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Pharmacology, Biochemistry and Behavior 86 (2007) 741 – 748
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
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Protective effects of Nicotiflorin on reducing memory dysfunction, energy metabolism failure and oxidative stress in multi-infarct dementia model rats

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Received 14 May 2006; received in revised form 4 March 2007; accepted 5 March 2007
Available online 14 March 2007

Abstract

The present study aimed to determine whether Nicotiflorin, a natural flavonoid extracted from coronal of *Carthamus tinctorius*, has a protective effect on cerebral multi-infarct dementia in rats. The multi-infarct dementia model rats were prepared by injecting man-made micro-thrombi into the right hemisphere. The administration groups were treated once daily with 30, 60 and 120 mg/kg Nicotiflorin (i.g.) from 5 days before ischemia operation to 3 days after the operation for biochemical examination, 10 days for Morris water maze study and morphological observations and 20 days for eight-arm radial maze task. 2,3,5-triphenyltetrazolium chloride (TTC) staining showed that infarct volume of each Nicotiflorin administration group was much smaller than that of vehicle-treated multi-infarct dementia group, and hematoxylin and eosin (HE) staining showed that histopathological abnormalities of each Nicotiflorin group were also much lighter than that of vehicle-treated multi-infarct dementia group. Each Nicotiflorin group showed much better spatial memory performance in Morris water maze tests and eight-arm radial maze task compared with the vehicle-treated multi-infarct dementia group, significantly attenuated the elevation of lactic acid and malondialdehyde (MDA) contents and the decrease in lactate dehydrogenase (LDH), Na^+K^+ATPase, $Ca^{2+}Mg^{2+}$ATPase and superoxide dismutase (SOD) activity in the brain tissue which was composed of striatum, cortex and hippocampus of the ischemia hemisphere at day 3 after ischemia operation. These results suggest that Nicotiflorin has protective effects on reducing memory dysfunction, energy metabolism failure and oxidative stress in multi-infarct dementia model rats.

Keywords: Nicotiflorin; Multi-infarct dementia; Energy metabolism; Oxidative stress; Flavonoid

1. Introduction

Carthamus tinctorius, a kind of traditional Chinese medicine, has been used extensively for treatment of cerebrovascular and cardiovascular diseases. Its extracts have been widely used as herbal medicine against stroke or vascular dementia, and have been demonstrated to possess neuroprotective actions (Robak and Gryglewski, 1988; Chen et al., 1989, 1990; Hotta et al., 2002; Lee et al., 2002; Tian et al., 2003; Wang et al., 2003; Loo et al., 2004; Jin et al., 2004; Liu et al., 2005; Luo et al., 2004; Zhao et al., 2004). It is possible that Nicotiflorin, a flavonoid extract, may have favorable effects on vascular dementia. Nicotiflorin (kaempferol-3-0-rutinoside), extracted from dried and powdered coronal of *C. tinctorius*, is a yellow amorphous powder soluble in acetoacetate, ethyl alcohol and methyl alcohol. The purity of Nicotiflorin was 99.5% by high performance liquid chromatography (HPLC) analysis. The molecular formula was determined to be $C_{27}H_{30}O_{15}$ and its chemical structure formula is shown in Fig. 1.

The present study aimed to determine neuroprotective effects of Nicotiflorin on vascular dementia in Sprague–Dawley rats. Vascular dementia is the most common form of cognitive deterioration after Alzheimer's disease. To our knowledge, this is the first time for this compound to be tested in the treatment of

Abbreviations: HPLC, high performance liquid chromatography; TTC, 2,3,5-triphenyltetrazolium chloride; HE, hematoxylin and eosin; MDA, malondialdehyde; LDH, lactate dehydrogenase; SOD, superoxide dismutase.

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0091-3057/$ - see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.pbb.2007.03.003

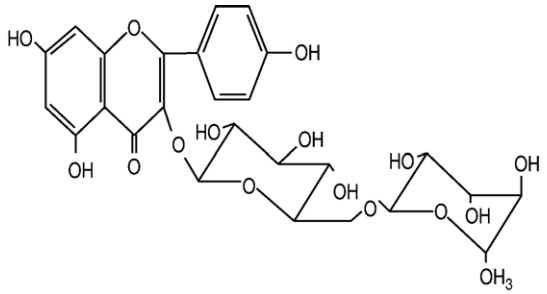

Fig. 1.

vascular dementia. Multi-infarct dementia is a major morphological type of vascular dementia (Hachinski et al., 1974; Jellinger, 2002). The rat model of vascular dementia we employed in the present study is a close mimic of clinical multi-infarct dementia.

