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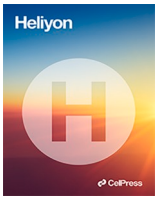

Research article

Quercetin improves the protection of hydroxysafflor yellow a against cerebral ischemic injury by modulating of blood-brain barrier and src-p-gp-mmp-9 signalling

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ABSTRACT

Protection of the structural and functional integrity of the blood-brain barrier (BBB) is crucial for treating ischemic stroke (IS). Hydroxysafflor yellow A (HSYA) and quercetin (Quer), two main active components in the edible and medicinal plant *Carthamus tinctorius* L., have been reported to exhibit neuroprotective effects. We investigated the anti-IS and BBB-protective properties of HSYA and Quer and the underlying mechanisms. They decreased neurological deficits in middle cerebral artery occlusion (MCAO) mice, while their combination showed better effects. Importantly, HSYA and Quer ameliorated BBB permeability. Their effects on reduction of both EB leakage and infarct volume were similar, which may contribute to improved locomotor activities. Moreover, HSYA and Quer showed protective effects for hCMEC/D3 monolayer against oxygen-glucose deprivation. Src, *p*-Src, MMP-9, and P-gp were associated with ingredients treatments. Furthermore, molecular docking and molecular dynamics simulations revealed stable and tight binding modes of ingredients with Src and P-gp. The current study supports the potential role of HSYA, Quer, and their combination in the treatment of IS by regulating BBB integrity.

1. Introduction

Recent World Health Organization (WHO) fact sheets estimate stroke as the second leading cause of death, accounting for approximately 11 % of total deaths, rising by more than 0.8 million to 6.094 million deaths in 2019 (https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death). Ischemic stroke (IS) is the main type of stroke, accounting for over 80 % [1]. The disease's elevated morbidity, high mortality, high disability rate, and high recurrence rate pose a serious threat to human health and impose a substantial strain on inhabitants and society [2]. However, therapeutic options for IS are currently very limited [3]. A new strategy for IS treatment is urgently required to reassess its management [4]. Research has revealed that cerebral ischemia may cause impaired function and increased permeability of the blood-brain barrier (BBB), resulting in a significant impact on disease prognosis. Therefore, the investigation into the structural and functional integrity of BBB is vital for IS treatment [4,5]. BBB

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permeability is associated with dynamic changes after cerebral ischemia, with various cells, transporters, and tight junctions (TJ) involved in this process [6]. Therefore, the protection of BBB structure and function, especially targeting TJ and transporters, has turned into a crucial approach towards treating IS [7].

P-glycoprotein (P-gp) is a crucial transporter in BBB, mainly located on the endothelial cell membrane surface on the lumen side. P-gp protects the brain by expelling toxins or drugs, albeit at the expense of pharmacological efficacy [8]. The decreased P-gp efflux transport activity has been reported to cause BBB dysfunction, which may be associated with P-gp internalization [9]. Cerebral ischemia activates Src signalling, which decreases P-gp efflux function through phosphorylation of Tyr-14 in caveolin-1 [10]. Src signalling also participates in the regulation of matrix metallopeptidase 9 (MMP-9) expression, and inhibition of Src can reverse the upregulation of MMP-9 after IS, which further increases the expression of TJ-related proteins (i.e., ZO-1, occludin, claudin-5, etc) and improves the BBB structure and function [11].

Given the current scarcity of effective therapeutic drugs for IS, natural compounds derived from food or medicinal plants may offer a promising alternative with potential for the treatment of IS. Quercetin (Quer), a well-known flavonoid, is widely found in fruits, vegetables, tea, and various medicinal plants, including Ginkgo biloba, etc [12]., and is among the most prominent dietary antioxidants. Although Quer displays certain protective effects against IS, but its therapeutic potential is hindered by the low bioavailability as well as the BBB structure [13]. Hydroxysafflor yellow A (HSYA), the main active ingredient in the medical and edible dual purpose plant safflower (*Carthamus tinctorius* L, Honghua as the Chinese name), possesses neuroprotective effects against IS injury [14,15]. However, the low bioavailability of HSYA hampers its clinical applications [15].

Carthamus tinctorius L, known as an important cash crop widely cultivated around the world (Fig. S1), is also a renowned traditional Chinese medicine (TCM) and recommended for promoting blood circulation and removing blood stasis in IS treatment. HSYA and Quer are the two main ingredients of *Carthamus tinctorius* L. Whether HSYA and Quer have synergistic protective effects against IS through regulation of the BBB requires further investigation.

Network pharmacology (NP) can effectively reveal the non-linear interactions between multiple components and complex diseases [16]. Study investigating the regulatory effects of the main active components in Honghua Injection on the cerebrovascular disease network concluded that HSYA and Quer were enriched in a cluster, implying the two ingredients may have synergistic protective effects against IS [17]. HSYA was reported to have protective potential against cerebral ischemia and reperfusion injury through multiple mechanisms [18]. Quer also showed BBB protective effects in middle cerebral artery occlusion/reperfusion (MCAO/R) rat models [19].

In the case of cerebral ischemia-reperfusion injury, the level of HSYA crossing BBB is increased, which is associated with BBB permeability and the expression of MMP-9 [20]. P-gp inhibitor enhanced the absorption of HSYA through Caco-2 monolayer [21]. Lexiscan, the FDA-approved adenosine receptor agonist, inhibits P-gp on BBB and facilitates the uptake of HSYA into the brain, enabling it to play an anti-inflammatory role while also protecting the endothelial layer [22]. As for Quer, it is capable of inhibiting P-gp activity [23]. By inhibiting P-gp at BBB, Quer enhances the concentration of drugs that have limited ability to cross the BBB, which is important in the treatment of brain disorders [24]. Thus, further research is necessary to elucidate the molecular mechanisms associated with the potential synergistic effects of HSYA and Quer in protecting the BBB in IS treatment. We hypothesized that HSYA and Quer may have synergistic protective effects against IS by modulating of BBB, the underlying molecular mechanisms may be associated with src-p-gp-mmp-9 signalling.

This study explored the protective effects of HSYA and Quer on BBB in mouse models following MCAO/R. Animal behaviour testing and TTC measurements were conducted to evaluate the potential synergistic protective effects of HSYA and Quer. Additionally, we applied hCMEC/D3 monolayer model to assess the protective effects of HSYA and Quer against resistance disruption in oxygen glucose deprivation (OGD). Western blotting (WB) analysis was used to evaluate the expression of Src, MMP-9 and P-gp. Furthermore, molecular docking and molecular dynamics simulation were performed to explore the potential interaction modes between ingredients and target proteins. Through the multi-level investigations, it is expected that Quer will improve the protection of HSYA against IS by modulating BBB and src-p-gp-mmp-9 signalling.

2. Materials and methods

2.1. Reagents and chemicals

Quercetin (purity >98 %) was purchased from Shanghai Winherb Pharmaceutical Technology Development Co., Ltd (Shanghai, China). Hydroxysafflor yellow A (purity >94 %) was provided by Dr. Kan from Zhejiang Yongning Pharmaceutical Co., Ltd (Zhejiang, China). Edaravone Injection (EDA) was purchased from China National Medicines Guorui Pharmaceutical Co., Ltd (Anhui, China). Sodium pentobarbital was obtained from Sigma-Aldrich (P3761, St Louis, MO, USA). PP2 (Src inhibitor) was bought from Beyotime (Shanghai, China). hCMEC/D3 was generously provided by Dr. Sun from School of Pharmacy, Hangzhou Medical College. Endothelial Cell Medium was purchased from ScienCell (ScienCell Research Laboratories, USA). Glucose free DMEM (Ref:11966-025) was obtained from Gibco (Grand Island, NY, USA). Cell Counting Kit (CCK-8) was bought from Yeasen Biotechnology (Shanghai) Co., Ltd. Pierce BCA Protein Assay Kit was purchased from Thermo Scientific (Rockford, IL, USA). Primary antibodies P-gp (22336-1-AP), MMP-9 (10375-2-AP) and Src (11097-1-AP) were purchased from proteintech (Hubei, China). Primary antibody *p*-Src (BM4307) was purchased from BOSTER (Hubei, China). Primary antibody GAPDH (14C10) (Cat 2118) was obtained from CST (MA, USA). HRP-labelled goat anti-Rabbit IgG(H + L) secondary antibody was purchased from Beyotime (A0208, Shanghai, China). TGX Stain-Free FastCast Acrylamide Kit (10 %) was obtained from Bio-Rad Laboratories, Inc. (USA). Biosharp ECL Chemiluminescence Kit was bought from Beijing Labgic Technology Co., Ltd (Beijing, China).

