

Therapeutic time window of YGY-E neuroprotection of cerebral ischemic injury in rats

Yongtong An, Zhen Zhao, Yuchen Sheng, Yang Min, Yuye Xia*

Shanghai Institute of Pharmaceutical Industry, Shanghai, China.

ABSTRACT: YGY-E is an active ingredient in traditional Chinese medical herbs which have anti-ischemic activity. The present work was designed to study its therapeutic time window in cerebral ischemic injury as well as its effect on neuronal apoptosis. Animals received an intravenous injection of YGY-E at 1, 3, and 6 h, respectively, after permanent focal cerebral ischemia induced by electrocoagulation of the middle cerebral artery. Infarct ratio and neurological function were employed to assess the effects of YGY-E on the therapeutic time window in this animal model. Furthermore, we evaluated effects of this compound on neuronal apoptosis and synthesis of Bcl-2 and Bax in ischemic brain tissue with *in situ* DNA end labeling (TUNEL), immunohistochemistry assay, and Western blot analysis. YGY-E (2-8 mg/kg) delivered at all the three time points dose-dependently decreased infarct ratio, neurological deficits, percentage of TUNEL-positive cells ($p < 0.01$) and Bax-positive cells ($p < 0.01$ or $p < 0.05$). In contrast, it increased the percentage of Bcl-2 positive cells ($p < 0.01$ or $p < 0.05$). These data demonstrated that YGY-E had protective effects against cerebral ischemia injuries in rats. But more importantly, they indicate that YGY-E has an unusually long (up to 6 h) therapeutic time window relative to classical drugs in treating cerebral ischemia. In addition, our results suggest that the anti-apoptotic effects of YGY-E are due to its regulation of the balance between Bcl-2 and Bax protein levels.

Keywords: YGY-E, permanent focal cerebral ischemia, therapeutic time window, apoptosis, Bcl-2, Bax

1. Introduction

Ischemic stroke is an enormous public health problem. It can cause permanent neurological damage, an area

of infarcted tissue, severe functional impairments, and even death if not managed quickly (1,2). Although several neuroprotectants with various mechanisms of action failed to improve neurological symptoms of patients, pharmacotherapy of ischemic stroke is still a promising treatment option (3-5).

Nowadays, thrombolytics including tissue plasminogen activator (tPA) are widely used for treating ischemic stroke, improving long-term functional recovery of patients (4). Only tPA is approved by the US Food and Drug Administration (FDA) for intravenous delivery within 3 h of symptom onset in treating ischemic stroke (6). However, only a small portion of ischemic stroke patients receive effective thrombolytic therapy due to the narrow therapeutic time window and the threat of hemorrhage (7). The earlier the intervention involvement, the more benefit the patient receives. It is often impossible for patients to receive timely treatment since the majority of strokes occur suddenly. Therefore, one of the potential approaches to increase treatment opportunities is to develop a novel drug with a longer therapeutic time window.

Chinese medicine phoenix-tail fern (Latin name: *Herba Pteridis Multifidae*) is known to have potent analgesic anti-inflammatory effects. We accidentally found that the medicine had a remarkable anti-ischemic injury activity (8). A series of pharmacodynamic screening experiments in an *in vivo* animal model of cerebral ischemia have revealed that YGY-E with a structure of apigenin-7-*O*- β -D-glucopyranosyl-4'-*O*- α -L-rhamnopyranosid (Figure 1) was the main active ingredient for stroke treatment.

YGY-E is a flavonoid glycoside that can be acquired from *Ranunculus* species, such as, *Ranunculus sieboldii* and *Ranunculus sceleratus*. The procedure for the separation, purification, and identification of this effective ingredient has been established (9). YGY-E can be purified using HPLC up to 99% pure, which is suitable for intravenous injection. Our recent studies have confirmed the neuroprotective and anti-ischemic activities of this compound (9). The present study aims to investigate its therapeutic time window in the permanent cerebral ischemia induced by electrocoagulating the middle cerebral artery (MCA).