Acute cerebral ischemia firstly result in energy metabolism failure with sharp decrease in adenosine triphosphate (ATP) formation (Tian et al., 2005) and activity of lactate dehydrogenase (LDH) and excessive accumulation of lactic acid (Yue et al., 1999; Hoxworth et al., 1999). ATP is a critical energy source for maintaining the ion pumping of Na^+K^+ATPase and $Ca^{2+}Mg^{2+}$ATPase, which regulate the ionic concentration gradients necessary to generate action potentials by neurons (Erecinska and Silver, 1989). The Na^+K^+ATPase and $Ca^{2+}Mg^{2+}$ATPase are very sensitive to even small changes in ATP formation (Mrsic-Pelcic et al., 2002).

The early stage of energy metabolism failure in cerebral ischemia injury can be studied by measuring the levels of lactic acid and activity of LDH and ATPases.

At the same time, energy metabolism failure is also accompanied by oxidative stress-enhanced formation of reactive oxygen radicals (ROS) such as superoxide anion, hydroxyl radical, and hydrogen peroxide in ischemia brain tissues. They attack lipids, protein and DNA of neurons in ischemia brain tissues. The neuronal cell membrane and the mitochondrial membrane constitute with high levels of polyunsaturated fatty acids which can be easily attacked by the ROS. ROS can be scavenged by endogenous antioxidant enzymes such as superoxide dismutase (SOD). Increased free radical formation coupled with a reduced antioxidant defense has been postulated to play a pivotal role in brain injury associated with multi-infarct dementia (Xu et al., 1999; McCord, 1985; Braughler and Hall, 1989).The main metabolite of polyunsaturated fatty acids-MDA accompanied with SOD has also become a significant marker of oxidative stress in ischemia brain tissues (Xu et al., 1999; Vajragupta et al., 2003; Tian et al., 2005).

In the course of screening tests, we attempted to clarify the effect of Nicotiflorin on multi-infarct ischemia-induced impairment of learning and memory in rats using the Morris water maze and the radial eight-arm maze. The effects on ischemia-induced energy metabolism failure and oxidative stress in the striatum, cortex and hippocampus have been determined by contents of LD and MDA and activities of LDH, ATPase and SOD by spectrophotometric assay.

2. Materials and methods

2.1. Isolation and purification (Zhang et al., 2002)

In brief, the dried and powdered coronal of *C. tinctorius* was extracted with 60–80% aqueous ethanol by infiltration. The solvent was evaporated under vacuum to afford crude extract, which was suspended in water and partitioned with petroleum ether, chloroform, ethyl acetate and aqua-saturated *n*-butanol successively. The ethyl acetate partition was subject to chromatography on a Sil–gel column eluting with a gradient mixtures of $CHCl_3$ and MeOH (5 to 50% MeOH) to yield Nicotiflorin.

2.2. Animals

Male Sprague–Dawley rats weighing 220–250 g (the Shanghai Experimental Animal Center of Chinese Academy of Sciences) were used in this study. Animals were allowed to acclimatize for at least 7 days prior to experimentation. Animals were housed at a room temperature of 22±2 °C and a relative humidity of 50±10%. Food and water were available ad libitum. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 80-23, revised 1996).

2.3. Experimental protocol

Three series of experiments were designed in the present study. First protocol is the behavior test, where animals with thrombi-infarct or in the sham operation group were subjected to Morris water maze tests from day 7 to day 10 after the operation and eight-arm radial maze task from day 7 to day 26. The second series of experiments is the determination of biochemical variables including lactic acid and MDA contents, LDH, ATPase and SOD activity in ischemia brain tissue of cortex striatum and hippocampus 3 days after ischemia operation. In the third series of experiments, morphological examination was performed by TTC and HE staining 10 days after the operation.

2.4. Ischemia operation

Preparation of micro-thrombi suspension: 10 ml of blood was obtained from the femoral artery in one male Sprague–Dawley rat and stored at 37 °C homeothermia drying incubator for clot formation for at least 7 days. Grind the clot into micro-thrombi. The micro-thrombi of 50–100 μm in diameter were sieved and were suspended into 6% dextran solution.

Micro-thrombi-induced cerebral infarction was induced by the modified methods of Miyake and Takagi (Takagi and Takeo, 2003; Miyake et al., 1993; Kaneko et al., 1985). In brief, rats were anesthetized (i.p.) with 10% chloral hydrate, 35 mg/100 g. The bifurcation of the right common carotid and external carotid artery was exposed and a temporary clip was applied with bulldog clamp to the external carotid artery just above its origin.

The infarct insult was induced by injecting with a dose of 1 mg/kg micro-thrombi into the internal carotid artery over 30 s through a disposable injection needles. After the needle had been removed, the puncture was repaired with surgical glue. The bulldog clamp occluding the right external carotid was released. The rats that underwent a sham operation were injected with the same volume of vehicle without dry micro-thrombi. Fifteen hours after the operation, the behavior of the operated rats was scored on the basis of paucity of movement, truncal curvature and forced circling during locomotion, which were considered to be typical symptoms of stroke in rodents (Furlow and Bass, 1976; McGraw 1977; Takagi et al., 1997). The score of each item was rated from 3 to 0 (3 very severe, 2 severe, 1 moderate, 0 little or none).

