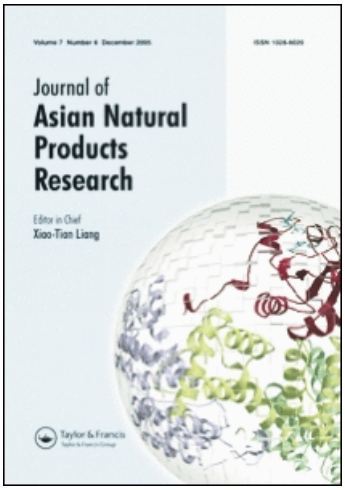

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

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Online publication date: 26 March 2010

To cite this Article Bie, Xiao-Dong , Han, Jue and Dai, Hai-Bin(2010) 'Effects of hydroxysafflor yellow A on the experimental traumatic brain injury in rats', Journal of Asian Natural Products Research, 12: 3, 239 — 247
To link to this Article: DOI: 10.1080/10286020903510636
URL: http://dx.doi.org/10.1080/10286020903510636

Journal of Asian Natural Products Research
Vol. 12, No. 3, March 2010, 239–247

ORIGINAL ARTICLE

Effects of hydroxysafflor yellow A on the experimental traumatic brain injury in rats

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(*Received 5 August 2009; final version received 25 November 2009*)

This paper explores the effects of hydroxysafflor yellow A (HSYA) on traumatic brain injury (TBI). Rats were divided into four groups: control, TBI, TBI combined with HSYA, and TBI combined with nimodipine. Saline, HSYA, or nimodipine was i.v. injected at 30 min before and 6 h after the onset of TBI. The contusion volume of brain, mitochondrial ATPase activity, brain malondialdehyde (MDA) content, and the concentrations of tissue plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) in the blood plasma were investigated. The results showed that the inhibitory rate of HSYA at a dose of 4 mg/kg was 59.2% compared with the TBI group. After the insult by TBI for 48 h, the activity of Na^+, K^+-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase decreased to 31, 35, and 38% of control group. HSYA increased these ATPase activities by 162, 96, and 131% of TBI group. HSYA also increased superoxide dismutase activity and decreased MDA content in the right parietal lobe adjacent to contusion foci in TBI rats. HSYA enhanced the t-PA activity by 64.64%, decreased the PAI-1 activity by 71.88%, and decreased the MMP-9 expression to 49.11% in the hippocampus of the TBI group at 12 h. In conclusion, HSYA may exert a potential therapeutic strategy to improve the outcome following TBI injury.

Keywords: hydroxysafflor yellow A; traumatic brain injury; ATPase; SOD; t-PA; MMP-9

1. Introduction

Traumatic brain injury (TBI) is a leading cause of death and disability, with an estimated annual cost of 60 billion dollars in the USA alone [1]. Aside from surgical hematoma evacuation, treatment remains largely supportive and is directed toward management of cerebral edema and intracranial hypertension with osmotic agents, hyperventilation, and ventricular drainage [2]. None of these interventions has been demonstrated to significantly improve the long-term functional outcome [3], and there remains a compelling need for more effective therapeutic options in this patient population.

TBI may cause contusion, intracranial hemorrhage, blood–brain barrier disruption, and diffuse axonal injury [4]. In addition, secondary damage is mediated by multifactorial mechanisms activated by the initial traumatic event, such as apoptosis, excitatory amino acids, inflammation, free radical production, hyperdepolarization, and the effects of altered levels of Ca^{2+} [5]. These secondary events

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ISSN 1028-6020 print/ISSN 1477-2213 online

DOI: 10.1080/10286020903510636
http://www.informaworld.com

may eventually lead to cell death, but may also provide a window of opportunity for therapeutic treatments, as they are of a delayed nature and possibly preventable or treatable [6].