2.2. Animals models

C57BL/6 male mice (20–24g) were purchased from Zhejiang Vital River Laboratory Animal Technology Co., Ltd. All animal procedures were performed as per the Guide for the Care and Use of Laboratory Animals published by the US NIH (the 8th edition, NRC 2011) and were approved by the Ethics Committee for the Use of Experimental Animals in Institutional Animal Care and Use Committee, ZJCLA (approval number: ZJCLA-IACUC-20010179).

MCAO/R mouse models were established using the intraluminal filament technique. After anaesthesia, surgical process was conducted under a stereoscopic surgical microscope. After carefully dissected the left common carotid artery (CCA), the left external carotid artery (ECA) and left internal carotid artery (ICA) were then separated and isolated. A silicon-coated monofilament nylon suture (RWD, Cat: MSMC19B100PK50) was slowly advanced 10 mm into the ICA to block the blood supply to MCA. After 90 min of occlusion, the filament was gently withdrawn for the reperfusion, and the incision was closed. The sham group was operated identically, except for no MCA occlusion. To prevent hypothermia, a homoeothermic blanket was used to maintain body temperature at 37 °C ± 0.5 °C during and after surgical periods.

Neurological scores were graded as 0–4 based on Longa's method. grade 0: no deficit. grade 1: The forelimb of the paralyzed side cannot be fully extended; grade 2: Circle to the paralyzed side while walking; grade 3: Fall on the paralyzed side while walking; grade 4: The mice could not walk autonomously, and there was a loss of consciousness.

Exclusion criteria are as follows: 1) obvious bleeding during the operation; 2) Neurological score grade 0.

Mice were divided into six groups: sham, model, EDA (5 mg/kg), HSYA 10 mg/kg, Quer 10 mg/kg, and HQ (HSYA 10 mg/kg combined Quer 10 mg/kg). All drugs were administered intraperitoneally. Regarding the concentrations of the active ingredients, we referred to previous literatures and found that 10 mg/kg was the potential concentration for HSYA to show neuroprotective, anti-oxidative, anti-nitrotyrosine formation, and dyskinesia ameliorating effects [14,25,26]. For Quer, 10 mg/kg showed neuroprotective, anti-oxidative, neurotransmitter-regulating effects [27–31].

2.3. Open field test (OFT)

The mouse open field is square (50 cm × 50 cm) with the walls and bottom are white. According to the VisuTrack software system (Shanghai, China), the bottom was divided into 16 grids, defining as the central area (4 grids in the middle), the peripheral area (8 grids in the periphery), the corner area (4 grids in the corners), and the wall area. A mouse was placed in the centre of the bottom, then start the VisuTrack recording program and capture the video continuously for 5 min. VisuTrack software system was applied to record locomotive activities, including the distance of each region (total, the total locomotor distance, central area distance, corners area distance, peripheral area distance), the ratio of the distance of each region to the total distance (central distance/total distance, corners distance/total distance, peripheral area distance/total distance), time in each area (time in central area, time in corner area, time in peripheral area), mean velocity, immobility time, numbers of crossing grids area, duration of supportive standing.

2.4. 2, 3, 5-Triphenyltetrazolium chloride (TTC) staining

The whole mouse brain was removed after sacrifice, and each brain was sliced into coronal sections (2 mm thickness for each slice) using a 0–175g cornal slices 1 mm pitch brain matrix from J&K Seiko electronic Co., Ltd (Guangdong, China). Brain slices were stained in 2 % TTC (Solarbio, Beijing, China) at 37 °C for 10 min and subsequently fixed in 4 % paraformaldehyde (Beyotime, Shanghai, China) for 24h. The slices were photographed with a digital camera and analysed using the ImageJ software (NIH, MD, USA).

2.5. BBB permeability evaluation

BBB integrity was assessed using evans blue (EB) leakage test. Mice were injected 4 % EB (2 ml/kg) through tail-vein 24 h before sacrifice. Mice brain was cut into 2 mm-thick coronal slices. The Caliper IVIS Spectrum system from Caliper Life Sciences (Hopkinton, MA, USA) was used for fluorescent imaging detection. EB leakage was quantified via calculating a total radiant efficiency [p/s]/[μW/cm^2].

2.6. Brain microvascular endothelial cell culture

hCMEC/D3 was cultured in Endothelial Cell Medium (ECM) (ScienCell Research Laboratories, USA) supplied with 10 % fetal bovine serum (ScienCell), 1 % endothelial cell growth supplement (ECGS, ScienCell), 1 % penicillin/streptomycin solution (P/S, ScienCell) in humidified air (5 % CO_2) at 37 °C. The cell culture medium should be protected from light both in storage and use.

2.7. hCMEC/D3 OGD model and drug treatment

Cells were plated in 96-well plate with 5000 cells in 100 μL medium per well. HSYA (0.5, 1, 2, 5, 10 μM) and Quer (0.5, 1, 2, 5, 10 μM) were pre-incubated with hCMEC/D3 for 24h. Then each well was replaced with serum-free and glucose-free DMEM in a hypoxia chamber (STEMCELL, USA) in 95 % N_2 and 5 % CO_2 at 37 °C for 20min, during which HSYA and Quer were co-incubated. In control group, cells were cultured in humidified air (5 % CO_2) at 37 °C.

2.8. Assessment of BBB integrity

hCMEC/D3 cells were seeded at a certain density into transwell inserts (Millicell® Standing Cell Culture Inserts, PIHP01250, with a pore size of 0.4 μM and an effective surface area of 0.6 cm^2). Then put the inserts into the wells of 24-well plate, cells can fully attach to the bottom surface of the inserts after 24 h. The cell monolayer integrity was assessed by Millicell® ERS-2 Electrical Resistance System (Millipore, MA, USA), and the transendothelial electrical resistance (TEER) values ($\Omega \cdot cm^2$) were calculated. After plant 50,000 cells per insert, the TEER plateau was formed 3–5 days after confluent monolayer was formed.

The 24-well plates were put in a hypoxia chamber for 20min to suffer OGD injury. HSYA, Quer, EDA, PP2, HSYA + Quer (HQ), HSYA + PP2 (HP) were pre-incubated for 24 h on the 3rd day after cell planting, in which PP2 was added into PP2 and HP groups at the time of 23 h after the initial pre-incubation of each group. PP2 only treated with cells for 1 h 24 h after initial-incubation, i.e. on the 4th day, each insert was replaced with serum-free and glucose-free DMEM in a hypoxia chamber in 95 % N_2 and 5 % CO_2 at 37 °C for 20min, during which the abovementioned groups were co-incubated with HSYA, Quer, EDA, serum-free glucose-free DMEM, HQ, HSYA, respectively. In control group, cells were cultured in humidified air (5 % CO_2) at 37 °C with normal medium.

2.9. Western blotting (WB)

hCMEC/D3 was seeded at a density of 1.2×10^5/ml into a 6 mm culture dish. Dishes were incubated in a CO_2 incubator for 24 h to allow complete cell attachment. EDA (50 μM), HSYA (1 μM), Quer (2 μM), and HQ (HSYA (1 μM) + Quer (2 μM)) were pretreated with cells for 24h. PP2, a commonly used Src inhibitor, was added to hCMEC/D3 cells 1 h before exposing cells to OGD. After 24h, each well was replaced with serum-free glucose-free DMEM in a hypoxia chamber filled with mixed gas (95 % N_2 and 5 % CO_2) at 37 °C for 20min, during which HSYA and Quer were co-incubated. In normal group, cells were cultured in humidified air (5 % CO_2) at 37 °C in normal medium. After 20min OGD treatment, the dishes were taken out of hypoxia chamber. After discarding cell culture medium, cells were washed rinsed three times with pre-cold PBS. Then the hCMEC/D3 in different group were used to extract proteins and BCA assay was applied to measure concentration of protein in each group.