2.5. Administration with Nicotiflorin

The animals were randomly divided into five groups before pretreatment with drugs, three Nicotiflorin-treated groups at doses of 30, 60, and 120 mg/kg, vehicle-treated multi-infarct dementia model and sham operation group. Nicotiflorin, suspended with 0.5% carboxyl methylcellulose, was administered into the stomach by gavage once daily, and the administration was initiated from 5 days before ischemia operation up to 10 days after the operation for the water maze test and morphological observations, 20 days for eight-arm radial task and 3 days for the biochemical examination.

2.6. Morris water maze test

The water maze test was performed according to the methods described previously (Morris et al., 1982). The test was performed on days 7–10 after the operation. All animals were tested in the water maze using a regimen of 4 trials/day. To exclude the rats that could not swim due to injury following operation, we performed the habituation study with the rats in the pool on day 6 after the operation. In the present study, no multi-infarct dementia model rats were ruled out due to a failure in swimming. The water maze apparatus (Shanghai Jiliang Software Technology Co. Ltd) was a circular pool of 180 cm in diameter, containing water of 30 cm in depth and at a temperature of 23 ± 1 °C.

In the escape trial, a black acrylic platform circle of 12 cm in diameter was placed 1.5 cm below the surface of the water and kept in a constant position in the center of one quadrant of the pool. The pool was surrounded by several cues external to the maze. When mounted on the platform, the rat was kept there for 30 s. If the rat did not climb onto the platform, it was transferred onto the platform by hand. Measurements were automated by an on-line video-tracking device (Shanghai Jiliang Software Technology Co. Ltd) designed to track the object in the field, that is, a white rat moving above the black bottom of the pool. Tracking was achieved by the system consisting of a monochrome video camera directly over the center of the pool. The tracker digitized coordinate values were sampled in turn using a personal computer. Escape latency, i.e., the time of climbing onto the platform, was recorded for each trial with a behavioral tracing analyzer. The cut-off time for each trial was set at 120 s. The mean latency of each trial on each day was recorded to find the hidden platform.

And then the rat received a 60-second probe trial in which the platform was removed. Performance of the test animals in each water maze trial was assessed using a personal computer for behavioral analysis. The time was recorded when each rat spent in the quadrant where the platform was located.

2.7. Eight-arm radial maze task (Lee et al., 2005)

The maze consisted of a central platform of 50 cm in diameter, with eight arms extending radially. Each arm was 70 cm in length and 10 cm in width, with clear acrylic sidewalls of 25 cm in height. The maze was located in a room containing many extra-maze visual cues. Radial arm maze task was performed once daily from day 7 after the operation and discontinued one day after each session of 3 days till all five sessions were over. Rats were inhibited to drink water for 23 h prior to the test. Subsequently, the rats were allowed to seek water and drink water for 10 min on the radial maze. The researcher blinded to rats quantified task performance of each rat in the eight-arm radial maze. The trial continued until the test animals entered all eight arms or 10 min had elapsed.

2.8. Biochemical examinations

At day 3 after ischemia operation for preparing the multi-infarct dementia model, the ischemia part of brain tissue including cortex, hippocampus and striatum was isolated for biochemical examinations over an ice cube. After weighing, the isolated brain issue was collected in 0.1 M phosphate buffer (pH 7.4) and homogenized. The homogenate was centrifuged for 30 min at 3000 ×*g* at 4 °C, and the supernatant was used. The content of protein in the supernatant was determined using the Bradford protein assay kit. The procedures quantifying lactic acid, MDA content, ATPase, LDH and SOD activity were carried out according to the description of the assay kits. All kits mentioned above were purchased from Nanjing Jiancheng Bioengineering Institute, China.

2.9. Estimation of cerebral infarct size

Infarct volume of each multi-infarct dementia model rat was analyzed at day 10 after ischemia operation. After anesthetization (i.p.) with 10% chloral hydrate, the brain of each rat was isolated and coronally sectioned into five slices (2 mm thick), and placed in 3% 2,3,5-triphenyltetrazolium chloride at 37 °C for 30 min. The images of brain sections were captured by a digital camera and hemispheric infarct volume was determined by summation of infarct volumes measured in each brain slice using Image-Proplus 5.0 (MEDIA CYBERNETICS, USA). The volumes measured by Image-Proplus program were not absolute value. Therefore, the relative infarct volume percentage was estimated by calculating the cerebral ischemic volume percentage by total stained volume (100 × total infarct volume / total stained volume).

