A Neuroprotective Polysaccharide from *Hyriopsis cumingii*

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A water-soluble polysaccharide from *Hyriopsis cumingii* (HCp) was isolated by hot-water extraction and ethanol precipitation. We investigated the preliminary neuroprotective activity of HCp on cerebral ischemia/reperfusion (I/R)-induced rat brain tissue injury using the middle cerebral artery occlusion (MCAO) model. The structural analysis showed that the major component of HCp (HP-I) is an α-(1→4)-D-glucan branched with a single α-D-glucose at C-6 every five residues on average and has a weight-average molecular weight of about 2.65×10^5. Pharmacological studies revealed that HCp improves and restores nerve function impairment, significantly increases brain tissue total superoxide dismutase activity, lowers malondialdehyde content effectively, and reduces brain levels of caspase-3 mRNA expression in a dose−effect manner, indicating that *H. cumingii* polysaccharide possesses significant neuroprotective effects.

Hyriopsis cumingii, which belongs to the family of freshwater pearl mussels, is widely cultivated in China for its production of high-quality pearls.[1] *H. cumingii* flesh is mainly used as animals' food or fertilizer after its pearls are harvested. In fact, mussel flesh is an important resource of interesting bioactive polysaccharides, many of which have been reported to possess various biological functions. For instance, mytilan isolated from the mantle of the mussel *Crenomytilus grayanus* displays immunomodulating activity;[2] a polysaccharide glycogen extracted from the NZ green-lipped mussel *Perna canaliculus* was found to possess anti-inflammatory activity;[3] heparin, a polysaccharide isolated from marine clam species *Anomalocardia brasiliana* and *Tivela mactroides*, exhibits antithrombin-binding properties;[4] and polysaccharide MP-I obtained from *Mytilus coruscus* has a protective function against acute liver injury.[5]

The polysaccharide from *H. cumingii* (HCp) was recently shown to have nutrient and pharmacological functions, such as antitumor, immunity-enhancing, anti-inflammatory, and antioxidant activities.[6,7] Previous investigations demonstrated that antioxidants could be used to treat cerebral ischemia/reperfusion (I/R) injury and attenuate oxidant stress in the brain.[8] However, no reports on the neuroprotective functions of HCp exist, and little structural information is known. The only published HCp structural information is from the polysaccharide HCLPS-1 from *H. cumingii*.[9] Therefore, further studies on the bioactivity, isolation, purification, and structure of HCp are desirable.

Herein, the neuroprotective functions and possible mechanisms of HCp on I/R-induced rat brain tissue injury are reported on the basis of our evaluation of the levels of neurological deficit, superoxide dismutase (SOD), malondialdehyde (MDA), and caspase-3 mRNA expression. In addition, the structural characterization of a primary fraction HP-I (different from HCLPS-1) is described on the basis of acid hydrolysis, methylation, Smith degradation, acetolysis, NMR spectroscopy, high-performance gel permeation chromatography (HPGPC), and infrared absorption spectrum (IR).

Results and Discussion

Extraction and Characterization of *H. cumingii* Polysaccharide. Prior to hot-water extraction, lipid extraction on the flesh of *H. cumingii* was carried out with 95% EtOH. After defatting, aqueous extraction was performed at 100 °C to yield HCp. The extraction yield of HCp reached approximately 2.5% of the fresh weight of *H. cumingii*. HCp was soluble in water and was obtained as a white powder.

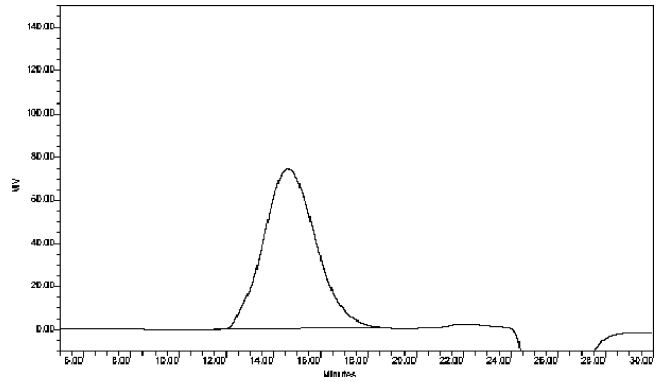

Figure 1. HPGPC profile of HP-I isolated from *H. cumingii*.

