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Research Article

Studies on the Protective Effects of Scutellarein against Neuronal Injury by Ischemia through the Analysis of Endogenous Amino Acids and Ca^{2+} Concentration Together with Ca^{2+}-ATPase Activity

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Scutellarin, which is extracted from the dried plant of *Erigeron breviscapus*, has been reported to protect the neural injury against excitotoxicity induced by ischemia. However, there are a few studies on the protective effects of scutellarein, which is the main metabolite of scutellarin in vivo. Thus, this study investigated the neuroprotective effects of scutellarein on cerebral ischemia/reperfusion in rats by bilateral common carotid artery occlusion (BCCAO) model, through the analysis of endogenous amino acids using HILIC-MS/MS, and evaluation of Ca^{2+} concentration together with Ca^{2+}-ATPase activity. The results showed that scutellarein having good protective effects on cerebral ischemia/reperfusion might by decreasing the excitatory amino acids, increasing the inhibitory amino acids, lowing intracellular Ca^{2+} level, and improving Ca^{2+}-ATPase activity, which suggested that scutellarein might be a promising potent agent for the therapy of ischemic cerebrovascular disease.

1. Introduction

Ischemic cerebrovascular disease is the main cause of disability and death among the elderly people [1]. The increasing evidences showed that the excessive release of various amino acid neurotransmitters is one pathogenesis during ischemia reperfusion [2], indicating that the amino acid levels could be diagnostic markers. The depolarization is mediated by the metabolic failure which is caused by ischemia, and the results indicate that there is an influx of Ca^{2+} via voltage-sensitive Ca^{2+} channels, thus initiate a flood of amino acid neurotransmitters, especially glutamate and aspartic acid [3], release into the synaptic cleft. The excitatory amino acids, which are the most important amino acid neurotransmitters, could induce a cascade of events leading to cell death [4, 5]. However, γ-aminobutyric acid (GABA), as an inhibitory amino acid, plays a neuroprotective role in vivo [6, 7]. Thus, the determination of these amino acid neurotransmitters is very important to evaluate the global cerebral ischemic injury.

Erigeron breviscapus (Vant.) Hand.-Mazz., which mainly grows in Yunnan province of China, has been used for centuries as an important Chinese traditional herbal medicine to treat ischemic cerebrovascular diseases. Previous studies have demonstrated that *breviscapine*, which is extracted from the dried whole plant of *Erigeron breviscapus*, has neuroprotective effect against glutamate-induced excitotoxicity, by inhibiting the accumulation of intracellular Ca^{2+} and upregulating X-linked inhibitor of apoptosis protein (XIAP) expression in hippocampal neurons [8]. Tao et al. found that the *breviscapus* ethanol extract could inhibit GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH) in the brain tissue to increase the GABA level

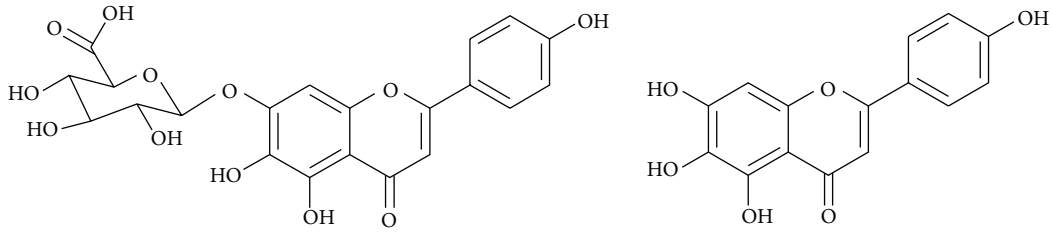

Figure 1: The chemical structures of scutellarin and scutellarein.

[9]. Scutellarin (Figure 1), the major bioactive constituent in *breviscapine*, possesses potent pharmacological effects similar to those of the herb. Many studies showed that scutellarin had a markedly neuroprotective activity against ischemia-induced injury by its anti-excitotoxicity, blocking Ca^{2+} channels, scavenging of reactive oxygen species, and so forth [10, 11]. Interestingly, some researchers found that scutellarin was mainly absorbed in the form of its hydrolyzed product scutellarein (Figure 1) by intestines [12], and scutellarein was much easier to be absorbed with the triple bioavailability, after oral administration of these two compounds in equal amount [13]. A recent research indicated that scutellarein had better protective effect than scutellarin in rat cerebral ischemia [10]. However, there are a few studies on the protective effect of scutellarein against excitotoxicity, which is induced by excitatory neurotransmitters. Therefore, the aim of this study was to evaluate the possible protective effect of scutellarein against neuronal injury, through the analysis of endogenous amino acids, and Ca^{2+} concentration together with Ca^{2+}-ATPase activity.