*Address correspondence to:

Dr. Yuye Xia, Shanghai Institute of Pharmaceutical Industry, Shanghai 200437, China.
e-mail: chengguoze1981@163.com

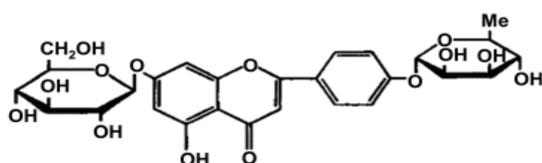


Figure 1. The structure of YGY-E.

Tissue damage following cerebral ischemia is caused by complicated pathophysiological processes such as glutamate excitotoxicity, membrane depolarization, inflammation, and especially, apoptosis (10,11). Apoptosis is a process of cell death which occurs under various kinds of pathological and physiological conditions. It has been known that apoptosis is one of the major neuronal death mechanisms in the experimental model of ischemia (12,13). In this study, we evaluated YGY-E's effect on apoptosis of neuron cells. Meanwhile, we investigated the expression of two proteins, Bcl-2 and Bax, the cardinal regulators in the process of apoptosis, to determine YGY-E's neuroprotective mechanisms in cerebral ischemic injuries.

2. Materials and Methods

2.1. Animals

Male Wistar rats (weight: 200-300 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd., Shanghai, China. The rats were housed with controlled temperature ($24 \pm 1^\circ\text{C}$), artificial light-dark cycle (light from 7 a.m. to 7 p.m., dark from 7 p.m. to 7 a.m.), and with free access to food and drinking water. All the animals used in the experiment received humane care. All surgical and experimental procedures were in accordance with institutional animal care guidelines.

2.2. Cerebral ischemia in rats

Permanent focal cerebral ischemia was induced by electrocoagulation of the MCA through a bone window exposure and electric coagulation technique according to a widely-used technique (14-17). Rats were anesthetized with 12% chloral hydrate (360 mg/kg, *i.p.*). The temporal muscle was excised and a 2 mm burr hole was drilled into the skull 2-3 mm rostral to the fusion of the zygomatic arch with the squamosal bone. The left MCA was exposed and occluded by electrocoagulation. YGY-E was administered intravenously *via* the ranine vein at 1, 3, and 6 h following surgery. Rats were returned to cages under the above mentioned conditions.

Twenty-four hours later, a single-blind behavioral test study was performed. The evaluation index included contralateral forelimb flexion, thorax twisting, and lateral push resistance (18,19). The most serious injury severity score was 10 points. Brain samples were

obtained and 2-mm thick coronal sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) (20). The infarct region was white, and the normal region was red. The whole brain and the infarct region were weighed separately, and calculated as follows: infarct region weight/whole brain weight $\times 100\%$.

2.3. Assessment of apoptosis

Rats were anesthetized with an overdose of 12% chloral hydrate 24 h after MCA electrocoagulation. Rat brains were perfused transcardially with 4% paraformaldehyde and then isolated and fixed in 10% formalin before being embedded in the paraffin. Brains were cut into 4-5 slices for apoptosis detection with TdT-mediated dUTP-biotin nick end labeling (TUNEL) (In Situ Cell Death Detection Kit; Roche Applied Science, Mannheim, Germany). The assay was performed according to the manufacturer's instructions. Briefly, after dewaxing, the slides were washed with 0.01 M phosphate-buffered saline and incubated in a 20 $\mu\text{g/mL}$ solution of proteinase K (Promega, Madison, WI, USA) at 37°C for 30 min. After the pretreatment, sections were blotted and incubated with a mixture containing TdT enzyme (Promega) and digoxigenin tagged dUTP (Boehringer Mannheim, Mannheim, Germany) for about 4 h, then were counterstained with nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) and mounted with neutral gum.

To quantify TUNEL-positive nuclei, positive cells were counted from five random high-power fields ($\times 200$) in each section, and apoptotic index was expressed as the percentage of positive cells (positive cells/total cells $\times 100\%$) in each animal.