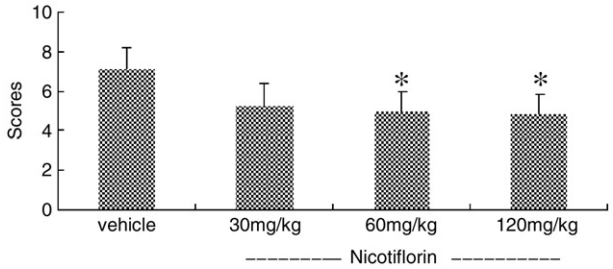

Fig. 2. Effects of Nicotiflorin on attenuating neurological deficits of MID rats. Rats in Nicotiflorin-pretreated groups were given i.g. Nicotiflorin (30, 60, 120 mg/kg) once a day, for 5 days prior to ischemia. Rats in MID group were administered i.g. 0.5% carboxyl methyl cellulose, ($n=20$). Significance was determined by one-way ANOVA followed by Dunnett t-test *$p<0.05$ The Nicotiflorin-treated groups vs. MID vehicle-pretreated group.

2.10. Histopathological examination

In histopathological examination, rats were sacrificed by decapitation, and the brains were taken out and transferred to 10% formalin. Frontal cortex sections were prepared (5 Am thick) and stained by hematoxylin and eosin. Stained sections were evaluated qualitatively (light microscopy) by an examiner blinded to experimental conditions.

3. Statistical analysis

All results were expressed as mean±SEM. Statistical analysis of data was performed by applying one-way analysis of variance (ANOVA) followed by Tukey test for biochemical parameters and infarct volumes, Dunnett t-test was applied for behavioral test. The p values less than 0.05 were considered as statistically significant.

4. Results

4.1. Neurological deficits

The neurological deficits scores of rats at 15 h after ischemia are shown in Fig. 2. The scores of pretreatment with Nicotiflorin (60, 120 mg/kg, i.g.) were significantly lower (5.9±1.1; 5.8±1.0; $p<0.05$) than those of vehicle-treated multi-infarct dementia group (7.1±1.2), but the difference was not significant in those with the low dose of 30 mg/kg administration (6.2±1.2).

4.2. Morris water maze test

Fig. 3A and B summarizes the day 4 results of Morris water tests. Though all rats located the hidden platform at the escape trial, the rats of vehicle-treated multi-infarct dementia group

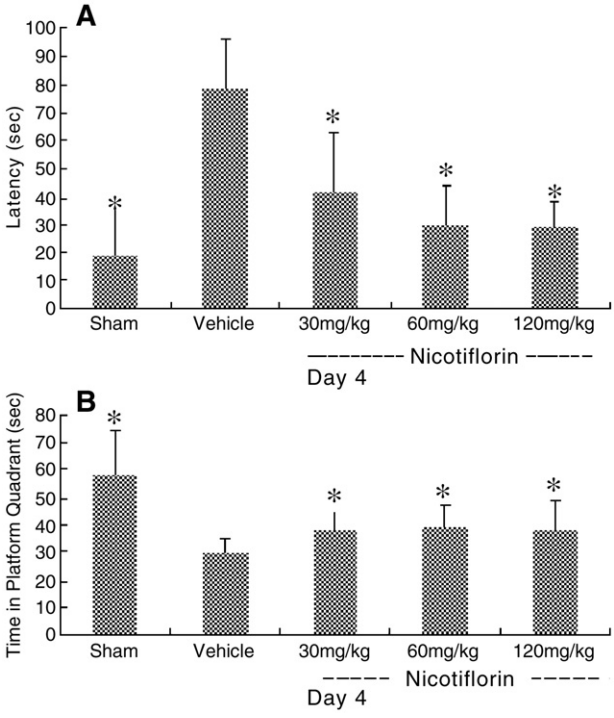

Fig. 3. Results of Morris water maze test. (A) (time in sec) shows the escape latency of each group for the submerged platform in last day of four successive days ($n=10$). *$p<0.05$, the vehicle-treated MID model group vs. sham and Nicotiflorin-treated groups (one-way ANOVA followed by Dunnett t-test); (B) (time in sec) shows the time spent in the quadrant of former platform position in the probe trial test ($n=10$). *$p<0.05$, The sham and Nicotiflorin-treated groups vs. MID vehicle-pretreated group (one-way ANOVA followed by Dunnett t-test).