The flower of the safflower plant *Carthamus tinctorius* L. is extensively used in the traditional Chinese medicine for the treatment of cardiovascular and cerebrovascular diseases. Extracts from *C. tinctorius* contain yellow and red pigments, including hydroxysafflor yellow A (HSYA; Figure 1), safflor yellow B, safflomin A and safflomin C, as well as other chemicals [7]. HSYA, the main chemical component of the safflower yellow pigments, was approved by the Chinese State Food and Drug Administration as a neuroprotective agent for the treatment of acute cerebral contusion in March 2005 (state drug permit document: Z20050146) [8]. The drug is demonstrated to be a good potential agent to treat focal cerebral ischemia [9], and the underlying mechanisms might be involved in its antioxidant action [10], inhibitory effects on mitochondrial permeability transition pore (mtPTP) opening [11], and thrombosis formation and platelet aggregation, as well as its beneficial action on the regulation of PGI2/TXA2 and blood rheological changes in rats [12]. Furthermore, HSYA inhibits the opening of mtPTP by a free radical scavenging action in the brain, and this may also contribute to the neuroprotective effect of HSYA [8]. But there is no report on the effects of HSYA on brain injury after TBI, and the mechanisms of its effect are unclear.

Numerous reports indicated that nimodipine exerted its neuroprotection by virtue of its strong inhibition on intracellular Ca^{2+} influx. Since intracellular influx of Ca^{2+} is associated with cell mortality, the net effect of this inhibition improves cell survival during TBI. It is presently unknown whether HSYA acts through a similar mechanism and the precise mechanism of the neuroprotective effect of nimodipine warrants further investigation. In this study, nimodipine is chosen as a positive control.

2. Results and discussion

2.1 Effects of HSYA on the contusion area in rats subjected to TBI

As a newly identified compound, HSYA showed promising neuroprotection against brain ischemic damage [9,12]. It was also

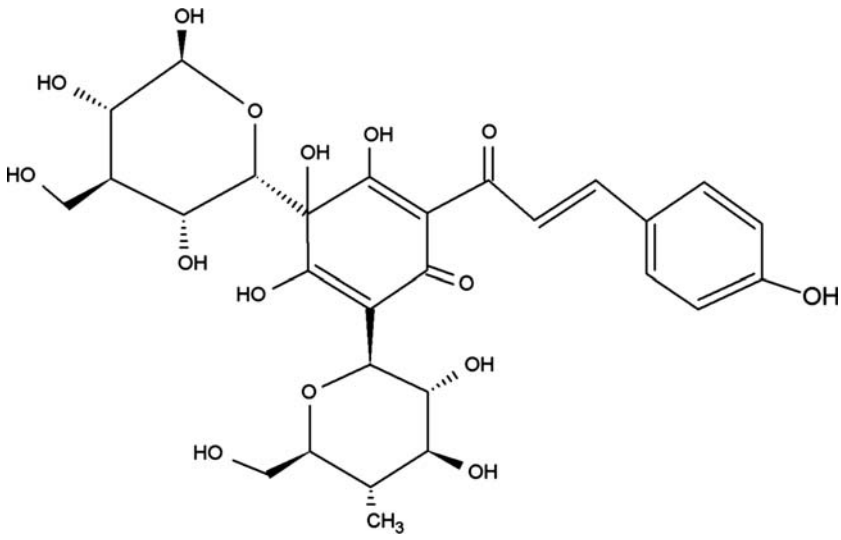

Figure 1. Structure of HSYA.