2.4. Detection of Bcl-2 and Bax

2.4.1. Immunohistochemistry assay

Twenty-four hours after cerebral ischemia, brain sections were fixed in 4% paraformaldehyde for 1 h before being preincubated for 30 min with methanol containing 0.5% hydrogen peroxide, and then blocked with goat serum for 30 min at room temperature. Slices were incubated with primary antibodies against Bcl-2 or Bax (Chemicon International, Temecula, CA, USA) at 37°C for 1 h until a biotinylated goat anti-rat IgG was added. Twenty minutes later, an avidin-horseradish enzyme complex was added and incubation lasted for another 20 min at 37°C (ABC Kit; Sino-American Biotechnology and Pharmaceutical Professionals Association (SABPA), San Diego, CA, USA). 3,3'-Diaminobenzidine (DAB; SABPA) was used as the chromogen. The result appeared as a brown peroxidase reaction product.

Five high-power fields ($\times 200$) per slide were chosen

for analysis. The percentage of positive proteins was determined using a computerized image analysis system attached to the microscope. The higher level of protein expression was determined by the more intense immunostaining observed.

2.4.2. Western blot analysis

The brain tissue samples were processed for cytosol and mitochondria fractions. After centrifugation, the supernatant was resuspended in loading buffer. The buffer was subjected to SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane, which was blocked with non-fat dry milk in buffer. The membrane was incubated with primary antibody against Bcl-2 and Bax antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and second antibody goat anti-mouse IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology). Thereafter the proteins were visualized by an electrochemiluminescence detection system (GE Healthcare Bio-Sciences, Uppsala, Sweden) and analyzed by Quantity One Analysis Software (Bio-Rad Laboratories, Hercules, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as protein loading control.

2.5. Therapeutic time window of YGY-E in cerebral ischemic injury

A total of 150 male Wistar rats were randomly divided into the following 5 treatment groups (30 rats/group): control group, YGY-E groups (2, 4, and 8 mg/kg, respectively), and nimodipine group (5 mg/kg). Each treatment group was further randomly divided into 3 timing subgroups (10 rats/subgroup). The 3 subgroups received corresponding treatments at 1, 3, and 6 h following cerebral ischemia, respectively. Effects were determined by infarct ratio and neurological function. Twenty-four hours after treatment, animal brains were sampled for analysis of infarct size. YGY-E was dissolved in 1 M sodium carbonate and diluted with sterile water to the appropriate concentration. Nimodipine and TTC were provided by Tianjin People's Pharmaceutical Industry (Tianjin, China) and China Pharmaceutical Group Shanghai Chemical Reagent Company (Shanghai, China), respectively.

2.6. Time window of YGY-E effect on neuronal apoptosis in cerebral ischemic injury

Forty male Wistar rats were randomly divided into the following 8 groups ($n = 5$ in each group): Sham-operated group, control group (NS, 2 mL/kg), YGY-E groups (4 mg/kg), and nimodipine groups (5 mg/kg). The latter two groups were redistributed into 3 subgroups which received YGY-E or nimodipine at 1, 3, or 6 h after MCA occlusion, respectively. Twenty-four hours later, brains were isolated and apoptosis was detected as described above.

2.7. Time window of YGY-E effect on Bcl-2 and Bax levels in cerebral ischemic injury

This experiment was performed in the same batch of rats divided into 8 groups as described above. Brains were treated to observe synthesis of Bcl-2 and Bax with the methods previously mentioned.

2.8. Statistics

Investigators were blind to the procedures during the determination of cerebral infarction, function evaluation, weighing, apoptotic cell counting, and synthesis of proteins observation. Data were expressed as mean \pm S.E.M. Unpaired Student's *t*-test was employed for statistical comparison between the control group and each treatment group. Statistical significance was accepted at less than 5%.