About 40 μg protein were separated with 10 % SDS-PAGE. The proteins were transferred onto 0.45 μm PVDF transfer membrane (Millipore, USA). The membrane was blocked with 5 % nonfat milk powder in 1 × TBST (BOSTER, Wuhan, China) for 1h at room temperature, and then incubated with primary antibodies (P-gp, MMP-9, Src, *p*-Src, GAPDH) overnight at 4 °C. After that, the membrane was washed with 1 × TBST, then incubated with HRP-conjugated secondary antibodies for 1.5 h at room temperature, and washed again by 1 × TBST. Protein bands were visualized using ECL Chemiluminescence Kit and photographed. Image J software was applied to analyse the gray value of the bands.

2.10. Molecular docking

3D target protein structures, i.e., p-gp (PDB ID: 6C0V) and Src (PDB ID: 4F5B), were obtained from the RCSB PDB database, and the target protein structures were pretreated by the application of AutoDock Tools (Version 1.5.6) to generate PDBQT files for molecular docking. mol2 files of HSYA and Quer were generated with ChemDraw and Chem3D software according to the structure in PubChem (HSYA PubChem CID: 91884958; Quer PubChem CID: 5280343). PDBQT files were further processed and prepared for molecular docking in AutoDock Vina software. Docking energy scores were used to evaluate the binding affinity between the drugs and the targets. LigPlot$^+$ (v.2.2.8) and Discovery Studio (v16.1.015350) were applied to analysis interaction modes between compound and target.

2.11. Molecular dynamics simulations

The protein-ligands complexes were subjected to molecular dynamics (MD) simulation using the Desmond software (version 2021-1, D. E. Shaw Research, New York, NY, USA, Education License Agreement). The system was solvated in a cubic box with TIP3P water molecules, and the net charge of the system was neutralized by adding Na^+ and Cl^- ions using the Desmond Ion Generator tool. The NPT ensemble temperature and pressure were set to 300 K and 1 atm, respectively. The OPLS-AA/L force field was employed to describe the protein and ligand. The MD simulation was performed for 100 ns with a time step of 2 fs, and the trajectory was saved every 10 ps.

2.12. Statistical analysis

All results are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA where appropriate, followed by performance of Tukey's post hoc test. Statistical significance was considered for P values < 0.05.

3. Results

3.1. Effects of HSYA and Quer on neurological deficits and cerebral infarction in MCAO mice

Fig. 1 illustrates that the model group exhibited a significant increase in neurological deficits in comparison to the sham group ($p < 0.01$). Administration of 10 mg/kg HSYA and 10 mg/kg Quer reduced neurological deficits compared with the model group.

Additionally, the ingredients combination further ameliorated neurological deficits in comparison to the model group ($p < 0.01$) (Fig. 1C).

TTC staining showed deep red in viable tissue and white in the infarcted hemisphere (Fig. 1A). No infarcts were observed in sham group, whereas significantly obvious infarcts were found in model group ($p < 0.01$, vs sham). 10 mg/kg HSYA and 10 mg/kg Quer treatment reduced infarct volume ($p < 0.05$, HSYA vs model). Two components combination further reduced infarct volume compared with model group ($p < 0.05$) (Fig. 1B).

3.2. HSYA and Quer treatment rescued MCAO mice against BBB leakage

In model group, it displayed strong EB fluorescence intensity ($p < 0.05$, model vs sham group) (Fig. 2B and C), which means the model mice have increased BBB leakage. 10 mg/kg HSYA and 10 mg/kg Quer treatment ameliorated BBB permeability compared with model group ($p < 0.05$, HSYA vs model). The ingredients combination further reduced BBB leakage ($p < 0.01$, vs model) (Fig. 2A–C).

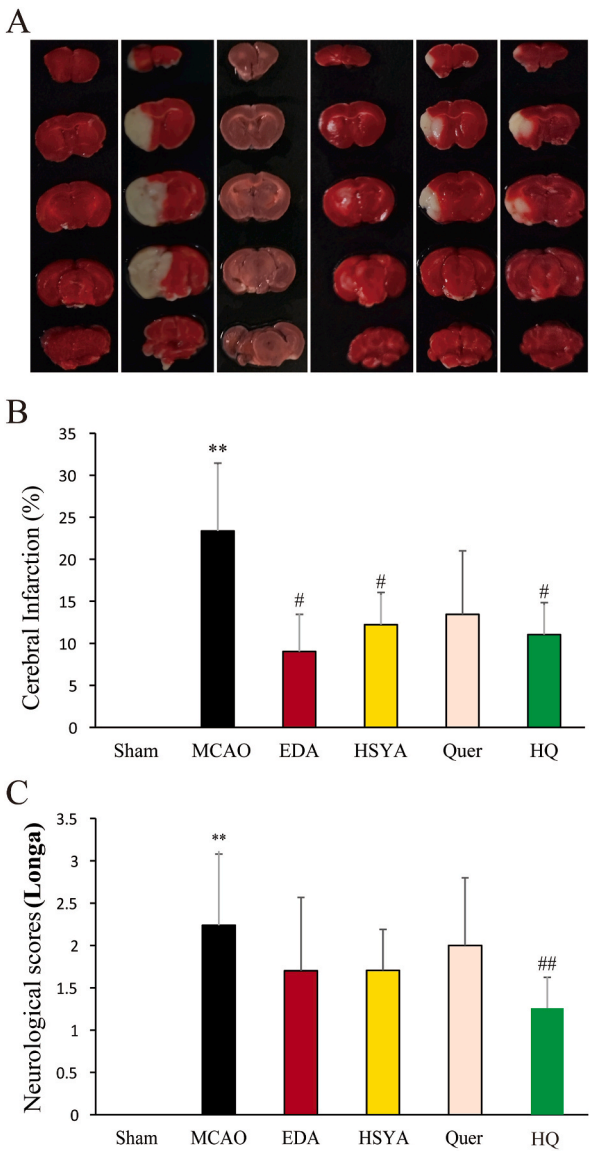

Fig. 1. Effects of HSYA and quercetin on neurological deficits and cerebral infarction in MCAO mice. (A) Representative TTC staining images. (B) 10 mg/kg HSYA and 10 mg/kg quercetin (Quer) treatment reduced infarct volume (#$p < 0.05$, HSYA vs model). Components combination further reduced infarct volume compared with model group (#$p < 0.05$). n = 4–7. (C) 10 mg/kg HSYA and 10 mg/kg Quer reduced neurological deficits in comparison to the model group. The ingredients combination further ameliorated neurological deficits (##$p < 0.01$). The data in the figures represent the averages ± SD. n = 6–14.

3.3. HSYA and Quer treatment improved locomotive activities of MCAO mice

In model group with comparing to sham group, mice presented significantly decreased total locomotor distance ($p < 0.01$). 10 mg/kg HSYA and 10 mg/kg Quer treatment increased total locomotor distance, and ingredients combination obviously improved total locomotor distance ($p < 0.05$ vs model) (Fig. 3A–G). Compared with model group, the total locomotor distance, central area distance, corners area distance, peripheral area distances were all improved in HSYA, Quer, and ingredients combination treatment groups. In the view of ratio of the distance of each region to the total distance (Fig. 3H-M), HSYA and Quer treatment reversed the changes observed in model group compared with sham.

On other hand, the mean velocity (mm/s) and the Nos. of crossing grid area in model decreased significantly compared with sham ($p < 0.01$). HSYA and Quer treatments increased the impaired activities of MCAO mice, while HSYA and Quer combined administration promoted obviously of the indexes ($p < 0.05$ vs model) (Fig. 4A–H).

As for the time of mice in the OFT, MCAO mice exhibited reduced duration in central area and peripheral area, increased time in corner area, which were all reversed by treatment with HSYA, Quer, and their combination (Fig. 5A–I).