HCp was purified and fractioned through fast-flow DEAE-Sepharose and Sephadex G-200 chromatography to afford the major component, HP-I, with a yield of about 1.5% based on the fresh weight of *H. cumingii*. Both gel filtration and HPGPC (Figure 1) gave a single and symmetrically sharp peak, indicating that the HP-I sample was homogeneous. According to the calibration curve, the weight-average molecular weight of HP-I is about 2.65×10^5.

HP-I showed absorption bands at 3386 (br), 2929, 1638, 1080, 1023, 931, 849, and 758 cm^{-1} in the IR spectrum. The bands at 3386 (br) and 2929 cm^{-1} were ascribed to the hydroxy and C−H stretching vibrations, respectively. The band at 1638 cm^{-1} was due to associated water,[10] whereas the band at 849 cm^{-1} in addition to the positive specific rotation $\{[\alpha]^{25}_D$ (*c* 0.4, water) +187$\}$ indicated the α-type glycosidic linkage of the polysaccharide.[11] The bands at 931 and 758 cm^{-1} were characteristic of an (1→4)-α-glucan in the pyranose form.[12]

For component analysis, the complete acid hydrolysis of HP-I was performed with 2 M TFA at 100 °C for 12 h. Paper chromatography (PC) analysis showed that only the monosaccharide glucose was present. After reduction and acetylation, the sample was analyzed by GC-MS. According to the GC-MS results of the alditol acetate derivatives of the components, HP-I is composed of only glucose monomers. Determination of the absolute configurations of the sugars present in HP-I was based on the GC-MS analysis of their acetylated (+)-2-butyl glycosides,[13,14] which indicated the D-configuration of the constructive sugars.

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Table 1. GC-MS Data of the Hydrolysis Products of MHP-I

O-methyl-D-glucose	retention time[a]	molar ratio of native MHP-I	linkage indicated
2,3,4,6-tetra-	1.00	1.00	Glc(1→
2,3,6-tri-	1.06	4.06	→4)-Glc(1→
2,3 -di-	1.11	0.84	→4)-Glc(1→ 6 ↑

[a] Retention times are given relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*- methyl-D-glucitol on HP-5.

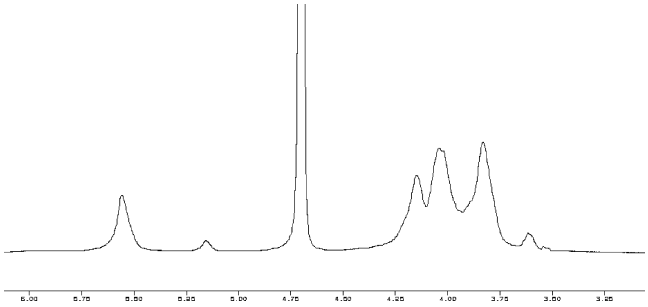

Figure 2. ^{1}H NMR spectrum of HP-I isolated from *H. cumingii* dissolved in D_2O at 50 °C.

Permethylated HP-I was obtained by repeating the Hakomori procedure five times. Next, the product was hydrolyzed, reduced, acetylated, and analyzed by GC-MS. The GC-MS results of the derived alditol acetates from the permethylated HP-I showed three separate peaks corresponding to 2,3,4,6-tetra-*O*-methyl-Glc, 2,3,6-tri-*O*-methyl-Glc, and 2,3-di-*O*-methyl-Glc derivatives in a relative molar ratio of 1.00:4.06:0.84 based on their respective peak areas (Table 1). These results suggested that HP-I is an α-(1→4)-D-glucan with branches at the *O*-6 glucosyl residues of the backbone. This conclusion was also confirmed by the results from periodate oxidation and Smith degradation (results not shown). After acetolysis, the resulting mixture of oligosaccharides was fractionated on a Sephadex G-15 column, yielding only two fractions (Ac1 and Ac2). The methylation data of Ac1 gave terminal glucose and 4-substituted glucose derivatives in a relative molar ratio of 1:12, consistent with (1→4)-linked oligosaccharides resulting from complete cleavage of the branched chains and probably from some cleavage of backbone (1→4) bonds. Ac2 is composed of only terminal glucose derivatives, demonstrating that the branched chain of HP-I is a single α-D-glucose moiety.