3. Results

3.1. Therapeutic time window of YGY-E in cerebral ischemic injury

The anti-ischemic effect of YGY-E administered at different time points after MCA electrocoagulation was expressed as its effect on the weight ratio of infarct regions to whole brains. The results are presented in Table 1. Example sections of brains are shown in Figure 2. Both YGY-E and nimodipine exhibited protective effects against cerebral ischemia injuries. YGY-E showed better overall effects than nimodipine, especially in drug groups administered at 6 h after ischemia (4 and 8 mg/kg). Compared with control group, the infarct ratio

Table 1. Therapeutic time window of YGY-E on permanent focal cerebral ischemia induced by MCA

Group	Dosage (mg/kg)	n	Infarct rate (%)			Neurological function evaluation		
			1 h	3 h	6 h	1 h	3 h	6 h
Control group	2 mL/kg	10	10.76 \pm 1.49	11.18 \pm 0.37	10.55 \pm 1.36	8.0 \pm 0.4	8.1 \pm 0.4	8.2 \pm 0.4
Nimodipine	5	10	5.68 \pm 0.90**	6.91 \pm 0.98*	7.86 \pm 1.37	5.9 \pm 0.5**	6.6 \pm 0.4*	7.0 \pm 0.5
YGY-E	2	10	6.31 \pm 0.75*	6.62 \pm 1.12*	7.19 \pm 0.90	6.5 \pm 0.3*	6.6 \pm 0.6*	7.1 \pm 0.4*
	4	10	5.48 \pm 0.95**	5.83 \pm 0.84**	6.45 \pm 0.92*	5.7 \pm 0.6**	6.2 \pm 0.4**	6.9 \pm 0.2*
	8	10	5.24 \pm 1.11**	5.75 \pm 1.07**	5.96 \pm 0.91*	5.7 \pm 0.5**	6.1 \pm 0.6**	6.4 \pm 0.4**

Data are presented as mean \pm S.E.M.; * $p < 0.05$, ** $p < 0.01$ vs. control group (NS).

was significantly decreased by nimodipine administered at 1 and 3 h after ischemia ($p < 0.05$ or $p < 0.01$). When administered at 6 h after ischemia, nimodipine slightly

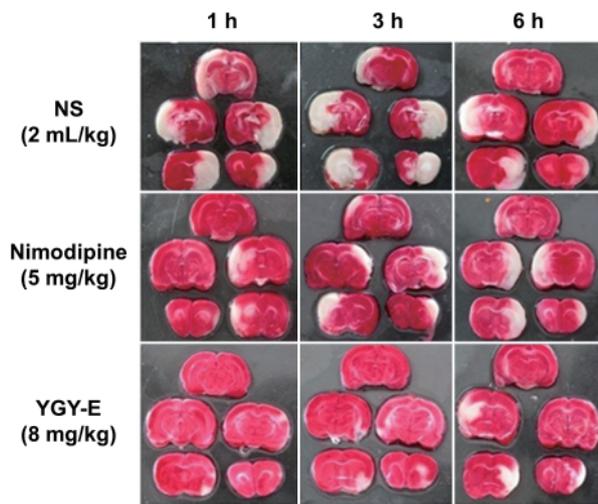


Figure 2. Typical sections of ischemic brain in rats subjected to 24 h of MCA electrocoagulation with or without nimodipine or YGY-E treatment. MCA occlusion produced an ipsilateral focal infarct (white area) as shown by TTC staining. *Upper*, control group (NS, 2 mL/kg); *Middle*, nimodipine treatment (5 mg/kg); *Lower*, YGY-E group (8 mg/kg). From left to right, administered at 1, 3, and 6 h after ischemia.

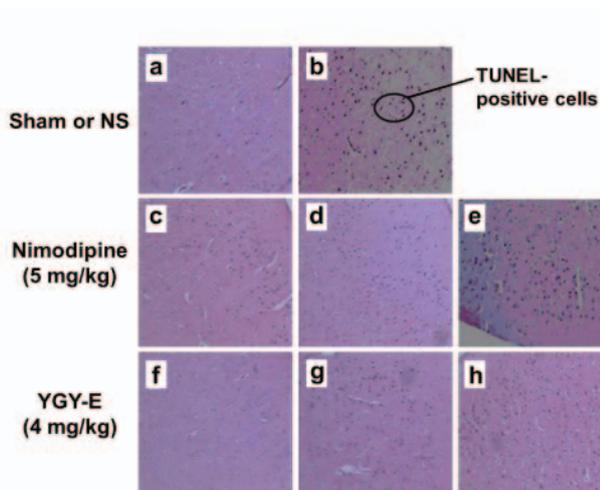


Figure 3. Photomicrograph of nerve cells with TUNEL-staining in the infarct region 24 h after MCA occlusion in rats with or without nimodipine or YGY-E treatment. a, sham-operated group; b, NS (2 mL/kg); c-e, nimodipine groups (5 mg/kg) administered at 1, 3, and 6 h after ischemia, respectively; f-h, YGY-E groups (4 mg/kg) administered at 1, 3, and 6 h after ischemia, respectively. Magnification, $\times 200$.