2. Materials and Methods

2.1. Materials. Scutellarein was prepared according to our previous procedure [14–16]. Chemicals standards of phenylalanine (Phe), γ-aminobutyric acid (GABA), leucine (Leu), valine (Val), methionine (Met), taurine (Tau), alanine (Ala), hydroxyproline (H-pro), glycine (Gly), glutamic acid (Glu), glutamine (Gln), serine (Ser), asparagines (Asn), citrulline (Cit), aspartic acid (Asp), arginine (Arg), and lysine (Lys) were purchased from Sigma-Aldrich (St. Louis, MO). The acetonitrile and formic acid were all of HPLC grade and purchased from Merck (Darmstadt, Germany). Ammonium formate (analytical grade) was purchased from the Shanghai Chemical Reagent Factory (Shanghai, China). Pure water for UHPLC analysis was purified using a Milli-Q water purification system (Millipore, Billerica, MA) and used for all solutions and dilutions. Other reagents and chemicals were of analytical grade.

All male Wister rats (240 ± 20 g, clean grade, Certification SCXK 2009-0001) were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). All rats were bred on 12 h dark-light cycle, with temperature of 23 ± 2°C and humidity of 60 ± 5%, and they have free access to the food and water. The rats were cared in accordance with the Guide for the Care and Use of Laboratory Animals (US National Research Council, 1996) and the related ethics regulations of our university.

2.2. Drug Administration and Surgery. The rats were randomly divided into six groups (n = 6/group) including sham-operation group, bilateral common carotid artery occlusion (BCCAO) rats without pretreatment (model group), and BCCAO rats pretreated with scutellarein (0.09, 0.17, 0.35 mmol/kg). The rats in the treatment groups were intragastrically administrated with scutellarein under body weight for 6 consecutive days, while the rats in the sham-operated and model group were intragastrically administrated with equal volume of 0.25% CMC-Na; 10% of choral hydrate (350 mg/kg ip) was used for anesthesia on the seventh day. The bilateral common carotid arteries were exposed and ligated with 0 thread for 30 min to induce cerebral ischemia; following cerebral ischemia, reperfusion was achieved by declamping the arteries for 15 min. Then repeated ischemia was allowed for 30 min and reperfusion was allowed for 22 h. While the rats which only had the bilateral isolation of bilateral common carotid arteries (without occlusion) served as the sham-operated group. Anesthetized rats were placed on a heating pad during recovery from anesthesia to maintain the body temperature at 37.0 ± 0.5°C after surgery [17, 18].

2.3. Preparation of Standard Solutions. A mixed standard solution containing 17 analytes was dissolved in water, and the concentrations of these analytes were shown as follows: GABA, 23.2 μg/mL; Gly, 51.6 μg/mL; Glu, 158 μg/mL; Tau, 20.4 μg/mL; Asp, 16.8 μg/mL; Leu, 172.0 μg/mL; Met, 20.0 μg/mL; Phe, 21.6 μg/mL; Ala, 122.0 μg/mL; Cit, 24.0 μg/mL; Gln, 19.2 μg/mL; Asn, 31.6 μg/mL; Ser, 233.6 μg/mL; Val, 21.6 μg/mL; H-pro, 32.8 μg/mL; Lys, 22.4 μg/mL; Arg, 22.8 μg/mL. These mixed standard solutions were then diluted to appropriate concentration for building calibration curves. The standard solutions were filtered through a 0.22 μm membrane prior to injection.