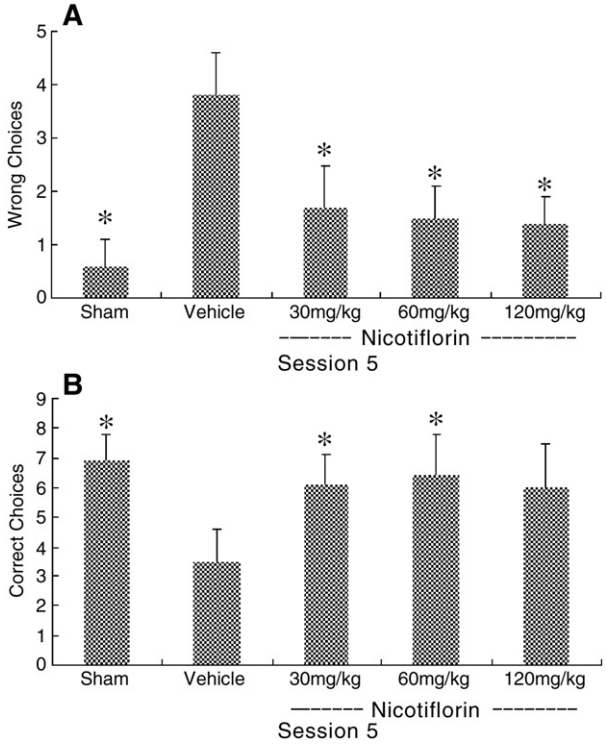

Fig. 4. Results of eight-arm radial maze task. (A) shows wrong choices of each group (i.g., $n=10$) in last session of 5 successive sessions. *$p<0.05$, the vehicle-treated MID model group vs. sham and Nicotiflorin-treated groups (one-way ANOVA followed by Dunnett t-test). (B) shows correct choices of each group (i.g., $n=10$) in the last session of 5 successive sessions. *$p<0.05$, The sham and Nicotiflorin-treated groups vs. MID vehicle-pretreated group (one-way ANOVA followed by Dunnett t-test).

Table 1
Effect of Nicotiflorin treatment (30 mg/kg, 60 mg/kg, 120 mg/kg; i.g.) on biochemical parameters of cortex, hippocampus and striatum of right hemisphere on day 3 after cerebral -ischemic operation ($n=10$)

Group	Lactic acid	LDH	Na^+K^+ATPase	$Ca^{2+}Mg^{2+}$ATPase	MDA	SOD
	mmol/gprot	U/gprot	U/mgprot	U/mgprot	nmol/mgprot	U/mgprot
Sham	0.18±0.06 *	8040±1085 *	6.21±0.98 *	5.42±0.89 *	3.63±0.67 *	64.8±19.1 *
Vehicle	0.36±0.10	5056±1803	2.91±0.82	2.37±0.74	6.28±1.47	45.5±20.0
Nicotiflorin (30 mg/kg)	0.25±0.06 *	6571±1016 *	4.97±1.20 *	4.83±0.77 *	3.94±1.04 *	54.5±15.8
Nicotiflorin (60 mg/kg)	0.24±0.08 *	6948±1752 *	5.63±1.08 *	5.06±0.91 *	3.48±0.78 *	60.0±13.2 *
Nicotiflorin (120 mg/kg)	0.25±0.06 *	6841±1316 *	5.24±0.85 *	4.98±1.14 *	4.39±1.09 *	62.1±18.2 *

Statistical analysis was done by one-way ANOVA followed by Tukey test.
* $p<0.05$, the vehicle-treated MID model group vs. sham or Nicotiflorin-treated groups.

required more time (77.9±17.7 s) than that of sham-operated controls (18.8±15.5 s). Three doses of Nicotiflorin treatment in the present study prevented this delay in escape latency (30 mg/kg: 41.1±21.9 s, 60 mg/kg: 29.6±14.7 s, 120 mg/kg: 29.3±8.1 s). All differed significantly when compared with the vehicle-treated multi-infarct dementia group. Analysis of swimming performance during the probe trial revealed that vehicle-treated multi-infarct dementia model rats spent less time (29.5±5.2 s) in quadrant of former platform position than sham-operated controls (57.3±17.5 s) and three doses of Nicotiflorin-treated rats (30 mg/kg: 38.3±6.8 s, 60 mg/kg: 39.4±8.6 s, 120 mg/kg: 38.2±11.0 s) did. Again, the groups differed significantly.

4.3. Eight-arm radial maze task

At day 17–19, three doses of Nicotiflorin treatment significantly reduced the increased wrong choices performance of dementia model rats and increased the correct choices in this task, as compared with the dementia model rats that received vehicle (Fig. 4A and B).

4.4. Biochemical observations

As shown in Table 1, lactic acid and MDA concentration significantly increased, LDH, Na^+K^+ATPase,$Ca^{2+}Mg^{2+}$ATPase and SOD activity decreased in the ischemia brain issue of cortex, hippocampus and striatum of vehicle-treated multi-infarct dementia model rats as compared to sham-operated controls. Each Nicotiflorin pretreatment significantly brought down increased levels of lactic acid and MDA, and raised the LDH, Na^+K^+ATPase, $Ca^{2+}Mg^{2+}$ATPase and SOD activity of multi-infarct dementia model rats on day 3 after ischemia operation. However, although 30 mg/kg Nicotiflorin treatment increased SOD activity of multi-infarct dementia model rats, the difference see no significant difference when compared with the multi-infarct dementia model rats treated with vehicle.