In the anomeric region of the ^{1}H NMR spectrum (Figure 2) of HP-I, two signals appeared at δ 5.56 and 5.16, which were assigned as the anomeric proton resonance of →4)-α-D-Glc*p*-(1→ (residue A) and α-D-Glc*p*-(1→ (residue C), respectively.[5] These results suggested that all of the residues are linked α-glycosidically, in agreement with the IR band at 849 cm^{-1}. The overlapping peaks in the δ 3.0−4.2 region were assigned to the protons of carbons C-2 to C-5 (or C-6) of the glycosidic rings. The ^{13}C NMR spectrum of HP-I confirmed the substitution patterns of the glucose units that were revealed by the methylation studies. Two signals at δ 100.64 and 99.38 correspond to the anomeric carbons of residues A and C, respectively. The signal at δ 78.53, compared with that of C-4 of the corresponding monosaccharide at δ 69.11, characteristic of C-4-substituted glucopyranosyl residues,[15] as well as signals at δ 61.38 and 71.20 characteristic of C-6-unsubsituted and -substituted glucopyranosyl residues,[16] indicate the (1→4)- and 6-linkages in the glucopyranosyl residues. The signal assignment of the ^{13}C NMR spectra (Figure 3, Table 2) took advantage of previously published results.[17−19]

Neuroprotective Effects of HCp on Cerebral Ischemia/Reperfusion Injury in Rat Brains. Recently, more attention has been paid to the factors involved in I/R-induced injury. Investigations showed that excessive reactive oxygen species are important

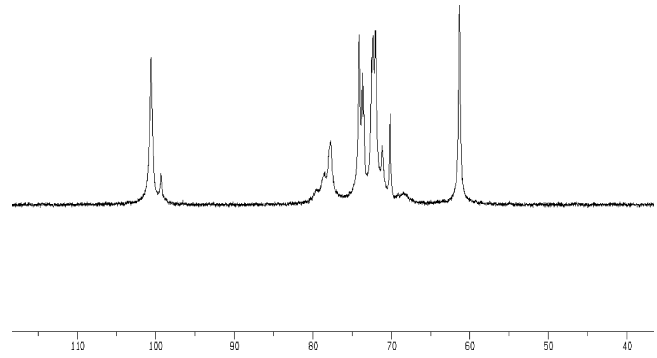

Figure 3. ^{13}C NMR spectrum of HP-I isolated from *H. cumingii* dissolved in D_2O at 25 °C.

Table 2. ^{13}C NMR Chemical Shifts of HP-I from *H. cumingii*

	chemical shift (ppm)[a]					
sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
A, →4)-α-D-Glc*p*-(1→	100.64	72.56	74.13	78.53	72.05	61.38
B, →4,6)-α-D-Glc*p*-(1→	100.64	72.36	74.13	77.74	73.53	71.20
C, α-D-Glc*p*-(1→	99.38	72.36	73.73	69.11	70.18	61.38

[a] In ppm downfield relative to the signal for Me_4Si.

Table 3. Effect of HCp on Rat Neurological Deficit after I/R ($\bar{X} \pm S$, $n = 8$)

		neurological score		
group	dose	24 h	48 h	72 h
sham-operated	NS	0 ± 0	0 ± 0	0 ± 0
I/R	NS	2.80 ± 0.32[a]	2.63 ± 0.43[a]	2.22 ± 0.35[a]
low dose	0.25 g/kg	2.47 ± 0.34Δ	2.23 ± 0.47Δ	1.99 ± 0.42Δ
middle dose	0.5 g/kg	2.19 ± 0.29Δ	1.83 ± 0.38Δ	1.62 ± 0.32Δ
high dose	1 g/kg	2.07 ± 0.42Δ	1.64 ± 0.43ΔΔ	1.48 ± 0.37Δ
nimodipine	1 mg/kg	2.10 ± 0.39Δ	1.99 ± 0.41Δ	1.56 ± 0.46Δ