2.4. Preparation of Sample Solutions. All rats were anesthetized with 10% choral hydrate and then sacrificed by decapitation. The complete cerebrum was removed from the skull, and the hippocampus tissue was carefully isolated; subsequently, the tissues were stored at −80°C. 0.1 g ischemia

tissue was homogenized in appropriate cold normal saline. The obtained supernatants were centrifuged at 3000 rpm for 10 min at 4°C. After vortex for 2 min and centrifugation at 13000 rpm for 10 min, the upper layer was transferred into another tube, and was then added 3 times of methanol. The concentrate was evaporated to dryness in a rotary evaporator at 25°C and then redissolved in 200 μL of 50% acetonitrile in water using vortex-mixing for 3 min; after being centrifuged at 13000 rpm for 15 min at 4°C, the upper layer was filtered through a 0.22 μm membrane prior to injection.

2.5. HILIC-UPLC–TQ-MS/MS Analysis Conditions. Chromatographic experiments were determined by a Waters ACQUITY UPLC system (Waters, Milford, MA) equipped with a binary solvent delivery system and autosampler. ACQUITY UPLC BEH column (2.1 mm × 100 mm, 1.7 μm, Waters) was applied for all analysis. A gradient of 10 mM ammonium formate and 0.15% formic acid in water (solvent A) and 2 mM ammonium and 0.15% formic acid in acetonitrile was used. A linear gradient elution was as follows: initial–6 min, 15–20% A; 6–10 min, 20–30% A; 10–12 min, 30–40% A; then the column was equilibrated for 6 min in the initial conditions. The flow rate of the mobile phase was set to 0.4 mL/min, an aliquot of 5 μL sample solution was injected into the BEH column, and the column temperature was maintained at 35°C. Mass spectrometry was carried out on a Waters Xevo TQ tandem quadrupole mass spectrometer (Micromass MS Technologies, Manchester, UK) using an ESI source operated in positive ion mode. The parameters in the source were set as follows: capillary voltage, 3.0 kV; source temperature, 150°C; desolvation temperature, 550°C; cone gas flow, 50 L/h; desolvation gas flow, 1000 L/h. The analyte detection was performed by using multiple reactions monitoring (MRM). The cone voltage and collision energy were optimized for each analyte. The dwell time was automatically set by the software.

2.6. Measurements of Ca^{2+} Concentration and Ca^{2+}-ATPase Activity. All rats were anesthetized with 10% choral hydrate and sacrificed by decapitation. The brain was separated and placed on ice; the brain cortex tissue was carefully isolated and homogenized with ice-cold normal saline to be 10% (w/v) homogenates and then stored at −80°C until assays were performed. The Ca^{2+} concentration and Ca^{2+}-ATPase activities were, respectively, determined using appropriative detection kits. Analysis of variance was used to determine the difference in Ca^{2+} concentration and the Ca^{2+}-ATPase activity. Repeated measurement or multivariate analysis of variance was used to compare the amino acids concentrations. All results were expressed as mean ± S.D, and a value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of Scutellarein on the Contents of Amino Acids. It was difficult to simultaneously analyze the endogenous amino acids in biological samples, because of their structure diversity, high polarity, and the absence of specific chromophores. In the past decade, many analytical methods have been used and they can be classified into two categories, the first approach for analysis of amino acids was the derivation method applying the reversed-phase high-performance liquid chromatography (RP-HPLC) or capillary electrophoresis (CE) and gas chromatography (GC) coupled with optical or MS detection. This derivation method used orthophthaldehyde (OPA), phenyl isothiocyanate (PITC), 9-fluorenylmethyl chloroformate (FMOC), and 1-fluoro-2,4-dinitrobenzene (DNFB) as derivation agents. Unfortunately, most of these derivation methods have some disadvantages including instable derivative, low yield, interferences caused by the reagent, or time-consuming derivatization procedures [19–22]. Another approach is ion-exchange HPLC coupled with electrochemical detection and CE-MS methods without derivatization. Most of these methods suffered from some drawbacks including lack of analyte specificity, low throughput, and comparatively poor reproducibility [19]. Our group [23] developed a sensitive and rapid method for the simultaneous determination of amino acids in fruits of *Ziziphus jujuba* without derivatization using hydrophilic interaction liquid chromatography coupled with tandem mass spectrometry (HILIC-MS/MS). The quantification of endogenous amino acids in brain tissues without derivatization had some advantages because it avoids instable derivatives and reagent interferences. So the 17 endogenous amino acids including Phe, GABA, Leu, Val, Met, Tau, Ala, H-pro, Gly, Glu, Gln, Ser, Asn, Cit, Asp, Arg, and Lys were analyzed by HILIC-MS/MS in this research.