4.5. Cerebral infarction

In the vehicle-treated multi-infarct dementia group, the cerebral infarction could be found on the cerebral cortex, hippocampus and striatum. Three doses of Nicotiflorin considerably

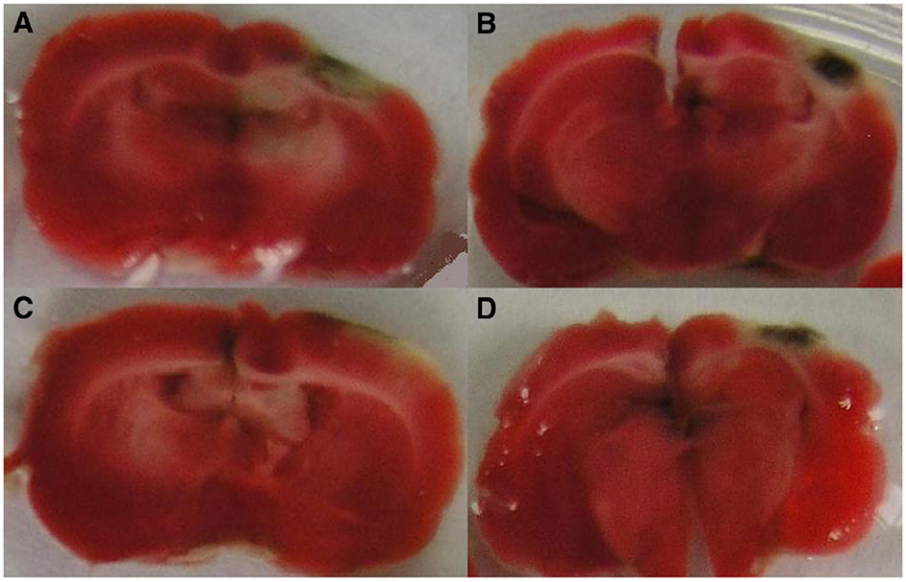

Fig. 5. Representative photographs of cerebral infarction ($n=6$). (A) Animals received the vehicle; (B) Nicotiflorin (30 mg/kg); (C) Nicotiflorin (60 mg/kg); (D) Nicotiflorin (120 mg/kg). The 2 mm thick coronal brain slices following reaction with a 3% TTC showing viable brain tissue in red and infracted brain tissue in white or black.

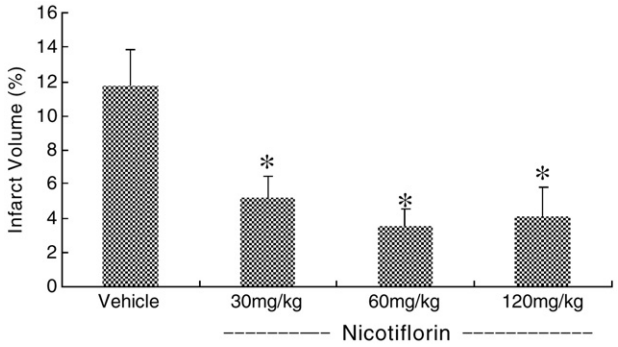

Fig. 6. Attenuation of infarct area by treatment with Nicotiflorin. The infarct size was quantified as described. Values are means ± SEM, $n = 6$. *$p < 0.05$, The sham and Nicotiflorin-treated groups vs. MID vehicle-pretreated group. Statistical analysis was done by one-way ANOVA followed by Tukey test.

reduced the cerebral infarction volume as compared with the vehicle-treated multi-infarct dementia group (Fig. 5A, B, C, D). When the relative percent of the cerebral infarction was compared between the groups, the Nicotiflorin-treated groups (30 mg/kg: 5.2 ± 1.2%; 60 mg/kg: 3.6 ± 0.9%; 120 mg/kg: 4.1 ± 1.7%) exhibited a significant reduction of the cerebral infarction volume compared to the vehicle-treated multi-infarct dementia group (11.7 ± 2.2%) (Fig. 6).

4.6. Histopathological observation

Fig. 7 shows the typical histopathological architecture of the brain in sham-operated animals. The brains subjected to multi-infarct ischemia revealed alterations in brain histology. There was an increase in the number of glial cells, presence of macrophages and inflammatory changes (Fig. 7B). Three doses of