Table 2. Effect of YGY-E on neuronal apoptosis in rat ischemic brain tissue

Group	Dosage (mg/kg)	n	The percentage of TUNEL-positive cells (%)		
			1 h	3 h	6 h
Sham-operated group		5	3.9 \pm 0.3**	–	–
Control group	2 mL/kg	5	39.6 \pm 1.1	–	–
Nimodipine	5	5	12.2 \pm 1.7**	21.8 \pm 0.9**	27.9 \pm 0.9**
YGY-E	4	5	11.1 \pm 0.2**	21.5 \pm 0.7**	23.8 \pm 2.2**

TUNEL: TdT-mediated dUTP-biotin nick end labeling; Data are presented as mean \pm S.E.M.; ** $p < 0.01$ vs. control group.

reduced the infarct ratio with no statistical significance ($p > 0.05$). YGY-E (4 and 8 mg/kg) delivered at 1, 3, and 6 h after cerebral ischemia significantly decreased the infarct ratio ($p < 0.05$ or $p < 0.01$). This effect was dose-dependent in the dose range from 2 to 8 mg/kg.

Compared with control group, the neurological function was significantly improved by nimodipine administered at 1 and 3 h after ischemia (Table 1). Nevertheless, the deficit scores were not significantly reduced by nimodipine administered at 6 h after ischemia. YGY-E (2 to 8 mg/kg) delivered at 1, 3, and 6 h after cerebral ischemia significantly improved neurological function in a dose-dependent manner.

3.2. Effect of YGY-E on neuronal apoptosis in cerebral ischemic rats

The cells were scored as apoptotic if TUNEL-staining is positive (brown staining), according to 5 fields observed at $\times 200$ magnification in the infarct region. The percentage of TUNEL-positive cells was remarkably decreased ($p < 0.01$) in all three YGY-E-treated subgroups compared with the control group (Figure 3 and Table 2). Fewer apoptotic cells were detected in the nimodipine group, as well. However, the effect of nimodipine was weaker than YGY-E.

3.3. Effect of YGY-E on Bcl-2 and Bax levels in ischemic brain tissue of rats

Compared with the control group, YGY-E administered at different time points after ischemia significantly increased the percentage of Bcl-2 positive cells ($p < 0.01$), indicating that YGY-E promotes the synthesis of Bcl-2 (Figure 4 and Table 3).

YGY-E remarkably decreased the percentage of Bax positive cells ($p < 0.01$), suggesting that it inhibits the synthesis of Bax (Figure 5 and Table 4). Nimodipine also reduced the percentage of Bax positive cells, but to a lesser extent compared with the YGY-E groups (Table 4).

4. Discussion

Focal cerebral ischemia is a common cause of death and serious long-term disabilities in many countries. It is caused by a sudden interruption of the blood supply to the brain (21). The mechanisms of ischemic neuronal injury include not only energy exhaustion, acidosis,