reported that HSYA protects the rat heart against myocardial contusion and attenuates pressure overload hypertrophy [10]. In this study, 24 h after the onset of TBI, contusion volume in TBI rats was $241.2 \pm 87.7\,mm^3$, indicating brain injury in the TBI model. Treatment with HSYA at doses of 2 and 4 mg/kg significantly reduced the contusion volume to 136.3 ± 50.2 and $98.4 \pm 31.8\,mm^3$, respectively, and the percentage of inhibitory rate was 43.5 and 59.2%, respectively, in the TBI group. HSYA at a dose of 4 mg/kg showed a similar potency compared with that of nimodipine at a dose of 0.4 mg/kg (Table 1). In the following tests, HSYA at a dose of 4 mg/kg and nimodipine at a dose of 0.4 mg/kg were chosen to explore further mechanism. Morphological evidence of cellular death caused by TBI is commonly detected by 2,3,5-triphenyl-tetrazolium chloride (TTC) staining. TTC is a sensitive histochemical indicator of mitochondrial respiratory enzyme function. Therefore, brain lesion identified by TTC staining indicated that tissues were irreversibly impaired in mitochondrial function and oxidative respiratory enzyme systems [13]. Our results revealed that HSYA can improve mitochondrial activity following TBI, and thus reduce contusion volume.

Table 1. Effects of HSYA on the contusion volume induced by experimental TBI in rats.

Group	Dose (mg/kg)	Contusion volume (mm^3)
TBI		241.2 ± 87.7
HSYA+TBI	1	185.7 ± 60.5 (23.0%)
	2	$136.3 \pm 50.2^{\#}$ (43.5%)
	4	$98.4 \pm 31.8^{\#}$ (59.2%)
Nimodipine+TBI	0.4	$102.8 \pm 35.3^{\#}$ (57.4%)

Notes: Animals received HSYA (1, 2, or 4 mg/kg, i.v.), nimodipine (0.4 mg/kg, i.v.), or saline at 30 min before and 6 h after the onset of TBI. Contusion volume was measured 24 h after TBI in consecutive brain slices taken. Contusion volumes were calculated as the percentage of contusion volumes over the total brain volumes in rats. Data were presented as mean ± SD of eight independent experiments. Groups were compared by Student's *t*-test for unpaired samples. Differences were considered significant at $P < 0.05$. $^{\#}P < 0.05$ vs. TBI group. The data in parentheses are the inhibitory rates (%).

2.2 Effects of HSYA on the ATPase activity in the brain mitochondria of rats subjected to TBI

Mitochondria are important and special cell organelles, which synthesize ATP and provide energy for cells. It is the main source of intracellular reactive oxygen species (free radicals). Ninety percent of the oxygen in the cell is processed by the mitochondria and 1–4% of it becomes reactive oxygen in the mitochondrial respiratory chain. There are many proteins and enzymes on the mitochondrial membrane and mitochondrial DNA inside the mitochondria which are related to the occurrence and development of diseases. Mitochondrial membranes also include abundant unsaturated fatty acids, which are a target of oxidative damage. Hence, the oxidative damage of mitochondrial membranes inevitably affects the normal physiological functions of the cell and may cause many diseases [14,15].

One of the first consequences of the resulting oxidative damage is the decrease in Ca^{2+}, Mg^{2+}-ATPase activity [16]. Calcium homeostasis is crucial to the survival of mitochondria, and the inability of mitochondria to maintain low intramitochondrial calcium is a classical marker of impending cell death. Na^+, K^+-ATPase is a membrane integral enzyme responsible for neuronal homeostasis through the membrane. This enzyme activity has been used as a potential indicator of membrane structure and function [17]. Its inhibition will result in a decline of Na^+ and K^+ electrochemical gradients and ultimately lead to cell death as a result of osmotic damage as well as the accumulation of intracellular Ca^{2+} secondary to a decrease

in active Ca^{2+} influx by the Na^{+}/Ca^{2+} exchange system [18]. Besides the action of the Na^{+}/Ca^{2+} exchange system, Ca^{2+}-ATPase is involved in the removal of cytosolic Ca^{2+}. Ca^{2+}-ATPase exists on both the sarcolemmal and sarcoplasmic membranes, and it contributes to the removal of Ca^{2+} from the cytosol to the extracellular space and sarcoplasmic reticulum, respectively.

