and macromolecule, HSYA is not expected to cross the BBB. In this study, the peak concentration of HSYA in control group is reached in about 10 min after a single dose and then declines gradually, and appreciable concentrations are found in brain tissue in less than 90 min. The findings revealed that HSYA could quickly cross the BBB after...
intravenous administration in physiologic condition, whereas the penetration was low and the HSYA concentration declined quickly.

BBB is prone to structure weakness and function instability after exposed to ischemic injury, the BBB ultrastructural changes was observed and expressed with the BCECs degeneration, pinocytotic vesicle increase, microvascular lumina stenosis, basement membrane lost astrocytic end-feet vacuolization as well as the adjacent astrocytic degeneration [13]. The BBB showed that an initial, acute disruption occurred at 3 h, and more widespread permeability increase at 24 h in ischemic hemisphere following cerebral ischemia-reperfusion, but there were no significant change was found in non-ischemic hemisphere [14]. Cerebral ischemia leads to disruption of blood flow, increased BBB permeability, and are associated with following factors:

(1) MMPs. MMPs comprise a family of endopeptidases which have similar primary structure and require Zn$^{2+}$ in a conserved cage for cleavage of specific ECM proteins, their inactive proforms are the most conveniently detected in physiological condition, and so far, direct evidence that MMPs actually degrades vascular or extravascular matrix during MCAO is reported [15–17].

(2) Oxygen free radicals elevation during cerebral ischemia-reperfusion cause lipid peroxidation of capillary endothelium and basal lamina layers, then destroy the integrity of BBB [18].

(3) During reperfusion, activated leukocytes interact with endothelial cells and plug capillaries, disrupt tight junctions through the release of neutrophil-derived oxidants and proteolytic enzymes, extravasate from capillaries and infiltrate brain tissue, and release bradykinin and thromboxane which mediate inflammation.

(4) Cytokines, such as TNF-α, IL-1β and platelet activating factor, released from glial cell and BCECs, which result in capillary endothelium and basal lamina layers injury and BBB permeability increase.

Increased BBB permeability is associated with severe ischemic injury, occurring with some delay after the insult. It may be helpful for the entry of therapeutic agents into CNS and treating ischemic cerebrovascular disease. In this study we found that the HSYA concentration in IR 3 h and IR 24 h groups increased significantly in ischemic hemisphere but had no dramatic changes in non-ischemic hemisphere. The peak HSYA concentration of ischemic hemisphere in IR 3 h group was attained at 30 min and later declined gradually but it was still could be detected at 90 min. The HSYA concentration of ischemic hemisphere in IR 24 h group has a gradually increased potency and peaked at 90 min in IR 24 h group. These results indicated that the HSYA penetration increased in ischemic hemisphere and seemed to be more long-lasting following IR.

In conclusion, the present findings showed that HSYA could penetrate across the BBB, and the content of penetration increased in ischemic hemisphere while the content of penetration had no obvious change in non-ischemic hemisphere. Based on the study and the literature previously reported, it is indicated that HSYA is an effective and promising medicine for treatment of ischemic cerebrovascular disease.
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Author’s Statements

Competing Interests
The authors declare no conflict of interest.

Animal Rights
The institutional and (inter)national guide for the care and use of laboratory animals was followed. See the Experimental part for details.

References