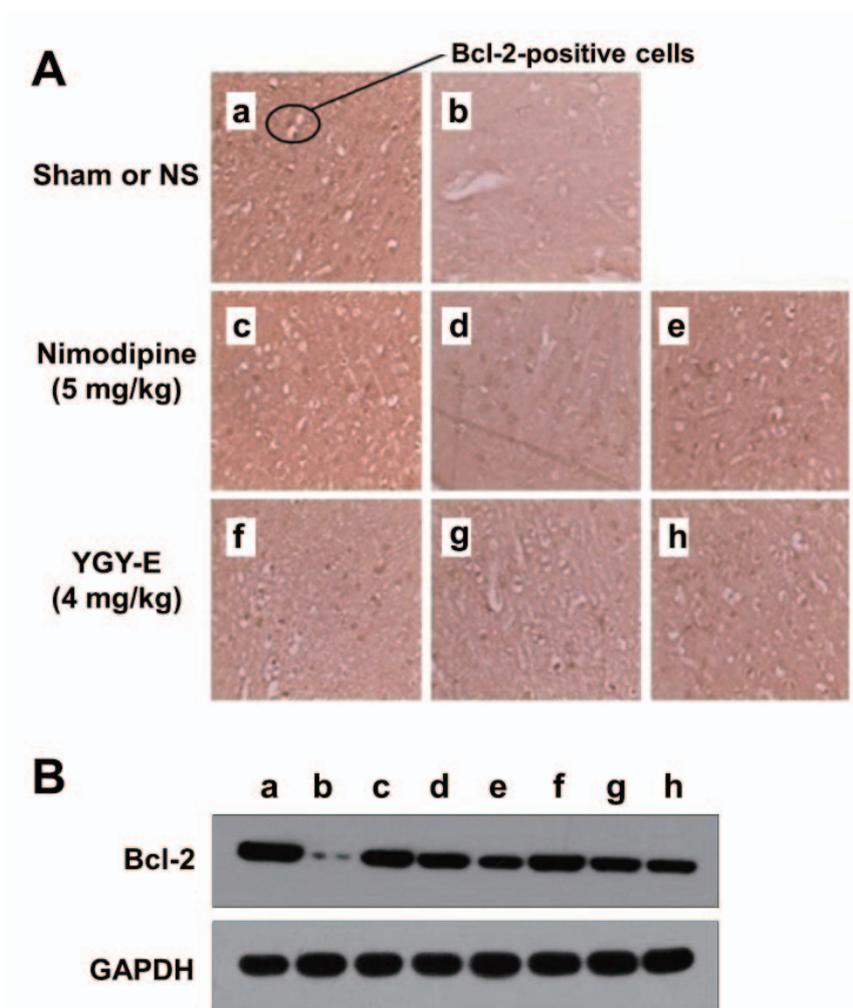


Figure 4. Effect of YGY-E on Bcl-2 expression in rat ischemic brain tissues. Twenty-four hours after cerebral ischemia, the brains tissues were subjected to Bcl-2 detection by immunohistochemical (A) and Western blot (B) analyses. **a**, sham-operated group; **b**, NS (2 mL/kg); **c-e**, nimodipine groups (5 mg/kg) administered at 1, 3, and 6 h after ischemia, respectively; **f-h**, YGY-E groups (4 mg/kg) administered at 1, 3, and 6 h after ischemia, respectively. Magnification in immunohistochemical analysis, $\times 200$.

Table 3. Effect of YGY-E on the expression of Bcl-2 in rat ischemic brain tissue

Group	Dosage (mg/kg)	n	Bcl-2 positive cells (%) (Immunohistochemical detection)			Expression level of Bcl-2 ^a (Western blot analysis)		
			1 h	3 h	6 h	1 h	3 h	6 h
Sham-operated group		5	23.3 \pm 0.3**	–	–	1.31 \pm 0.02*		
Control group	2 mL/kg	5	12.0 \pm 0.4	–	–	0.33 \pm 0.01*		
Nimodipine	5	5	20.0 \pm 0.3**	17.6 \pm 0.3**	15.8 \pm 0.7**	0.88 \pm 0.02*	0.68 \pm 0.03*	0.51 \pm 0.03*
YGY-E	4	5	22.5 \pm 0.2**	20.9 \pm 0.2**	17.5 \pm 0.5**	0.97 \pm 0.03**	0.69 \pm 0.02**	0.64 \pm 0.02*

^a The expression level of Bcl-2 (%): the density of electrophoretic strip of Bcl-2/the density of electrophoretic strip of GAPDH (%); Data are presented as mean \pm S.E.M.; * $p < 0.05$, ** $p < 0.01$ vs. control group (NS).

cellular ion imbalance, intracellular Ca^{2+} increase, but also apoptosis, inflammation, and mitochondrial damage.