So, the effects of HSYA on the mitochondrial ATPase subjected to TBI were first investigated. After the insult by TBI for 12 h, the activities of Na^{+}, K^{+}-ATPase and Ca^{2+}-ATPase were significantly decreased to 1.23 and 1.30 mol/mg protein/h, respectively, which was 58 and 56% compared with control groups. After 24 h, the activity of Mg^{2+}-ATPase was also significantly decreased to 0.87 mol/mg protein/h, which was 41% when compared with control groups. When treated with HSYA at 30 min before and 6 h after the onset of TBI, the activities of Na^{+}, K^{+}-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase were significantly enhanced, especially at 24 and 48 h, compared with TBI groups. On the other hand, the activities of Na^{+}, K^{+}-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase were also reversed by nimodipine (Table 2).

2.3 Effects of HSYA on the activity of superoxide dismutase and the content of malondialdehyde in the brain homogenate of rats subjected to TBI

On the other hand, oxidative stress leads to the accumulation of the lipid peroxidation product malondialdehyde (MDA) in the brain, which results in cell membrane

Table 2. Effects of HSYA on the ATPase activity in the brain mitochondria of rats subjected to experimental TBI.

		ATPase activity (mol/mg protein/h)		
Group	Time (h)	Na^{+}, K^{+}-ATPase	Ca^{2+}-ATPase	Mg^{2+}-ATPase
Control	12	2.12 ± 0.43	2.33 ± 0.49	1.92 ± 0.35
	24	2.26 ± 0.36	2.17 ± 0.53	2.12 ± 0.40
	48	2.49 ± 0.54	2.54 ± 0.86	1.89 ± 0.32
TBI	12	1.23 ± 0.34*	1.30 ± 0.26*	1.67 ± 0.22
	24	0.95 ± 0.32*	1.05 ± 0.35*	0.87 ± 0.32*
	48	0.78 ± 0.23*	0.91 ± 0.29*	0.72 ± 0.25*
HSYA+TBI	12	1.63 ± 0.53 (32.56%)	1.58 ± 0.44 (21.54%)	1.78 ± 0.39 (6.59%)
	24	1.56 ± 0.36$^{\#}$ (64.21%)	1.79 ± 0.37$^{\#}$ (74.48%)	1.78 ± 0.39$^{\#\#}$ (104.60%)
	48	2.05 ± 0.43$^{\#\#}$ (162.82%)	1.78 ± 0.39$^{\#}$ (95.60%)	1.67 ± 0.38$^{\#}$ (131.94%)
Nimodipine+TBI	12	1.67 ± 0.41 (35.77%)	1.60 ± 0.28 (23.08%)	1.69 ± 0.37 (1.20%)
	24	1.59 ± 0.34$^{\#}$ (67.37%)	1.77 ± 0.51$^{\#}$ (68.57%)	2.01 ± 0.47$^{\#\#}$ (131.03%)
	48	1.88 ± 0.21$^{\#}$ (141.03%)	1.93 ± 0.14$^{\#}$ (112.09%)	1.96 ± 0.23$^{\#}$ (172.22%)

Notes: Animals received HSYA (4 mg/kg, i.v.), nimodipine (0.4 mg/kg, i.v.), or saline at 30 min before and 6 h after the onset of TBI. Brains were quickly removed and then isolated mitochondria for the determination of Na^{+}, K^{+}-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase activity as described. Data of ATPase activity were presented as mean ± SD of eight independent experiments. Groups were compared by one-way ANOVA. Differences were considered significant at $P < 0.05$. $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. TBI. The data in parentheses are the enhancement rates (%).

breakdown and subsequent cell swelling. Superoxide dismutase (SOD), as an endogenous free radical scavenging enzyme, protects cells against the toxic effects of superoxide radicals [19]. SOD can catalyze the conversion of superoxide radicals to H_2O_2, which is decomposed into water and oxygen by catalase [20].