The content changes of the neurotransmitter amino acids in hippocampus were shown in Table 1. It was found that Glu, Tau, Asp, Leu, Met, Phe, Cit, Gln, H-pro, Lys, and Arg in the model group changed significantly in comparison to the sham-operated group, such as the Glu content in the Sham group being 42.66 μg/g; however, the Glu content in the model group was 85.52 μg/g. From Table 1, it was clearly showed that 0.09 mmol/kg scutellarein could decrease significantly the contents of excitatory amino acids including Gly, Glu, Asp, Leu, Met, Phe, Ala, Cit, Gln, Asn, Val, H-pro, Lys, and Arg by 3.863, 69.72, 4.737, 19.93, 4.897, 16.85, 23.10, 2.391, 134.5, 4.391, 9.013, 3.122, 3.832, and 8.831 μg/g, respectively, compared with the model group. By contrast, 0.09 mmol/kg scutellarein significantly increased the content of inhibitory amino acid such as GABA by 121.8 μg/g. After treatment with scutellarein, the contents of Glu, Asp, Leu, Met, Phe, Cit, Gln, Asn, Val, H-pro, Lys, and Arg decreased greatly (68.95, 3.641, 17.73, 3.845, 15.71, 2.122, 126.4, 3.472, 8.912, 2.821, 3.553, and 8.262 μg/g, resp.) at the dose of 0.17 mmol/kg and fell slightly at the dose of 0.35 mmol/kg. In contrast, the GABA and Tau contents significantly increased and reached the highest level (135.9 μg/g and 112.1 μg/g) at the dose of 0.35 mmol/kg.

3.2. Determination of Ca^{2+} Concentration and Ca^{2+}-ATPase Activity. The Ca^{2+} concentration and Ca^{2+}-ATPase activities were, respectively, determined using appropriative detection kits. Results presented in Table 2 showed the Ca^{2+}

Table 1: Effects of scutellarein on contents of 17 amino acid neurotransmitters in brain of cerebral ischemic/reperfusion rats (μg/g, $\bar{x} \pm s$, $n = 6$).

Analyte	Sham (μg/g)	Model (μg/g)	Scutellarein (0.09 mmol/kg)	Scutellarein (0.17 mmol/kg)	Scutellarein (0.35 mmol/kg)
GABA	111.6 ± 0.2579	119.9 ± 0.8276$^{\triangle\triangle}$	121.8 ± 1.263*	124.1 ± 1.191**	135.9 ± 6.187*
Gly	3.332 ± 0.1681	4.607 ± 0.2127$^{\triangle}$	3.863 ± 0.1112*	3.961 ± 0.2612*	3.558 ± 0.1295**
Glu	42.66 ± 4.420	85.52 ± 5.436$^{\triangle}$	69.72 ± 3.356*	68.95 ± 11.10	61.80 ± 4.438**
Tau	55.86 ± 3.225	96.11 ± 2.247$^{\triangle\triangle}$	81.11 ± 2.153	91.54 ± 3.421*	112.1 ± 5.386*
Asp	2.413 ± 0.3432	5.214 ± 0.2840$^{\triangle\triangle}$	4.737 ± 0.2623	3.641 ± 0.2933*	3.133 ± 0.2145**
Leu	11.35 ± 1.211	21.84 ± 1.342$^{\triangle\triangle}$	19.93 ± 0.8321**	17.73 ± 1.231**	15.50 ± 2.320**
Met	3.432 ± 0.1210	5.041 ± 0.2140$^{\triangle\triangle}$	4.897 ± 0.3254**	3.845 ± 0.0231*	3.792 ± 0.1100*
Phe	10.08 ± 2.113	18.92 ± 2.513	16.85 ± 2.653*	15.71 ± 1.216**	12.53 ± 0.3245**
Ala	18.78 ± 2.165	25.52 ± 3.245$^{\triangle}$	23.10 ± 0.4265*	24.17 ± 3.254**	20.00 ± 0.6530
Cit	1.813 ± 0.2311	2.562 ± 0.4301$^{\triangle}$	2.391 ± 0.1254**	2.122 ± 0.4322*	2.023 ± 0.3251**
Gln	107.1 ± 3.248	140.7 ± 1.342$^{\triangle}$	134.5 ± 6.231**	126.4 ± 4.563*	117.0 ± 3.258*
Asn	3.072 ± 0.1281	4.713 ± 0.4381$^{\triangle}$	4.391 ± 0.4351	3.472 ± 0.1043**	3.533 ± 0.2549**
Ser	27.13 ± 1.151	29.44 ± 1.432	28.57 ± 4.324	28.95 ± 3.980*	28.38 ± 1.543**
Val	8.201 ± 0.3505	9.192 ± 0.5319$^{\triangle}$	9.013 ± 0.5600**	8.912 ± 0.5341**	8.732 ± 0.4533**
H-pro	2.301 ± 0.2321	3.303 ± 0.5432$^{\triangle}$	3.122 ± 0.1120**	2.821 ± 0.2004**	2.573 ± 0.1043*
Lys	1.562 ± 0.2210	4.071 ± 0.2311$^{\triangle\triangle}$	3.832 ± 0.1240*	3.553 ± 0.1043	2.231 ± 0.2034**
Arg	4.582 ± 0.1211	10.65 ± 0.3218$^{\triangle}$	8.831 ± 0.5421**	8.262 ± 0.5902**	6.801 ± 0.6420**