What's more, in the OFT, mice in model group showed significantly increased immobility time and obviously decreased duration of supportive standing ($p < 0.05$, $p < 0.01$, respectively, vs sham). HSYA, Quer, and the two ingredients combination administration can

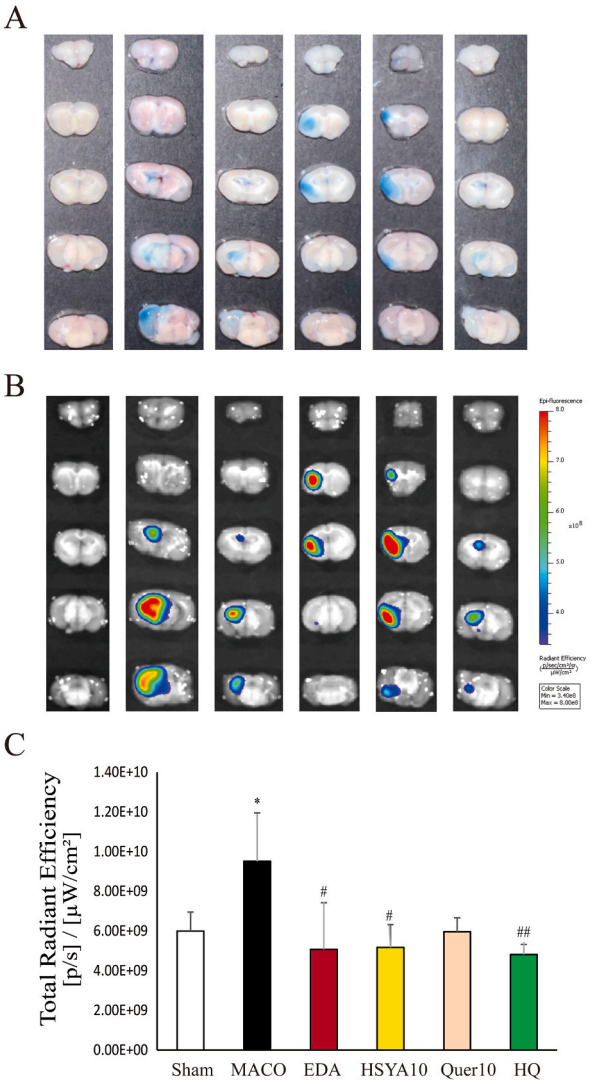

Fig. 2. HSYA and quercetin treatment rescued MCAO mice against BBB leakage. (A) and (B) Representative evans blue (EB) leakage test images. (C) Model group displayed strong EB fluorescence intensity (*$p < 0.05$, vs sham group). 10 mg/kg HSYA and 10 mg/kg quercetin (Quer) treatment ameliorated BBB permeability (#$p < 0.05$, HSYA vs model). Ingredients combination further reduced BBB leakage (##$p < 0.01$, vs model). The data in the figures represent the averages ± SD. n = 3–5. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

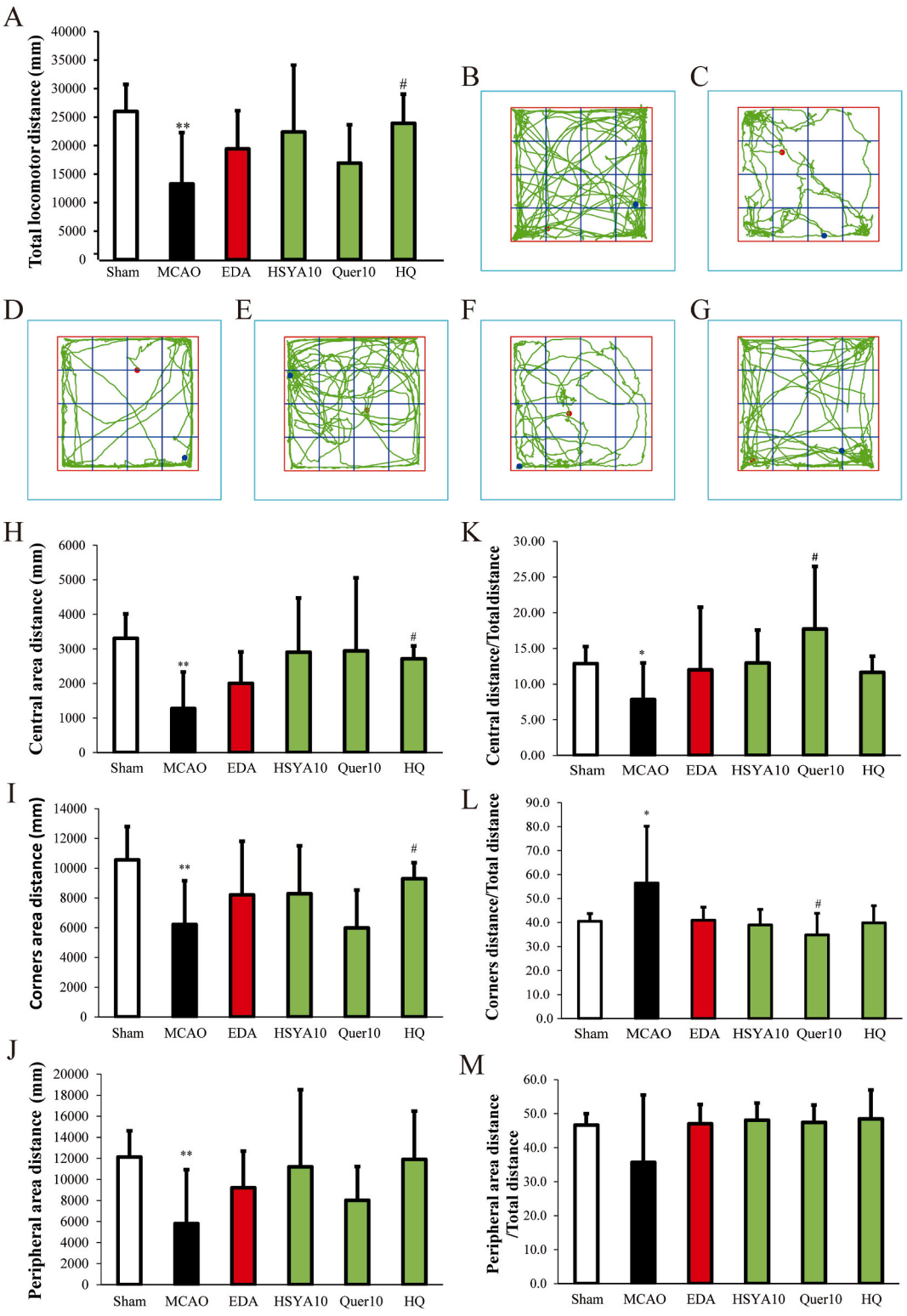

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Fig. 3. HSYA and quercetin treatment improved locomotor distance of MCAO mice. (A) MCAO mice presented significantly decreased total locomotor distance (**p < 0.01). 10 mg/kg HSYA and 10 mg/kg quercetin (Quer) treatment increased total locomotor distance, and ingredients combination obviously improved total locomotor distance (#p < 0.05 vs model). (B) (C) (D) (E) (F) (G) are the representative motion track of Sham, MCAO, EDA, HSYA, Quer, and HQ, respectively. n = 5–12. (H, I, J) Central area distance, corners area distance, peripheral area distances were all improved in HSYA, Quer, and ingredients combination treatment groups. (K, L, M) As the ratio of the distance of each region to the total distance, HSYA and Quer treatment reversed the changes observed in model group compared with sham. n = 5–12.

mitigate the impairment induced by MCAO (Fig. 5J and K).

3.4. Cytoprotective effects of HSYA and Quer against hCMEC/D3 OGD injury

OGD 20min induced hCMEC/D3 injury with cell viability about 40 %. HSYA (0.5, 1, 2, 5, 10 μM) and Quer (0.5, 1, 2, 5, 10 μM) incubation ameliorated OGD induced injury, in which 1 μM HSYA and 2 μM Quer obviously enhanced cell viability against OGD-treated group (p < 0.05, vs model) (Fig. 6A and B).

3.5. BBB integrity evaluation

After 3–5 days, hCMEC/D3 in transwell inserts of 24-well plate formed tight monolayer with TEER value of 38.3 ± 6.23 Ω cm^2. TEER value significantly decreased in hypoxia group compared to normoxia group (13.7 ± 3.97 Ω cm^2 vs 38.3 ± 6.23 Ω cm^2, p < 0.01). 5 μM PP2, 1 μM HSYA and 2 μM Quer increased TEER versus hypoxia group, HSYA and Quer combination further enhanced TEER (28.7 ± 7.53 Ω cm^2 vs 13.7 ± 3.97 Ω cm^2, p < 0.05). HSYA and PP2 also significantly enhanced TEER compared to hypoxia group (26.3 ± 7.53 Ω cm^2 vs 13.7 ± 3.97 Ω cm^2, p < 0.05) (Fig. 6C). The results indicated that HSYA and Quer can prevent the impairment of OGD on BBB permeability.