Then, whether HSYA can reverse TBI-induced enhancement of SOD activity and MDA content in the brain was investigated. As shown in Table 3, the activity of SOD was significantly decreased to 156.45 U/mg protein, which was 57% to control groups after the insult by TBI for 24 h, and the content of MDA was significantly immediately enhanced to 347 nmol/mg protein, which was 273% to control groups after the insult by TBI for 12 h. When treated with HSYA and nimodipine, the activity of SOD increased to 69.22 and 68.65% compared with the TBI group after 48 h (Table 3). Changes in these markers can indicate both oxidative damage and the antioxidant protective effect of drugs [21].

When mitochondria are attacked by TBI, the mitochondrial content of MDA, an index of lipid peroxidation, was increased to 347 nmol/mg protein, which was only 126.80 nmol/mg protein after the insult for 12 h. When treated with HSYA and nimodipine, the content of MDA decreased to 38.73 and 36.99% compared with the TBI group after 12 h (Table 3). This means that HSYA may restore MDA content to protect from TBI injury. Increased reactive oxygen species decrease mitochondrial SOD. MDA links with phospholipids and proteins to reduce the fluidity and increase the permeability of membranes, and this will inevitably affect the structure and function of mitochondria [15,22]. HSYA could protect mitochondria from TBI-induced damage. They could also restore SOD and ATPase activity and preserve membrane structure as evidenced by decreasing MDA.

2.4 Effects of HSYA on the activity of tissue plasminogen activator and plasminogen activator inhibitor-1 in the serum of rats subjected to TBI

Tissue plasminogen activator (t-PA) is the major physiological plasminogen activator and is synthesized by the endothelium.

Table 3. Effects of HSYA on the activity of SOD and the content of MDA in the brain homogenate of rats subjected to experimental TBI.

Group	Time (h)	SOD activity (U/mg protein)	MDA content (nmol/mg protein)
Control	12	285.51 ± 32.11	126.80 ± 13.22
	24	275.67 ± 31.22	134.57 ± 18.23
	48	288.25 ± 37.15	153.14 ± 20.43
TBI	12	203.23 ± 36.26	346.51 ± 38.62*
	24	156.45 ± 23.43*	367.19 ± 41.18*
	48	146.23 ± 28.21*	388.41 ± 35.73*
HSYA + TBI	12	236.17 ± 33.25 (16.21%)	212.32 ± 26.10$^{\#}$ (38.73%)
	24	235.59 ± 24.27$^{\#}$ (50.58%)	231.85 ± 31.68$^{\#}$ (36.86%)
	48	247.45 ± 29.55$^{\#}$ (69.22%)	239.44 ± 43.11$^{\#}$ (38.35%)
Nimodipine + TBI	12	229.56 ± 43.23 (12.96%)	218.35 ± 34.32 (36.99%)
	24	232.47 ± 23.74$^{\#}$ (48.59%)	229.71 ± 45.21$^{\#}$ (37.44%)
	48	246.62 ± 31.60$^{\#}$ (68.65%)	235.41 ± 23.14$^{\#}$ (39.39%)

Notes: Animals received HSYA (4 mg/kg, i.v.), nimodipine (0.4 mg/kg, i.v.), or saline at 30 min before and 6 h after the onset of TBI. Brains were quickly removed and then homogenated for the determination of the activity of SOD and the content of MDA as described. Data of enzyme activity were presented as mean ± SD of eight independent experiments. Groups were compared by one-way ANOVA. Differences were considered significant at $P < 0.05$. *$P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. TBI. The data in parentheses are the enhancement rates of SOD activity and inhibitory rate of MDA content, respectively.

Plasminogen activator inhibitor-1 (PAI-1) is the major endogenous inhibitor of both t-PA and urokinase-type plasminogen activator, and therefore plays a dominant role in the control of fibrinolysis; it is also synthesized within the endothelium. t-PA can cause thrombus fibrinolysis, whereas PAI-1 can lead to thrombus formation by inhibiting t-PA activity [23].