Model groups compared with their sham groups, respectively ($^{\triangle}P < 0.05$, $^{\triangle\triangle}P < 0.01$), compared with model groups ($^{**}P < 0.01$, $^{*}P < 0.05$).

Table 2: The content of Ca^{2+} and the activity of Ca^{2+}-ATPase in ischemia tissue.

	Ca^{2+} (mmol/L)	Ca^{2+}-ATPase (molpi/mgprot/hour)
Model group	34.13 ± 6.873$^{\triangle\triangle}$	4.215 ± 0.4531$^{\triangle\triangle}$
Sham group	12.18 ± 1.268	6.544 ± 0.3465
Scutellarein (0.09 mmol/kg)	29.30 ± 1.439*	4.699 ± 0.3851*
Scutellarein (0.17 mmol/kg)	25.83 ± 3.438*	5.111 ± 0.1554*
Scutellarein (0.35 mmol/kg)	19.21 ± 2.059**	5.917 ± 1.171*

Model groups compared with their sham groups, respectively ($^{\triangle\triangle}P < 0.01$), compared with model groups ($^{**}P < 0.01$, $^{*}P < 0.05$).

concentration and the Ca^{2+}-ATPase activities in the sham-operated group, model group, and ischemic rats treated with various doses of scutellarein. The model group significantly increased the Ca^{2+} concentration in comparison to the sham-operated rats, indicating that ischemic-reperfusion significantly induced Ca^{2+} overload in the BCCAO model rats. After administration of scutellarein, the Ca^{2+} concentration decreased greatly in a dose-dependent manner compared to the model group. Ca^{2+}-ATPase played a critical role in regulation of the intracellular calcium level. In the present study, we found that the activity of Ca^{2+}-ATPase in the model group decreased significantly compared to the sham-operated group. However, the Ca^{2+}-ATPase activity in the scutellarein pretreated group increased in a dose-dependent manner. These results indicated that the protective effect of scutellarein might be due to its cerebral neuroprotection by inhibiting Ca^{2+} overload and enhancing Ca^{2+}-ATPase activity.

4. Discussion

The interstitial contents of neurotransmitter amino acids, especially excitatory amino acids and inhibitory amino acids, are often recognized as an index of neuronal injury. Excitatory amino acids were the important stimulant medium after receptor activation, which can be classified into two categories: the ionotropic receptor and metabotropic receptor, the former includes N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-methyl-4-isoxazole propionic acid (AMPA), and kainate (KA), and the latter includes L-(+)-2-amino-4-phosphonobutyric acid (L-AP4), 1-amino-1,3-dicarboxycyclopentane (ACPD), and L-quisqualic acid (L-QA). During cerebral ischemia, the excitatory amino acids were released pathologically from dying cells [24] and glutamate was recognized as a predominant neurotransmitter involved in the excitotoxicity process [25]. Firstly, the glutamate which was at the postsynaptic membrane bond to AMAP receptor and then led to Na^{+} influx thus resulted in cerebral edema [26]. Simultaneously, Glu activated NMDA receptor leading to the entry of external Ca^{2+} and inositol triphosphate (IP_3) stimuli further induced the release of internal Ca^{2+} that was stored within mitochondria and endoplasmic reticulum [27, 28]. It was widely believed that Ca^{2+}, as a second messenger, played a key role in the development of ischemic cell damage [29, 30]. Ca^{2+}-ATPase, which was an important modulator of the intracellular calcium level, could pump Ca^{2+} back into the endoplasmic