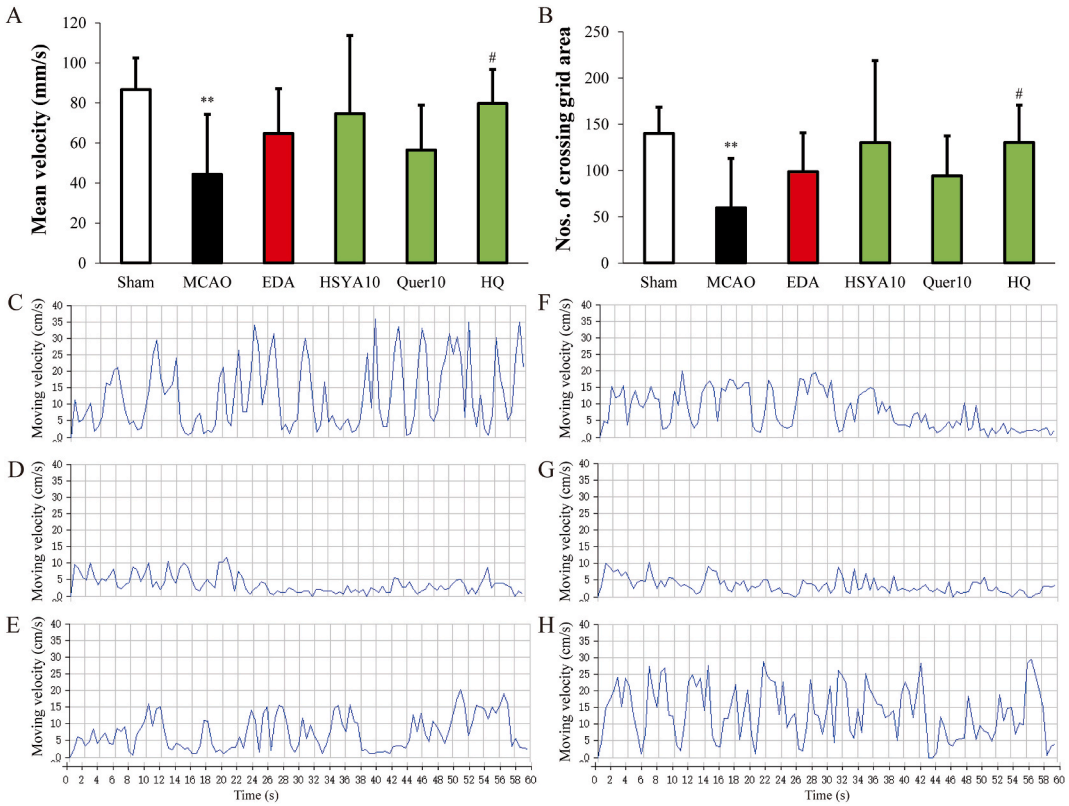

Fig. 4. HSYA and quercetin treatment improved locomotor activity of MCAO mice. (A, B) The mean velocity (mm/s) and the Nos. of crossing grid area in model decreased significantly (**p < 0.01, vs sham). HSYA and quercetin (Quer) treatments increased the impaired activities of MCAO mice, while HSYA and Quer combination promoted obviously of the indexes (#p < 0.05, vs model). (C) (D) (E) (F) (G) (H) are the representative motion velocity of group Sham, MCAO, EDA, HSYA, Quer, and HQ, respectively. n = 5–12.

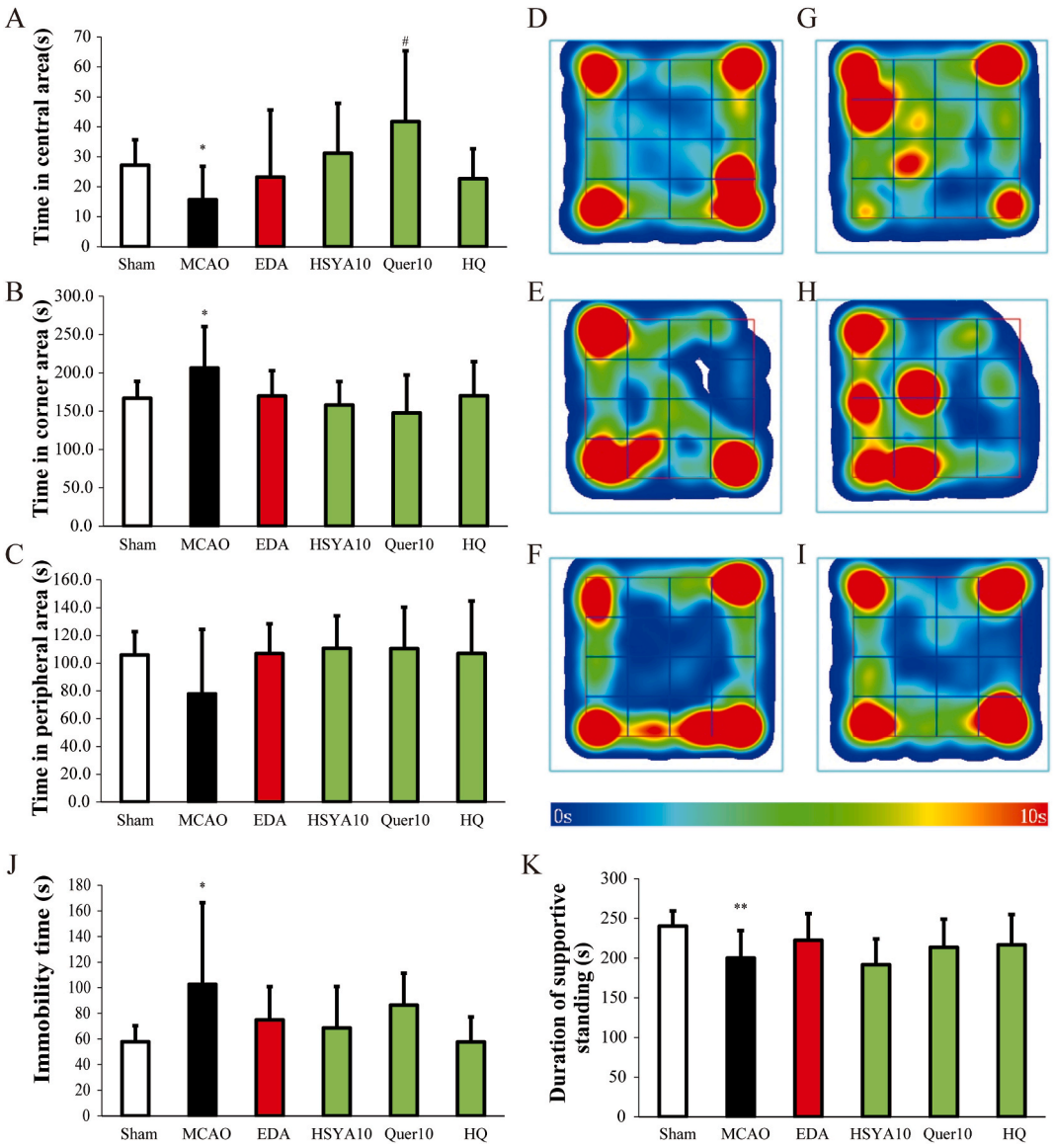

Fig. 5. HSYA and quercetin treatment improved locomotor time, immobility time and duration of supportive standing of MCAO mice. (A, B, C) show the time of mice in different areas of OFT testing arena, MCAO mice exhibited reduced duration in central area and peripheral area, increased time in corner area, which were all reversed by treatment with HSYA, quercetin (Quer), and their combination. (D, E, F, G, H, I) are the representative heatmaps of mice in different areas of OFT testing arena. n = 5–12. (J, K) Model group showed significantly increased immobility time and obviously decreased duration of supportive standing (*$p < 0.05$, **$p < 0.01$, respectively, vs sham). HSYA, Quer, and ingredients combination mitigate the impairment induced by MCAO. n = 5–12.