Then, the effects of HSYA on the activity of t-PA and PAI-1 in the serum was investigated. The activity of t-PA was significantly decreased to 56% compared with control groups after the insult by TBI for 12 h, while the activity of PAI-1 was increased to 138% compared with control groups. When treated with HSYA at 30 min before and 6 h after the onset of TBI, the enhancement rate of t-PA activity was 64.64%, the inhibitory rate of PAI-1 activity was 7.59% after 12 h compared with TBI groups, and the inhibitory rate of the PAI-1/t-PA ratio decreased to 71.88%. At that time, the activity of t-PA was also significantly enhanced to 62.97% and the activity of PAI-1 was decreased to 7.70% by nimodipine in the experiments. Even after 48 h, HSYA and nimodipine have the potential to restore the t-PA and PAI-1 activity which is impaired by TBI (Table 4). Our results show that HSYA could downregulate t-PA activity and upregulate PAI-1 activity in TBI insult. These additional antithrombotic and fibrinolytic effects of HSYA may promote important benefits in TBI injury.

2.5 Effects of HSYA on the MMP-9 expression in the rat hippocampus subjected to TBI

Matrix metalloproteinases (MMPs) are a family of calcium-requiring, zinc-containing proteolytic enzymes, which degrade the molecules of the extracellular matrix. Among MMPs, gelatinase A (MMP-2) and gelatinase B (MMP-9) are able to digest the components of the basement membrane. The increase in MMPs is implicated in the pathogenesis of several central nervous system diseases, such as cerebral ischemia [24,25]. In this experiment, the gelatinase expression of MMP-9 detected in the hippocampus at 12 h was markedly

Table 4. Effects of HSYA on the activity of t-PA and PAI-1 in the serum of rats subjected to experimental TBI.

Group	Time (h)	t-PA (U/ml)	PAI-1 (U/ml)	PAI-1/t-PA
Control	12	0.856 ± 0.018	0.668 ± 0.022	0.78
	24	0.887 ± 0.022	0.682 ± 0.029	0.769
	48	0.874 ± 0.035	0.685 ± 0.033	0.784
TBI	12	0.478 ± 0.043**	0.922 ± 0.043*	1.929
	24	0.511 ± 0.032**	0.859 ± 0.018*	1.649
	48	0.583 ± 0.021*	0.844 ± 0.023*	1.413
HSYA+TBI	12	0.787 ± 0.025$^{\#}$ (64.64%)	0.852 ± 0.021 (7.59%)	1.083 (71.88%)
	24	0.819 ± 0.036$^{\#\#}$ (60.27%)	0.727 ± 0.032$^{\#}$ (15.37%)	0.888 (46.15%)
	48	0.833 ± 0.045$^{\#}$ (42.88%)	0.714 ± 0.027$^{\#}$ (5.40%)	0.868 (38.57%)
Nimodipine+TBI	12	0.779 ± 0.023 (62.97%)	0.851 ± 0.043 (7.70%)	1.09 (43.49%)
	24	0.808 ± 0.051$^{\#}$ (58.12%)	0.729 ± 0.012$^{\#}$ (51.30%)	0.90 (45.42%)
	48	0.829 ± 0.038$^{\#}$ (42.19%)	0.718 ± 0.037$^{\#}$ (14.93%)	0.866 (38.71%)

Notes: Animals received HSYA (4 mg/kg, i.v.), nimodipine (0.4 mg/kg, i.v.), or saline at 30 min before and 6 h after the onset of TBI. Blood was collected in tubes from rats just prior to sacrificing the animals, centrifuged at 4°C at 1200*g* for 10 min and the supernatant serum was used for determination of t-PA and PAI-1 activity as described. Data of enzyme activity were presented as mean ± SD of eight independent experiments. Groups were compared by one-way ANOVA. Differences were considered significant at $P < 0.05$. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. TBI. The data in parentheses are the enhancement rates of t-PA activity and inhibitory rate of PAI-1 activity and PAI-1/t-PA ratio, respectively.