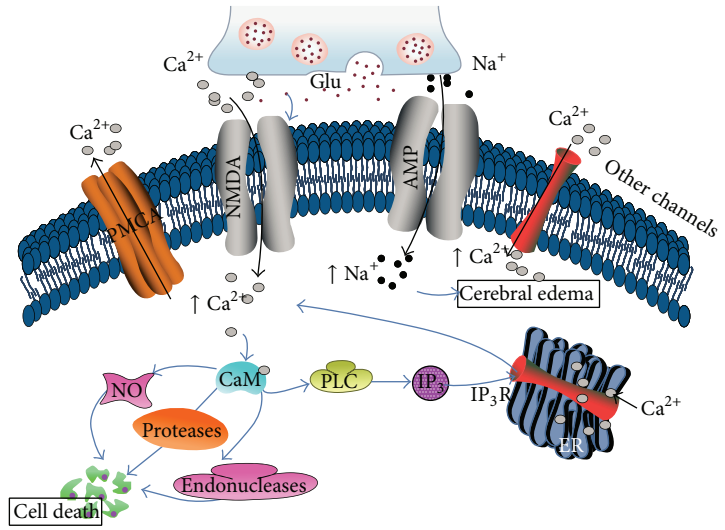

Figure 2: The possible mechanism of excitotoxicity during ischemia/reperfusion.

reticulum against a steep concentration gradient [31]. However, endoplasmic reticulum (ER) calcium homeostasis was disturbed during ischemia, because ATP was needed to fuel the ER Ca^{2+}-ATPase [32]. Intracellular Ca^{2+}-overload could activate calcium-dependent protein kinases, such as calpain, which ultimately destroyed cellular integrity (Figure 2) [33, 34]. In this process, we knew that the inhibition of the release of excitatory amino acids neurotransmitters might be expected to reduce cerebral ischemic injury.

In this study, we clearly found that scutellarein could significantly decreased the contents of excitatory amino acids including Gly, Glu, Asp, Leu, Met, Phe, Ala, Cit, Gln, Asn, Val, H-pro, Lys, and Arg. Furthermore, the contents of the inhibitory amino acids such as GABA and Tau increased significantly after treatment with scutellarein; these results suggested that scutellarein might attenuate the impairment of neuronal by BCCAO [35–37] via decreasing the excitatory amino acids (such as Gly, Glu, and Asp) contents and enhancing the inhibitory amino acids (GABA and Tau) contents. The measurement of the intracellular Ca^{2+} levels and the activity of Ca^{2+}-ATPase in the brain tissues showed that there was sharp rise of Ca^{2+} and great decline of Ca^{2+}-ATPase activity after ischemia/reperfusion; this phenomenon indicated that scutellarein might slow down the influx of Ca^{2+} and reduce the intracellular Ca^{2+} concentration during ischemia.

5. Conclusion

In summary, a reliable, simple, and sensitive method was developed for the identification and quantification of 17 endogenous amino acids in the brain tissue by using UPLC-MS/MS. The Ca^{2+} concentration and the Ca^{2+}-ATPase activity were also evaluated. These results showed that scutellarein might ameliorate the brain injury in the rats of cerebral ischemia reperfusion through decreasing the excitatory amino acids, increasing the inhibitory amino acids, lowing intracellular Ca^{2+} level, and improving Ca^{2+}-ATPase activity. All these results indicated that scutellarein might have good protective effect on cerebral ischemia/reperfusion.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Hao Tang, Ze-Xi Dong, Ting Gu, Qian-Ping Shi, and Peng-Xuan Zhang performed the experiments. Nian-Guang Li and Jian-Ming Guo designed the experiments. Hao Tang and Nian-Guang Li wrote the paper. Yu-Ping Tang and Jin-Ao Duan revised the paper.

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