3.6. Effects of HSYA and Quer on MMP-9, P-gp and src expression in regulation of BBB integrity

To investigate the mechanism of the protective effects of HSYA and Quer on BBB function regulation, the protein levels of MMP-9, P-gp, Src and *p*-Src in whole cell extracts were examined. WB results showed that OGD increased MMP-9 expression, however the effect was alleviated by HSYA and Quer treatment, and the ingredients combination further decreased MMP-9 expression. Src inhibitor PP2 also inhibited MMP-9 expression, HSYA further decreased MMP-9 level based on PP2 treatment (Fig. 7A and B).

OGD stimulated increased P-gp expression, and HSYA treatment decreased P-gp expression (Fig. 7C and D). The significantly enhanced phosphorylation of Src (***$p < 0.001$, vs control) was alleviated by PP2 treatment, and HSYA in combination with PP2 further decreased *p*-Src level ($^{\#\#}p < 0.01$, vs model) (Fig. 7E and F).

Src, MMP-9 and P-gp involved signalling pathways may play potential roles in HSYA and Quer treatments induced protective effects against BBB permeability.

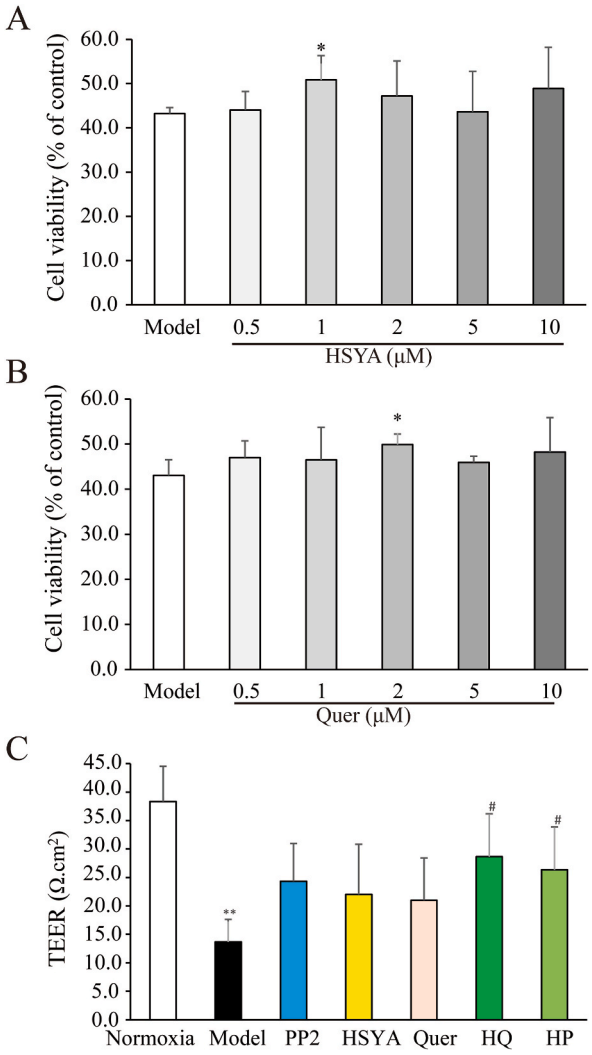

Fig. 6. Cytoprotective effects of HSYA and quercetin against hCMEC/D3 OGD injury, and the improvement of BBB integrity. (A, B) HSYA (0.5, 1, 2, 5, 10 μM) and quercetin (Quer, 0.5, 1, 2, 5, 10 μM) co-incubation ameliorated OGD 20min induced injury. 1 μM HSYA and 2 μM Quer obviously enhanced cell viability (*$p < 0.05$, vs model). Each experiment was repeated three times independently. (C) TEER value significantly decreased in hypoxia group (**$p < 0.01$, vs normoxia). 5 μM PP2, 1 μM HSYA and 2 μM Quer increased TEER, HSYA and Quer combination further enhanced TEER (#$p < 0.05$, vs model). HSYA and PP2 also significantly enhanced TEER (#$p < 0.05$, vs model). Each experiment was repeated three times independently.

3.7. Molecular docking

To explore the binding modes of HSYA with P-gp and Src, molecular docking was applied to decipher the potential interactions. Results showed that hydrogen bonds were formed between HSYA and Gln438, Gln441, Glu476, Arg905, Thr906, Thr1174, Lys1181 of P-gp, Pi-alkyl interactions were formed with Val478 and Arg905 of P-gp, and unfavorable receptor-receptor interaction formed with Gln1175, showing a strong affinity of −10 kcal/mol (Fig. 8A and B).

As cerebral ischemia activates Src signalling, which participates in the regulation of P-gp and MMP-9 expression and function. Molecular docking of HSYA and Src showed a certain binding affinity of −6.2 kcal/mol (Fig. 8C and D).

3.8. MD simulation

MD simulation has become a popular technique for assessing the structural features of protein-ligand systems and for investigating the stability of protein-ligand binding. In the range of 0–30 ns, the system of HSYA and P-gp is constantly fluctuating. In the range of 30–100 ns, the RMSD value (right Y-axis, Ligand RMSD (Å)) is in a stable state with little changes, revealing that the binding between HSYA and P-gp has been equilibrated. As for HSYA and Src system, there are two stable periods, in the range of 10–25ns and the time

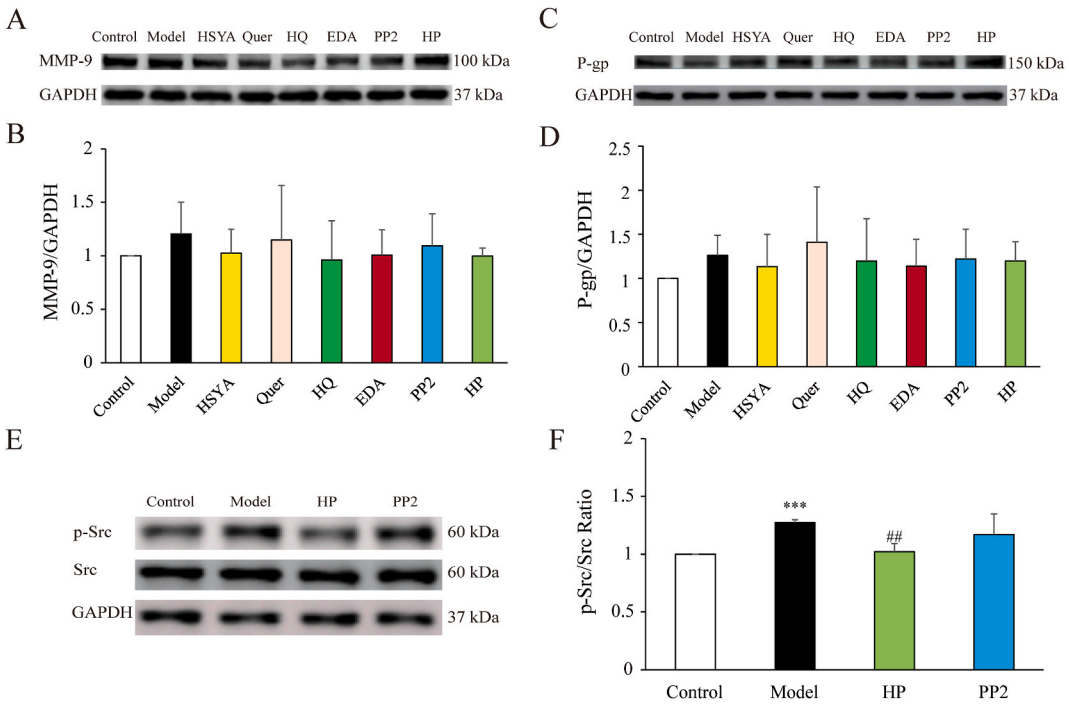

Fig. 7. Effects of HSYA and quercetin on MMP-9, P-gp, and Src expression in regulation of BBB integrity. (A, B) OGD increased-MMP-9 expression was alleviated by HSYA and quercetin (Quer) treatment, and the ingredients combination further decreased MMP-9 expression. PP2 inhibited MMP-9 expression, PP2 pretreatment for 1h followed HSYA co-incubation further decreased MMP-9. (C, D) HSYA and PP2 treatment alleviated the enhanced expression of P-gp induced by OGD stimulation, and HSYA in combination with PP2 further decreased P-gp level. Each experiment was repeated three times independently. (E, F) OGD stimulated significantly increased phosphorylation of Src (***$p < 0.001$, vs control). The enhanced *p*-Src was alleviated by PP2 treatment, and HSYA in combination with PP2 further decreased *p*-Src level (##$p < 0.01$, vs model). Each experiment was repeated three times independently. PP2 is Src inhibitor.

after 80 ns towards 100ns, respectively. Collectively, MD simulation results are consistent with the findings of molecular docking that HSYA can bind well with P-gp and Src (Fig. 9A and B).