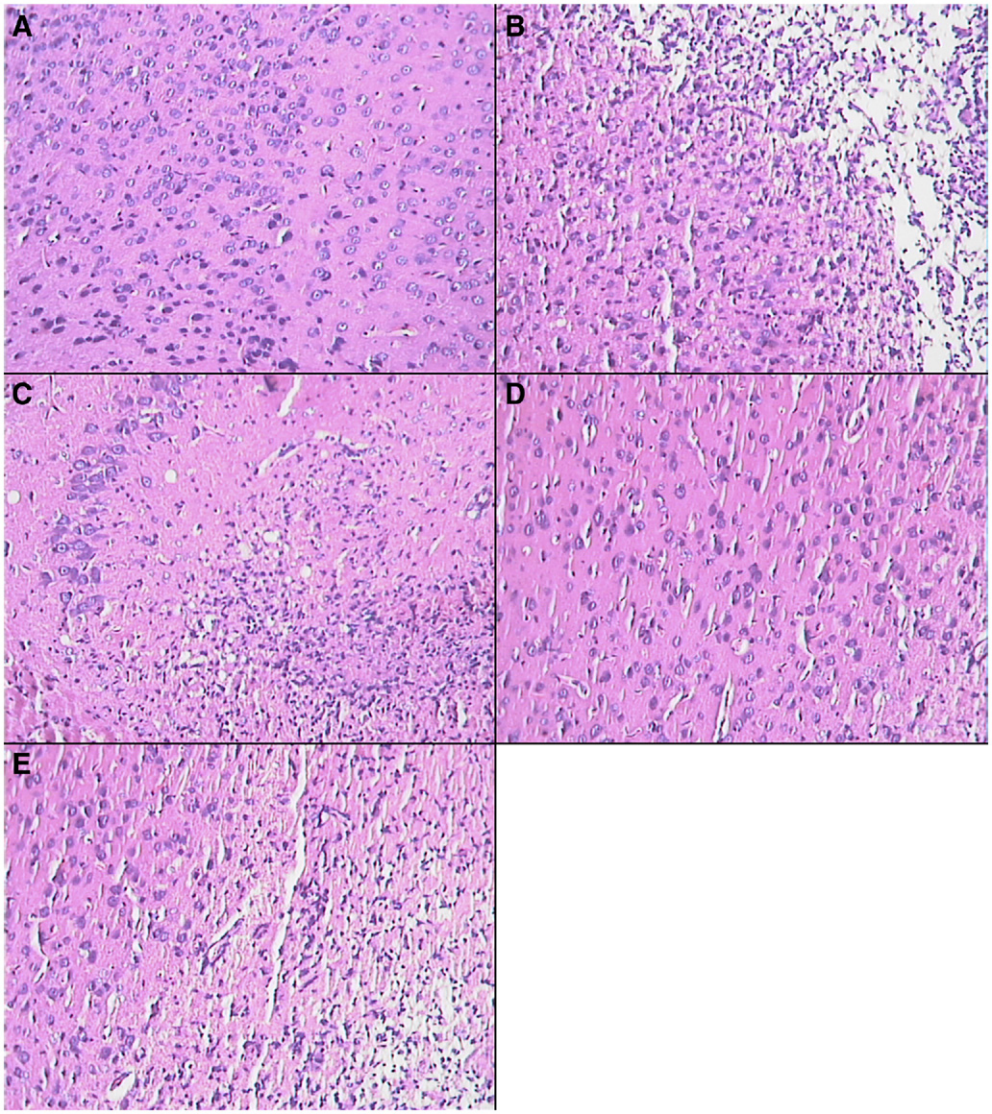

Fig. 7. Representative photographs of histopathological observation (HE × 200) in the rat cortex of each group ($n = 6$). (A) Sham-operated control rats. Note the normal architecture of the rat brain. (B) vehicle-treated MID group rats. Reactive changes in the form of gliosis, inflammatory infiltration, cellular edema and recruitment of macrophages are seen. (C) 30 mg/kg, (D) 60 mg/kg, (E) 120 mg/kg Nicotiflorin-treated MID rats. Note that severity of reactive changes is significantly reduced.

Nicotiflorin treatments in the present study reduced ischemia-induced histological abnormalities, presenting as less inflammatory changes, milder lymphocytic infiltration and less glial cell proliferation (Fig. 7C, D, E).

5. Discussion

We prepared multi-infarct dementia model rats by injecting micro-thrombi into the right hemisphere of the brain. Previous study suggested that vascular dementia was mainly the result of large hemispheral infarcts of over 100 ml of the brain tissue (Loeb et al., 1998). Some researchers chose thrombi of 200 μm in diameter for injecting (Chen et al., 1994; Hu et al., 1998). Recent data indicate that cognitive decline is commonly associated with widespread small ischemic or vascular lesions (microinfarcts) throughout the brain (Jellinger, 2002; Eszter and Paul, 2001). Researchers chose styrene divinybenzene microsphere of 47.5 μm in diameter for injecting (Takagi et al., 2003). But styrene divinybenzene microsphere could not mimic the situation of thrombolysis, which is much important in screen drugs of thrombolysis. The thrombi of 50–100 μm in diameter we employed in the present study were much more feasible to induce widespread, small embolic infarcts, especially in the cerebral cortex, striatum and hippocampus and were a much closer mimic of clinical vascular dementia type of multi-infarct dementia (Kaneko et al., 1985).

The blood forming the thrombi was extracted from the same group of the rats. And our pre-test showed that 1 mg/kg was the best injection dose for preparing the ischemia model in rats. In the pre-test of the present study, 0.5 mg/kg dose of thrombi injection induced average neurological deficits scores of rats in vehicle-treated multi-infarct dementia model group, which were lower than 5 points 15 h after the ischemia operation, while 2 mg/kg dose of injection induced a mortality of over 50%. Mortality and neurological scores of 1 mg/kg injection group were 10% and 7.6±1.3 (unpublished data) respectively.