[a] $p < 0.01$ in comparison with sham-operated group; $\Delta p < 0.05$, $\Delta\Delta p < 0.01$ in comparison with I/R group.

in initiating the brain injury process.[20,21] Therefore, the potent radical-scavenging properties or lipid peroxidation-inhibiting ability of some natural products demonstrated that they may be valuable therapeutic agents for the treatment of I/R-induced injury. In addition, proteins of the caspase family, which are associated with the signaling pathway, ultimately lead to programmed cell death (apoptosis).[22,23] Therefore, reduction of caspase-3 mRNA expression can inhibit the mitochondrial-related caspase-3 apoptotic pathway. Middle cerebral artery occlusion (MCAO) was performed to understand the mechanisms of therapeutic protocols, because it may represent the most prevalent form of stroke in humans.[24−26]

The neuroprotective effects of HCp on I/R-induced brain injury of rat were assessed initially. The neurological deficit, SOD activity, MDA content, and caspase-3 mRNA expression in brains of rats were investigated after I/R.

Depending on the neurological score, the neurological deficit after 24, 48, and 72 h of reperfusion period was significantly increased in the model I/R rats compared to the sham-operated ones ($P < 0.01$). Treatment with all doses of HCp obviously reduced the neurological deficit ($p < 0.01$ or $p < 0.05$) in contrast to nimodipine (1 mg/kg) ($P < 0.01$) (Table 3) compared with the model I/R animals.

Determination of SOD activity and MDA content was carried out to estimate the level of oxidative stress in brain tissues. Compared to the sham-operated group, significantly increased MDA content and decreased SOD activity were observed in the brain tissues of model I/R rats ($P < 0.01$). High, middle, and low doses of HCp all significantly increased the activity of SOD and reduced the MDA content in the brains of ischemic rats compared with those of the model I/R rats ($p < 0.01$ or $p < 0.05$) in a dose−effect manner.

Table 4. Effects of HCp on SOD Activity and MDA Content in Brain Tissues of I/R Rats ($\bar{X} \pm S$, $n = 8$)

group	dose	SOD (U/mg protein)	MDA (nmol/mg protein)
sham-operated	NS	55.75 ± 3.26	5.07 ± 1.32
I/R	NS	26.59 ± 3.42[a]	19.07 ± 1.62[a]
low dose	0.25 g/kg	31.57 ± 2.74Δ	16.07 ± 2.03Δ
middle dose	0.5 g/kg	34.32 ± 2.36ΔΔ	14.03 ± 1.98ΔΔ
high dose	1 g/kg	38.35 ± 3.02ΔΔ	11.98 ± 2.21ΔΔ
nimodipine	1 mg/kg	35.91 ± 3.29ΔΔ	12.65 ± 2.79ΔΔ

[a] $p < 0.01$ in comparison with sham-operated group; $\Delta p < 0.05$, $\Delta\Delta p < 0.01$ in comparison with I/R group.

Table 5. Effect of HCp on Caspase-3 mRNA Expression in Brain Tissues of I/R Rats ($\bar{X} \pm S$, $n = 8$)

group	dose	caspase-3 mRNA expression ($2^{-\Delta Ct}$)
sham-operated	NS	0.171 ± 0.091
I/R	NS	1.000 ± 0.000[a]
low dose	0.25 g/kg	0.937 ± 0.078Δ
middle dose	0.5 g/kg	0.875 ± 0.091Δ
high dose	1 g/kg	0.746 ± 0.087ΔΔ
nimodipine	1 mg/kg	0.806 ± 0.097ΔΔ

[a] $p < 0.01$ in comparison with sham-operated group; $\Delta p < 0.05$, $\Delta\Delta p < 0.01$ in comparison with I/R group.

Nimodipine (1 mg/kg b.w.) also significantly elevated the activity of SOD ($p < 0.05$) and lowered the MDA content ($p < 0.05$) compared with the model I/R rats (Table 4).