Current treatments for ischemic strokes include intravenous thrombolytics, endovascular approaches, anticoagulation, neuroprotection, anti-platelet aggregation, *etc.* (21,22). Even if the efficacy of some treatment strategies has been proven, the number of acute stroke patients successfully treated remains disappointingly low because of the narrow therapeutic

time window for each approach. In ischemic brain injury, the tissue of the ischemic region center would turn to necrosis 10 min after arterial occlusion meanwhile a strip of neurons and edema in the surrounding areas remain recoverable through the collateral circulation, known as penumbra. Ischemic brain tissue in penumbra is reversible within 3-6 h after symptom onset. However, after this time period, it will cause irreversible damage and severe degeneration or even necrosis, infarction expansion, and nerve injury

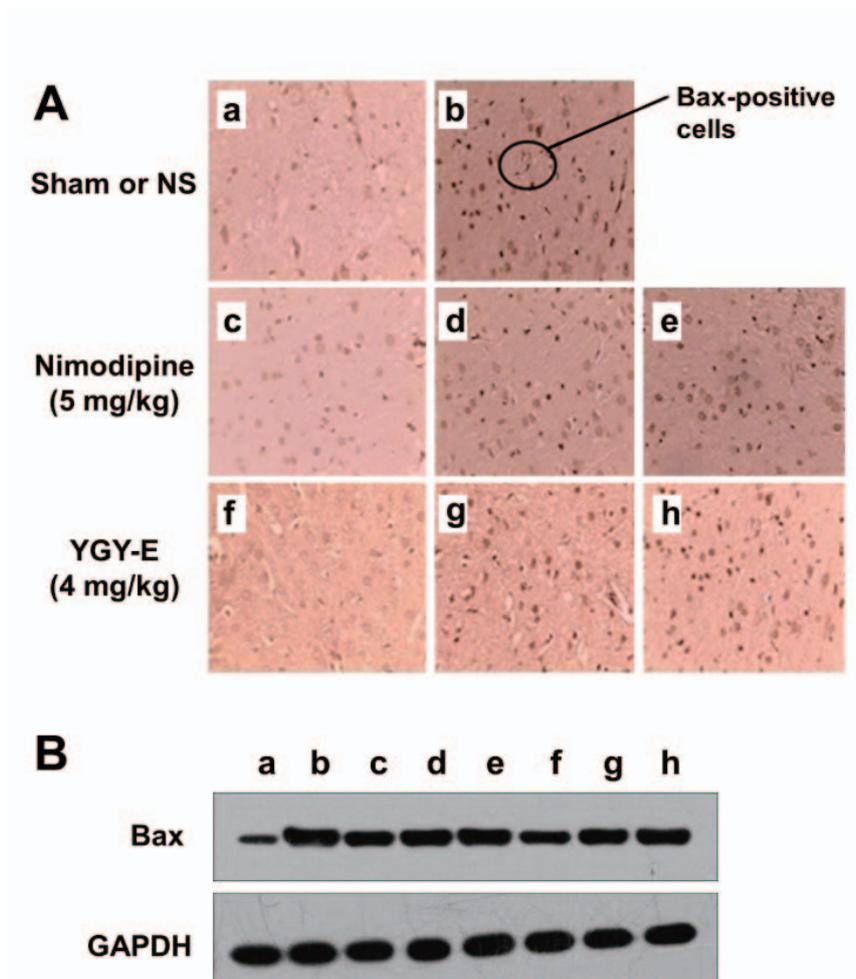


Figure 5. Effect of YGY-E on Bax expression in rat ischemic brain tissues. Twenty-four hours after cerebral ischemia, the brains were subjected to Bax detection by immunohistochemical (A) and Western blot (B) analyses. **a**, sham-operated group; **b**, NS (2 mL/kg); **c-e**, nimodipine groups (5 mg/kg) administered at 1, 3, and 6 h after ischemia; **f-h**, YGY-E groups (4 mg/kg) administered at 1, 3, and 6 h after ischemia. Magnification in immunohistochemical analysis, $\times 200$.