Previous study about *C. tinctorius* mainly concentrated on its mixed extracts (Loo et al., 2004; Jin et al., 2004; Liu et al., 2005; Hotta et al., 2002; Lee et al., 2002; Luo et al., 2004; Zhao et al., 2004; Tian et al., 2003; Wang et al., 2003). The results suggest that *C. tinctorius* and its extracts have effects of improving functions of cardiac contraction and dilation, increasing coronary blood flow (Zhang and Jiang, 2004), calcium antagonistic effects (Liu et al., 2005) and antioxidant effects (Hotta et al., 2002; Lee et al., 2002; Kanehira et al., 2003). However, further information is scarce concerning the functional roles of a single compound extract from this traditional Chinese medicine.

Intake of flavonoids is inversely associated with risk of stroke and cardiovascular diseases, indicating that it may contribute to vasorelaxant effect, antioxidant and anti-platelet properties of flavonoids (Robak and Gryglewski, 1988; Chen et al., 1990; Takizawa et al., 2003). HSYA, a nature flavonoid extracted from *C. tinctorius* has recently been proved to be a good potential agent to treat focal cerebral ischemia, and the underlying mechanisms exerted by HSYA might be involved in its inhibitory effects on thrombosis formation and platelet aggregation in rats (Zhu et al., 2003, 2005). Our results suggest that Nicotiflorin may also have neuroprotective effects in the treatment of ischemia injury in primary stage of vascular dementia.

In the present study, we scored the neurological deficit by the way of Furlow and McGraw (Furlow et al., 1976; McGraw, 1977) 15 h after the ischemia operation. We found that the scores of all Nicotiflorin pretreatment groups were much lower than that of vehicle-treated multi-infarct dementia group, indicating that pretreatment with Nicotiflorin attenuate neurological deficits induced by thrombi-infarct ischemia.

Morris water maze is a very popular apparatus for testing spatial memory, and eight-arm radial maze for testing the working memory of rodent animals. We employed eight-arm radial maze task with some modification instead of food tablet as bait in the present study (Lee et al., 2005). The results suggest that they are as effective as food tablet. The results of these two behavioral tests show that treatment with Nicotiflorin significantly improved the memory impairment occurred in multi-infarct dementia model rats.

Regarding the biochemical determination, energy metabolism failure occurred in the cortex, striatum and hippocampus ischemic brain hemisphere because of the shortage of oxygen and glucose. As a result, LDH activity decreased and lactic acid accumulated in the ischemic brain tissue. All the activity of ATP-dependent ion channel or enzyme decreased, including $Na^{+}K^{+}$ATPase and $Ca^{2+}Mg^{2+}$ATPase. Treatment of Nicotiflorin reversing all these changes indicates its protective effects on improving energy metabolism. We examined the MDA contents and SOD activity in the brain tissue of cortex, striatum and hippocampus in the right hemisphere of each rat. The results suggest that pretreatment with Nicotiflorin could prevent the increase of MDA level and decrease of SOD activity.

Our findings also provided direct and visible evidence of the existence of an anti-infarction role of Nictoflorin using TTC staining. It is a useful technique for the measurement of cerebral ischemia infarction since TTC can react to mitochondrial cytochrome oxidases in the visible tissue to produce a deep red color, whereas the infracted, dead tissue cannot been stained. As found in the present study, multi-infarct ischemia induced an infarction with a volume of 11.7±2.2% in the brain. Treatments of different doses of Nicotiflorin significantly decreased the infarction volume (30 mg/kg: 5.2±1.2%; 60 mg/kg: 3.6±0.9%; 120 mg/kg: 4.1±1.7%).

Histopathological changes, such as the increase of the number of glial cells, presence of macrophages and inflammatory changes reflect ischemia-induced abnormalities in rat brain. The microglia is rapidly activated in response to pathological stimuli and plays an important role in immune defense mechanism of brain. The microglia-derived pro-inflammatory cytokines such as IL-1h and TNF-α may be detrimental and may then cause secondary damage to nervous tissue. The presently observed reactive morphological changes in multi-infarct dementia model rats reflect the negative outcome of multi-infarct ischemia, and treatments of three doses of Nicotiflorin significantly reduced the proliferation of the microglia and other abnormalities in fontal cortex of ischemia hemisphere.

In summary, in the present study we found that Nicotiflorin attenuates memory dysfunction, energy metabolism failure and

oxidative stress induced by multi-infarct ischemia injury. In addition, it also reduces infarct volume and inflammatory reactions caused by multi-infarct ischemia. Nicotiflorin can therefore inhibit a number of deleterious processes known to be involved in ischemia damage, which can explain its marked neuroprotective effects on multi-infarct dementia.

Acknowledgement

Thanks for the funding support from National High-tech R&D Program (863 Program).

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