Caspase-3 mRNA expression was measured to evaluate apoptosis in brain tissues. The similar peaks within the melting-curve analysis suggest the absence of contamination, mispriming, or primer−dimer artifacts, in the FQ-RT-PCR experiments (results not shown). A remarkable increase in caspase-3 mRNA expression was found in the brain tissues of model I/R rats compared to the sham-operated group ($p < 0.01$). Compared with model I/R rats, HCp ($p < 0.01$ or $p < 0.05$) and nimodipine ($p < 0.05$) treatment reduced caspase-3 mRNA expression, but all of the results were not significant (Table 5).

The results showed that I/R caused serious injury in rat brain tissue, leading to a significant increase of MDA content and decrease of SOD activity, consistent with the neurological score. Administration of HCp and nimodipine elevated the SOD activity and reduced the MDA contents in rat brains.

Moreover, HCp suppressed the caspase-3 mRNA expression to some extent, indicating that it can also inhibit apoptosis. Cell death is considered to occur by only two basic mechanisms: necrosis and apoptosis. When widespread necrosis occurs within the area of injury, many features of apoptosis are observed in brain tissue of animal models with focal ischemia, so apoptosis is also thought to play an important part in ischemic cell death.[27−29] Administration of HCp could constrain the caspase-3 mRNA expression, preventing brain injury.

In conclusion, the present study demonstrated that HP-I with a weight-average molecular weight of about 2.65×10^5 is an α-(1→4)-D-glucan, branched with a single α-D-glucose at the C-6 position every five residues on average (Figure 4). HCp can significantly protect the brain tissue of rat from I/R injury in a dose−effect manner. Elevating the brain tissue SOD activity and reducing the MDA content might provide an important protective effect against necrosis of neurons. In addition, HCp can restrain caspase-3 mRNA expression and prevent apoptosis, all these effects being strongly related to the patient clinical end point. We verified that these neuroprotective functions are associated with the antioxidant and antiapoptotic activities of HCp. In the future, additional experiments will be carried out to investigate whether HP-I is responsible for the neuroprotective activity.

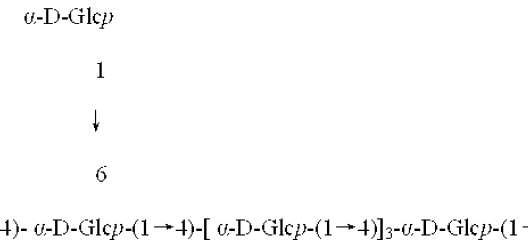

Figure 4. Proposed structure of HP-I.

Experimental Section

General Experimental Procedures. The optical rotation was measured with a JACSO P-1030 polarimeter, and UV absorptions were determined on a JASCO V-530 UV/via spectrophotometer. The IR spectra (KBr pellets) were recorded on a Beckman Acculab 10 instrument. The NMR spectra were obtained with a Bruker 500 instrument. For ^{1}H and ^{13}C NMR spectroscopy at 50 and 25 °C, respectively, HP-I (50 mg) was dissolved in 1 mL of 99.99% D_2O. The molecular weight of HP-I was determined by HPGPC (Waters 515 instruments). GC-MS was carried out on a Trace DSQ GC-MS instrument (Thermofisher) using an HP-5 column (30 m × 0.25 mm i.d., Agilent). The ionization potential was 70 eV, and the temperature of the ion source was 200 °C.

Extraction and Isolation of Polysaccharide from *H. cumingii*. Fresh, commercially obtained *H. cumingii* was used for the isolation of mussel polysaccharide.

After homogenization, 1 kg of mussel meat was extracted twice with deionized H_2O (3 L) for 1 h at 100 °C. The suspension was centrifuged at 3600 rpm for 10 min. The combined extracts were concentrated to 500 mL using a rotary evaporator at 50 °C under vacuum, and 3 volumes of 95% EtOH was added into the supernatant. Twelve hours later, the precipitate was collected and washed with acetone and 95% EtOH three times. The residue was dissolved in deionized H_2O (100 mL) and kept in a water bath at 37 °C for 8 h. The protein was removed with Sevage reagent (*n*-BuOH−$CHCl_3$ at a ratio of 1:5, v/v) several times by the Sevage procedure.[30] The resulting aqueous fraction was extensively dialyzed for 2 days against deionized H_2O with a tubular membrane (MWCO: 6000−10 000 Da, Huamei Bioengineering Company, Shanghai, China) to remove small molecules and was then concentrated and lyophilized to yield 25 g of HCp.