(maximally) expressed at 24 h, and remained strongly 48 h after TBI injection. On the other hand, HSYA significantly decreased the MMP-9 expression.

After the insult by TBI for 12 h, the percent of MMP-9 positive cells in the rat hippocampus enhanced to 49.93%, which is only 11.38% in the control group (Table 5). When treated with HSYA and nimodipine at 30 min before and 6 h after the onset of TBI, the percent of MMP-9 positive cells decreased to 25.41 and 24.96% after 12 h, respectively, which was significantly different from the TBI groups, and the inhibitory rates of HSYA and nimodipine were 49.11 and 50.01, respectively (Table 5).

Table 5. Effects of HSYA on the MMP-9 expression in the rat hippocampus subjected to experimental TBI.

Group	Time (h)	MMP-9 positive cells (%)
Control	12	11.38 ± 2.06
	24	12.76 ± 3.25
	48	12.23 ± 0.52
TBI	12	49.93 ± 10.71*
	24	52.98 ± 12.08*
	48	51.28 ± 13.12*
HSYA + TBI	12	25.41 ± 7.03$^{\#}$ (49.11%)
	24	22.25 ± 6.36$^{\#\#}$ (58.00%)
	48	22.88 ± 7.19$^{\#\#}$ (55.38%)
Nimodipine + TBI	12	24.96 ± 6.71$^{\#}$ (50.01%)
	24	22.76 ± 5.36$^{\#\#}$ (57.04%)
	48	21.99 ± 7.41$^{\#\#}$ (57.12%)

Notes: Animals received HSYA (4 mg/kg, i.v.), nimodipine (0.4 mg/kg, i.v.), or saline at 30 min before and 6 h after the onset of TBI. About 30 μm coronal sections were cut through the entire dorsal hippocampus with cryotome as described. Data of MMP-9 positive cells were presented as mean ± SD of eight independent experiments. Groups were compared by one-way ANOVA. Differences were considered significant at $P < 0.05$. *$P < 0.05$ vs. control, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. TBI. The data in parentheses are the inhibitory rates (%).

In conclusion, HSYA may exert potential therapeutic strategies to improve the outcome following TBI injury. These effects may be, in at least part, based on improving mitochondrial activity, antioxidant activity, and antithrombotic or fibrinolytic effects.

3. Experimental

3.1 Animals and TBI

Male Sprague–Dawley rats (260–300 g; Grade II, Certificate No. SCXK2003-0001, Experimental Animal Center, Zhejiang Academy of Medical Science, Hangzhou, China) were maintained in individual cages with a 12 h light–dark cycle (lights on from 8 am to 8 pm). All experiments were carried out in accordance with the ethical guidelines of the Zhejiang University Animal Experimentation Committee and were in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Water and food were given *ad libitum*.

After chloral hydrate anesthesia (400 mg/kg, i.p.), the rat scalps were cut along the midline and spread laterally. A stainless helmet was sewed onto the skull to prevent direct damage from blows. Rats were placed prone on the bottom plate of the weight-drop device. The device consisted of a long plastic tube extruding upward. A 400 g weight scale was put on the top of the tube (about 1.5 m) and slid through the tube to deliver a blow to the exposed skull of a rat to induce TBI [24].

In the experiments, rats were divided into four groups: control, TBI, TBI combined with HSYA (Zhejiang Yongning Pharmaceutical Co., Ltd, Ningbo, China; purity > 99%), and TBI combined with nimodipine. Animals without spontaneous respiration died within 3 min after TBI; only those showing spontaneous respiration were used in this study ($n = 32$). Rats were administered via tail-vein injection with HSYA (1, 2, or 4 mg/kg) or nimodipine (0.4 mg/kg)

at 30 min before and 6 h after the onset of TBI. Control rats always received saline. Blood sample (500 μl) of the animals in each group was taken from arteria carotis communis, and then the animals were decapitated at 12, 24, or 48 h after TBI.