Table 4. Effect of YGY-E on the expression of Bax in rat ischemic brain tissue

Group	Dosage (mg/kg)	n	Bax positive cells (%) (Immunohistochemical detection)			Expression level of Bax ^a (Western blot analysis)		
			1 h	3 h	6 h	1 h	3 h	6 h
Sham-operated group		5	1.97 \pm 0.05**	–	–	0.33 \pm 0.01*		
Control group	2 mL/kg	5	6.12 \pm 0.68	–	–	1.10 \pm 0.03*		
Nimodipine	5	5	3.21 \pm 0.28**	5.56 \pm 0.23*	6.35 \pm 0.15**	0.54 \pm 0.03**	0.78 \pm 0.03*	0.92 \pm 0.04*
YGY-E	4	5	3.43 \pm 0.24**	5.46 \pm 0.26**	6.03 \pm 0.20*	0.55 \pm 0.02**	0.64 \pm 0.02*	0.84 \pm 0.02*

^a The expression level of Bax (%): the density of electrophoretic strip of Bax/the density of electrophoretic strip of GAPDH (%); Data are presented as mean \pm S.E.M.; * $p < 0.05$, ** $p < 0.01$ vs. control group (NS).

aggravation (23-25). Therefore, "therapeutic time window" is critical in determining the effectiveness of clinical treatment of ischemia. The central challenge for enhancing drug efficacy seems to be developing a new generation of medicine with a longer therapeutic time window. Our results show that YGY-E was effective as long as 6 h in permanent focal cerebral ischemia at the doses used in rats, suggesting that the medicine has a better clinical value.

Apoptosis is important in maintaining a stable

cellular environment, which involves a series of gene activation, expression and regulation events, and it plays an important role in hypoxic-ischemic brain injury by acting as an important form of delayed neuronal death (26). The Bcl-2 protein family, a principal regulator of mitochondrial membrane integrity and function, is classified into the following three subgroups based on their structural homology: *i*) anti-apoptotic proteins including Bcl-2, Bcl-XL, and Bcl-w; *ii*) pro-apoptotic proteins including Bax and Bak; and *iii*) BH3-only

proteins (27-29). The anti-apoptotic effect of Bcl-2 relies on its prevention of cytochrome c release into the cytoplasm (30). Thus, regulation of the expression of Bcl-2 related proteins provides an important target for developing drugs of anti-ischemic neuronal apoptosis (31). Bax is oligomerized and activated after interacting with other Bcl-2 family proteins. The activated Bax then triggers release of apoptotic proteins stored in the mitochondrial intermembrane space and leads to neuronal apoptosis (32). Bcl-2 family proteins form homodimers or heterodimers, suggesting that they act through a competitive mechanism to regulate the apoptosis signaling pathway (33). In this study, we demonstrated that YGY-E administered at different time points after ischemia effectively decreased neuronal apoptosis. Consistently, YGY-E increased Bcl-2 positive cells and decreased Bax-positive cells, suggesting that YGY-E suppresses neuronal apoptosis by regulating the balance between Bcl-2 and Bax levels. We propose that YGY-E executes its neuroprotection in focal cerebral ischemia by suppressing neuronal apoptosis.

In conclusion, YGY-E, a novel active ingredient, extracted from Chinese medical herbs had a remarkable neuroprotective action in focal cerebral ischemia. These findings provide solid experimental evidence for development of YGY-E as a promising drug candidate in treating clinical cerebral ischemia.

5. Conclusion

YGY-E has a strong protective action against cerebral ischemia injuries in rats with a therapeutic time window unusually up to 6 h. This neuroprotection may be due to YGY-E's suppression of neuronal apoptosis by promoting Bcl-2 synthesis and inhibiting Bax synthesis.

Acknowledgements

This study was supported by "Traditional Chinese Medicine Modernization Project" (No. 06DZ19711) grants from Shanghai Science and Technology Commission of PR China.

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(Received December 21, 2010; Revised March 22, 2011; Re-revised April 14, 2011; Accepted April 15, 2011)