HCp (500 mg) was dissolved in deionized H_2O (2.0 mL) and centrifuged. The resultant supernatant was applied to a fast-flow DEAE-Sepharose column (Pharmacia) (2.6 × 40 cm) and eluted successively with deionized H_2O, 0.05 M NaCl, 0.2 M NaCl, and 0.5 M NaCl (300 mL each). The content of the polysaccharide was detected by the phenol−H_2SO_4 spectrophotometric method.[31] Based on the elution profile, the main fraction obtain from the 0.05 M NaCl elute was dialyzed, concentrated, and lyophilized. The product was further purified on a Sephadex G-200 column (Pharmacia, 2.6 × 40 cm) with 0.05 M NaCl. The appropriate fractions were combined, dialyzed, and lyophilized to give HP-I.

Purity Determination of HP-I.

(a) Gel Filtration. A solution of HP-I (5.0 mg) in 0.05 M NaCl (1.0 mL) was applied to a DEAE-cellulose-32 column (Pharmacia, 2.6 × 40 cm) using 0.05 M NaCl as the eluent. The elution profile was obtained by the phenol−H_2SO_4 method. (b) HPGPC. HPGPC was carried out on a Waters 515 instrument (Milford MA), equipped with a Waters 2410 refractive index detector and two columns in series (Ultrahydrogel 120 and Ultrahydrogel 500). The mobile phase consisted of 0.1 M $NaNO_3$, and the sample concentration was 5 mg/mL. T-series Dextran standards with definite molecular masses ranging from 10 to 2000 kDa were used to calibrate the HPGPC system. During the experimental process, the columns were kept at 40 °C. All data obtained by the system were collected and analyzed using the Waters Millenium 32 software package.

Component Analysis. HP-I (2.5 mg) was boiled with 2 M TFA (3 mL) in a water bath for 12 h, and the acid was removed at 50 °C in a rotary evaporator under reduced pressure. These products were reduced with $NaBH_4$ for 12 h and acetylated with Ac_2O−pyridine (1 mL each) at room temperature for 10 h. Finally, the alditol acetates were analyzed by GC-MS.

Methylation Analysis. The Hakomori method[32] was adopted in the methylation analysis. HP-I (14.5 mg) dissolved in dry DMSO (3 mL) was methylated with CH_3I (1 mL) and NaOH (20 mg). The process was repeated four times. On the basis of the IR spectrum, no OH existed in the methylated product. After hydrolysis with 90% HCO_2H for 10 h and then with 2 M TFA for 6 h in a boiling water bath, the methylated HP-I (MHP-I) was reduced with $NaBH_4$, converted into partially methylated alditol acetates, and analyzed by GC-MS.[33]

Paper Chromatogramic Analysis. PC was performed on Whatman No. 1 and 3 mm filter papers using two solvent systems: (a) *n*-BuOH−pyridine−H_2O (6:4:3 v/v) and (b) EtOAc−HOAc−H_2O (3:1:3 v/v). Aniline−diphenylamine and *p*-anisidine were used for detection. Authentic standards including D-glucose, D-mannose, D-galactose, D-arbinose, D-xylose, L-rhamnose, and D-glucuronic acid were used as references.[34]

Periodate Oxidation and Smith Degradation. HP-I (19.3 mg) in 0.040 mol/L sodium metaperiodate (25 mL) was kept at 4 °C in a dark environment. The periodate consumption was monitored at intervals by the Fleury−Lange method.[35] Ethylene glycol was added to reduce excess periodate after the consumption of periodate was complete (2 days), and the liberated HCO_2H was titrated with 0.01 M NaOH.

The resulting solution was dialyzed against deionized H_2O for 48 h. The residue was concentrated to 2 mL and reduced with $NaBH_4$ (80 mg) at room temperature for 10 h; next the excess $NaBH_4$ was decomposed with HOAc. The product was hydrolyzed with 2 M TFA at 100 °C for 6 h, acetylated, evaporated to dryness, and analyzed by GC-MS as described above.