3.2 TTC staining

Animals were all sacrificed 24 h after trauma. The brain tissue was then removed, immersed in cold saline for 5 min, and sliced into 2.0 mm sections with a tissue slicer. The brain slices were incubated in 2% TTC dissolved in phosphate buffered saline (PBS) for 30 min at 37°C, and then transferred to 5% formaldehyde solution for fixation. The volume of contusion, as revealed by negative TTC staining indicating dehydrogenase-deficient tissue, was measured in each slice and summed using computerized planimetry (PC-based Image Tools software). The volume of contusion was calculated as 2 mm (thickness of the slice) × (sum of the contusion area in all brain slices (mm^2)) [25].

3.3 Brain homogenate, mitochondria isolate, and sample treatment

After decapitation, the brains were quickly removed and the right parietal lobes adjacent to the contusion foci were chopped into small pieces and placed in ice-cold isolation buffer for mitochondria (10 mM Tris–HCl, pH 7.4, 250 mM sucrose, 0.5 mM EDTA, and 0.5% bovine serum albumin). After being homogenized, the homogenate was centrifuged at 750*g* for 10 min. Next, 2 ml of the supernatant was stored at −80°C and used for measurement. The remainder was centrifuged at 10,000*g* for 10 min. The mitochondrial pellet was washed twice with isolation buffer, and then re-suspended in the same buffer solution. The freshly prepared mitochondria were used to determine the ATPase. Protein content was determined according to the Coomassie blue protein-binding method, using bovine serum albumin as a standard. ATPase activity in the mitochondria was determined by measuring the liberated Pi and MDA content, and SOD activity in the homogenate was determined by the TBA method using kits obtained from Jiancheng Bioengineering Ltd, Nanjing, China.

3.4 Measuring the concentration of t-PA and PAI-1 in the blood plasma

The concentrations of t-PA and PAI-1 in the blood plasma were determined by the ELISA method. t-PA and PAI-1 assay kits were purchased from Shanghai Sun Biology Company, Shanghai, China.

3.5 Immunofluorescence staining and cell counting

Animals were anesthetized and perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were immediately removed and immersed in 4% paraformaldehyde for 24 h and then placed in 25% sucrose solution.

About 30 μm coronal sections were cut through the entire dorsal hippocampus (bregma −2.3 to −4.5 mm) with cryotome (Leica, CM1900, Wetzlar, Germany). Sections were collected in PBS (0.01 M, pH 7.4), washed three times for 10 min and then pre-incubated with a blocking solution of PBS containing 1% albumin bovine and 4% normal goat serum (Vector Labs, Burlingame, CA, USA) for 1 h at room temperature. Subsequently, the sections were incubated in primary antibody (mouse anti-MMP-9 monoclonal antibody, 1:400; NeoMarkers, Fremont, CA, USA) solutions of PBS containing 2% normal goat serum and 0.25% Triton-X 100 for 24 h at 4°C. After rinsing three times for 10 min, the sections were incubated in Alexa 488-conjugated secondary antibody solutions (goat anti-mouse in PBS; Molecular Probes, Eugene, OR, USA) for 2 h at room temperature in the dark. The sections were then washed and mounted in mounting

medium. Finally, they were examined with a fluorescent microscope (BX5, Olympus, Tokyo, Japan), and prepared without primary antibody samples which were used as specificity controls. No staining was observed in such experiments.

3.6 Data presentation and statistical analysis

All data are expressed as mean ± SD. Groups were compared by Student's *t*-test for unpaired samples. Statistical significance was set at $P < 0.05$.

Acknowledgements

This project was supported by grants from the Zhejiang Provincial Natural Science Foundation of China (Y207256), the Zhejiang Provincial Scientific Foundation of China (2008C33061), and the Zhejiang Province Traditional Chinese Medicine Foundation of China (2004C092, 2008YA021).

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