On the other hand, the system of Quer and P-gp is fluctuating. However, the changes of the order are limited in 1–3 Å and this is perfectly acceptable according to Desmond software. After 80 ns, the Quer and P-gp complex converged to RMSD of P-gp. The RMSD of Quer and P-gp complex ranged in 3–4 Å from 90 ns to 100ns. The binding system between Quer and Src is relatively stable in the range of 0–100ns (right Y-axis, Ligand RMSD (Å)), ligand Quer has not diffused away from its initial binding site and the RMSD values stabilize around a fixed value of 2.5 Å (Fig. 9C and D).

4. Discussion

The protection of BBB structure and function integrity has turned into a crucial approach towards treating IS. *Carthamus tinctorius* L., with medicinal and edible value, is also a renowned TCM and recommended for promoting blood circulation and removing blood stasis in IS treatment. In this study, HSYA and Quer, the two main dietary ingredients of *Carthamus tinctorius* L., were found to have synergistic protective effects against IS through regulation of BBB.

In the current study, HSYA and Quer reduced neurological deficits of MCAO mice, and the two ingredients combination further ameliorated neurological deficits. The protective effects are consistent with the reduction of cerebral infarction in MCAO mice treated with HSYA and Quer. Importantly, HSYA and Quer treatment ameliorated BBB permeability and rescued MCAO mice against BBB leakage. It showed similar effects of HSYA and Quer on the reduction of EB leakage and infarct volume in ischemic ipsilateral hemisphere, and which may contribute to improved locomotive activities of MCAO mice.

As cerebral ischemia usually leads to behaviors change compared to normal conditions. In experimental cerebral ischemic animal models, such as rodents, the model group animals show reduced locomotor activities with reduced travel distances and reduced entries in the central area, reduced mean velocities, while staying at corners may be safe for model group animals [32,33]. Another parameter used to assess locomotive activities is immobility time. Consistent with increased time in corner area and reduced staying time in central area, the model group showed increased immobility time compared to normal group and drug administration groups. That is, time in each area (time in central area, time in corner area, time in peripheral area) together with other parameters, including the distance of each region (total, the total locomotor distance, central area distance, corners area distance, peripheral area distance), the ratio of the distance of each region to the total distance (central distance/total distance, corners distance/total distance, peripheral

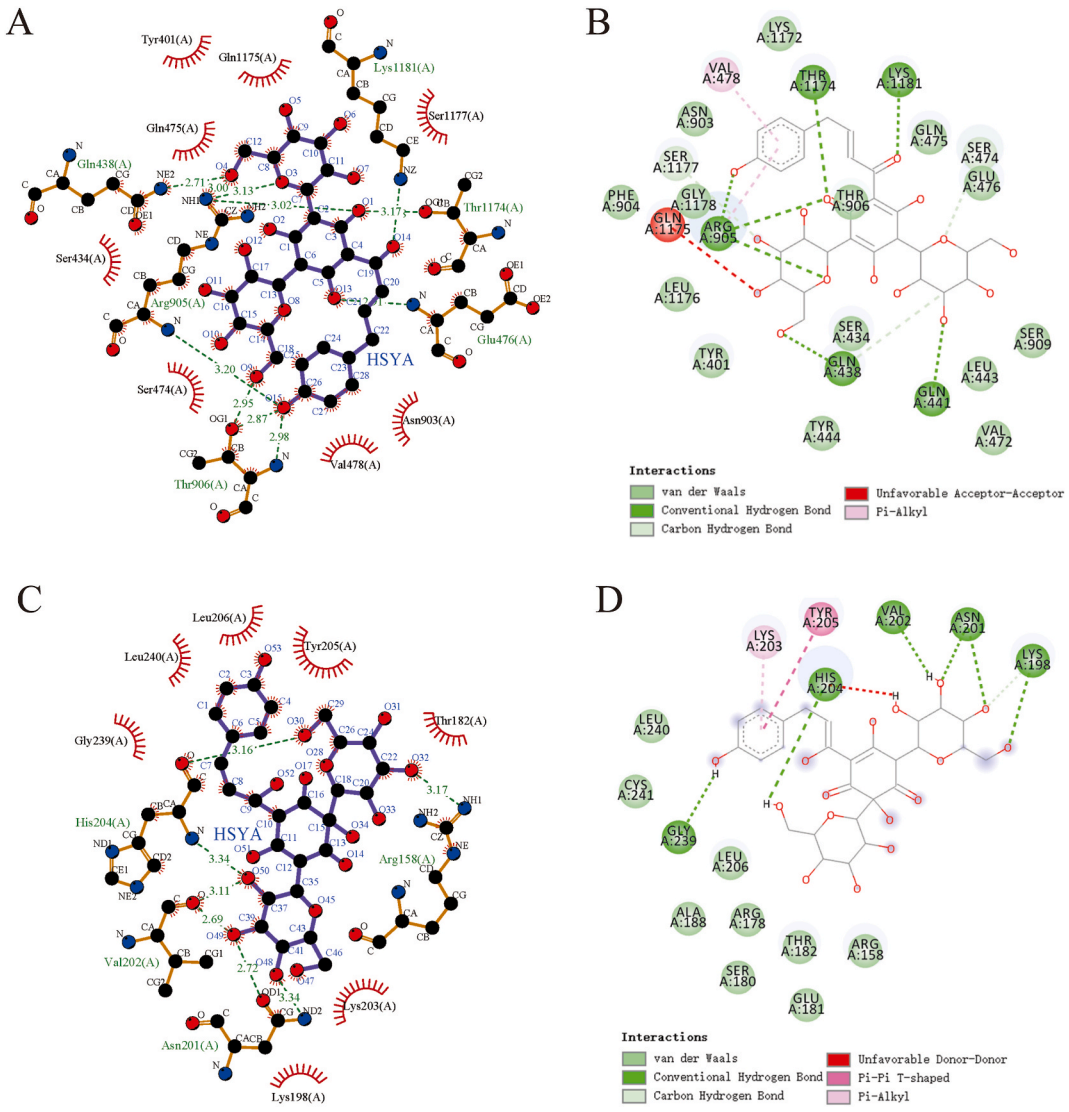

Fig. 8. Molecular docking of HSYA with P-gp and Src. (A, B) Hydrogen bonds were formed between HSYA and amino acid residues of P-gp, showing a strong affinity of −10 kcal/mol (C, D) Molecular docking of HSYA and Src showed a certain binding affinity of −6.2 kcal/mol, hydrogen bonds were formed between HSYA and amino acid residues of Src.

area distance/total distance), mean velocity, immobility time, numbers of crossing grids area, and duration of supportive standing, etc, are all applied to evaluate the locomotive activities of animals with cerebral ischemic injury, which are useful to further evaluate potential drug effects against cerebral infarction and neurological deficits. The current OFT evaluation results indicated the enhanced locomotive activities after treatment with HSYA and Quer, including the total locomotor distance, the mean velocity, the Nos. of crossing grid area, the immobility time, and the duration of supportive standing, etc. The cerebro-protective effects of HSYA and Quer observed in this study align with previous reports [13,15]; Furthermore, our investigations revealed that Quer may improve the anti-ischaemic stroke effects of HSYA.

Further studies in the classic brain vascular endothelial cell hCMEC/D3 demonstrated the cytoprotective effects of HSYA and Quer in protecting against OGD injury, with a significant improvement in cell viability. After 3–5 days, hCMEC/D3 in transwell inserts of 24-well plate formed tight monolayer as in vitro BBB integrity assessment model [34]. MMP-9, P-gp and Src are vital elements for maintaining the structural and functional integrity of the BBB. Src signalling pathways are crucial for regulating of MMP-9 and P-gp, thus affecting BBB functionality [10,11]. WB results showed that the Src inhibitor PP2 decreased the enhanced expression of P-gp in OGD stimulated hCMEC/D3, and HSYA-PP2 combination further reversed the P-gp level compared with HSYA or PP2 treated alone. MMP-9 expression was promoted in OGD condition, but PP2 treatment reversed the change. As for TEER value, PP2 promoted the decreased TEER in OGD stimulation, and HSYA combined with PP2 further increased TEER. This illustrates that the Src signalling may play an important role in HSYA-mediated protective effects against BBB injury through regulations of P-gp and MMP-9.

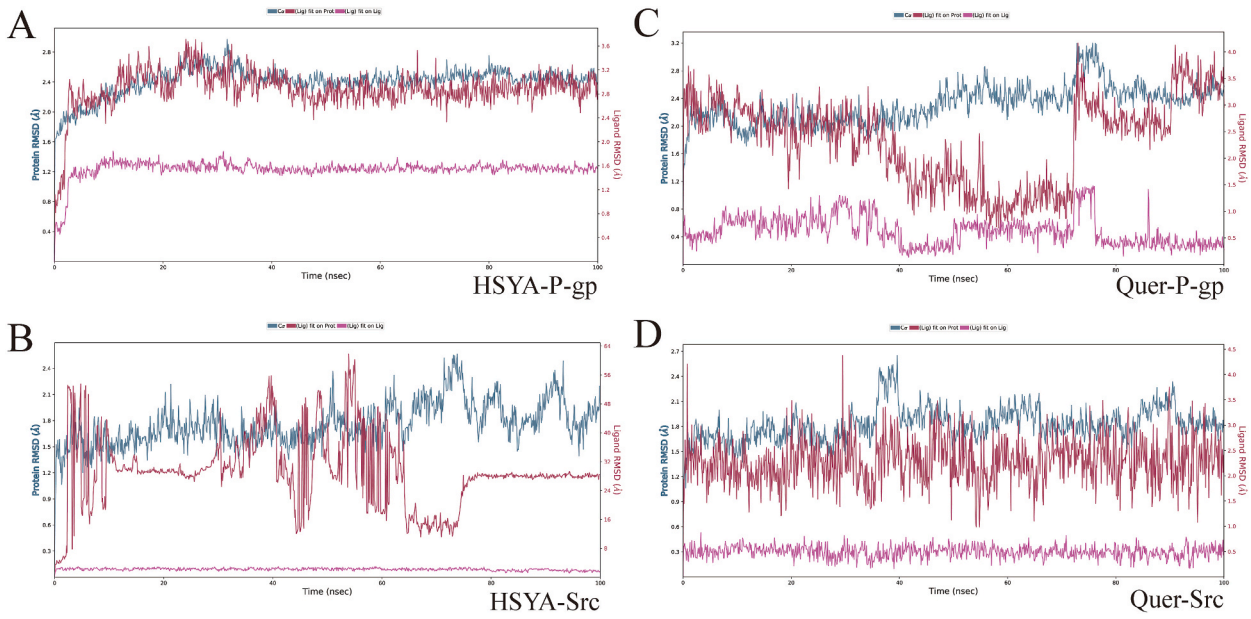

Fig. 9. MD simulation of HSYA, quercetin with P-gp, Src. (A) The RMSD value of HSYA and P-gp system. In the range of 30–100 ns, the RMSD value (right Y-axis, Ligand RMSD (Å)) is in a stable state with little changes. (B) After 80 ns towards 100ns, HSYA can bind well with Src. (C) The RMSD of quercetin (Quer) and P-gp complex ranged in 3–4 Å from 90 ns to 100ns. (D) The binding system between Quer and Src is relatively stable in the range of 0–100ns.

Molecular docking and MD simulation assessments were applied to decipher the potential modes of actions between ingredients and targets. Docking results showed that the binding of HSYA and Quer with P-gp is strong with low binding energy, revealing the combination is stable. HSYA and Quer also showed a certain binding affinity with Src. Moreover, MD simulation also indicated the extremely stably binding between HSYA and P-gp, together with Quer and Src. While it also showed relatively stable systems between HSYA and Src, Quer and P-gp. The stable combinations between ligands and target proteins may be associated with interactions formed in the binding systems, including hydrogen bonds, Pi-alkyl interactions, and unfavorable receptor-receptor interactions, etc.

These findings may suggest that HSYA, Quer, and combination of the two active ingredients from *Carthamus tinctorius* L. may act as regulators of Src, P-gp, MMP-9 signalling to protect BBB integrity in IS treatment.

In addition, we've addressed the issues of potentially low bioavailability of Quer and HSYA [13,15]. A potentially interesting issue to discuss is how to overcome low bioavailability in future studies and medical applications. We know that due to the presence of the BBB, it is usually not easy for substances to penetrate and distribute into the brain. There are several ways to overcome the problems of low bioavailability. Firstly, the BBB becomes easier to cross in conditions such as ischaemic, oxidative, inflammatory, traumatic brain injury, etc. In the case of cerebral ischemia-reperfusion injury, the amount of HSYA crossing the BBB is increased [20]. P-gp inhibitor enhanced the absorption of HSYA through Caco-2 monolayer [21]. And Quer is capable of inhibiting P-gp activity [23], by inhibiting P-gp at BBB, Quer enhances the concentration of drugs that have limited ability to cross the BBB, which is important in the treatment of brain disorders. Secondly, it will be useful to improve the bioavailability of Quer through the design of drug delivery systems, modification of drug formulations and their applications. Quer aglycone [35] and Quer stabilized by microencapsulation with pectin/casein polymers [36] are two examples. Quer in its nano formulations is another way to enhance bioavailability and pharmacological effects [27]. Thirdly, the absorption of HSYA was enhanced using a self-double-emulsifying drug delivery system. P-gp inhibitor could enhance the absorption of HSYA through Caco-2 monolayer [21], while Lexiscan, the FDA-approved adenosine receptor agonist, inhibits P-gp on BBB and facilitates the uptake of HSYA into the brain, enabling it to play an anti-inflammatory role while also protecting the endothelial layer [22]. Designing of polydrug containing P-gp inhibitor or adenosine receptor agonist together with HSYA may be a promising way to improve bioavailability and pharmacological effects.

In summary, in the current study, we demonstrated that HSYA and Quer can protect against cerebral ischemic injury through the modulations of BBB integrity and Src-P-gp-MMP-9 signalling. Our study may further support the potential use of HSYA, Quer and their combination as effective therapeutics against IS. And further research is warranted to explore the effects of HSYA and Quer on the structure and function of the BBB, as well as to clarify the molecular mechanisms underlying these processes.

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Ethics statement

All animal protocols were approved by the Ethics Committee for the Use of Experimental Animals in Institutional Animal Care and Use Committee, ZJCLA (approval number: ZJCLA-IACUC-20010179).

Data availability statement

Data associated with our study has not been deposited into a publicly available repository. The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Xiang Li: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Yuanxiao Yang:** Writing – original draft, Investigation. **Pinpin Feng:** Investigation. **Hongwei Wang:** Supervision. **Mingzhi Zheng:** Investigation. **Yiliang Zhu:** Investigation. **Kai Zhong:** Investigation. **Jue Hu:** Formal analysis. **Yilu Ye:** Formal analysis. **Linhuizi Lu:** Investigation. **Qinqin Zhao:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31002.

Xiang Li and Qinqin Zhao designed the study and revise the manuscript; Xiang Li, Yuanxiao Yang, Pinpin Feng, Mingzhi Zheng, Yiliang Zhu, Kai Zhong and Linhuizi Lu performed the experiments; Qinqin Zhao, Jue Hu and Yilu Ye analysed the experimental data; Xiang Li, Qinqin Zhao and Yuanxiao Yang prepared the manuscript. Hongwei Wang supervised the data. All authors have read and approved the final version of the manuscript.

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