Acetolysis. Acetolysis was performed as previously described.[36,37] The deacetylated oligosaccharides were applied to a Sephadex G-15 (Pharmacia) column (2.0 × 100 cm) and eluted with deionized H_2O. The appropriate fractions were pooled, lyophilized, methylated, hydrolyzed, reduced, acetylated, and characterized by GC-MS.

Animal Experiment. Adult male Sprague−Dawley rats (8 weeks old) weighing 280 ± 10 g were used. The rats were brought from the Center Animal House of Zhejiang Chinese Medical University and kept in cages at an ambient temperature of 22 −25 °C and relative humidity of 60−70% with free access to water and food. All of the procedures were performed according to the Ethics Committee for the Use of Experimental Animals at Zhejiang University. After we established the corresponding model, male Sprague−Dawley rats were selected and divided into six groups (eight rats per group): a sham-operated group; I/R group; high dose (1 g/kg), middle dose (0.5 g/kg), and low dose (0.25 g/kg) of HCp groups; and a nimodipine group (1 mg/kg). Different concentrations of HCp in normal saline were administered by intraperitoneal injection at 6, 24, 48, and 72 h after MCAO. The sham-operated group and the I/R group received the same volume of solvent as the other groups.

Middle Cerebral Artery Occlusion. To investigate the neuroprotective effects of HCp in an experimental model of I/R, the method of Longa et al.[38] was used to produce a rat MCAO model. Briefly, the rats were anesthetized with 10% chloral hydrate (0.4 mL/100 g, ip). Next, a nylon thread with a rounded tip was inserted from the bifurcation to the MCAO until a slight resistance was felt. After 1 h, the filament was slowly withdrawn for reperfusion until the tip reached the external carotid artery. The sham-operated animals were subjected to the same procedure, but the filament was not inserted. The procedure-related mortality rate was about 6% throughout the whole experiment.

Assessment of Neurological Deficit and Sampling. The neurological deficit in I/R groups was assessed after 24, 48, and 72 h of reperfusion according to a published method.[38] The neurologic findings were scored on a 5-point scale: a score of 0 indicates no neurologic deficit, a score of 1 (failure to extend left forepaw fully) corresponds to a mild focal neurologic deficit, a score of 2 (circling to the left) indicates a moderate focal neurologic deficit, and a score of 3 (falling to the left) corresponds to a severe focal deficit. Rats with a score of 4 did not walk spontaneously and had a depressed level of consciousness. Rats with a neurologic deficit scoring less than 1 were removed from the study.

After completion of the neurobehavioral experiment, the brain of each animal was removed. One third of the frontal pole of brain tissue was stored in liquid N_2 for real-time fluorescence quantitative polymerase chain reaction (FQ-RT-PCR). The remainder was homogenized to produce 10% tissue homogenate and kept at −80 °C until used for determination of SOD activity and MDA content.

Determination of SOD Activity and MDA Content. The MDA level and SOD activity were determined using commercial kits (Jiancheng Bioengineering Institute, Nanjing, China). A 10% tissue homogenate was prepared according to the user manual. Briefly, brain tissue was homogenized in 10 vol (w/v) of normal saline. Then the homogenate was centrifuged at 3000 rpm for 10 min, and the supernatant was used in further experiments (Table 3).

Determination of Caspase-3 mRNA Expression in Brain Tissue. According to reported literature,[22,39] caspase activation, especially caspase-3, played a key role in apoptotic cell death, and caspase-3 mRNA expression steadily increased following MCAO. The total RNA was isolated from 0.1 g of brain tissue from a different group with 1 mL of Trizol reagent according to the manufacturer's instruction. Then, 0.1 μg of each total RNA sample was added to a reverse transcription reaction containing dNTP, oligodT18 primer, RNAsin, and M-MLV reverse transcriptase reagent. Reverse transcription was performed at 42 °C for 60 min and was followed by enzyme inactivation at 70 °C for 5 min to synthesis first strand cDNA. FQ-RT-PCR was carried out in an MJ Mini personal thermal cycler (Bio-Rad, USA) with 1 μL of cDNA in a final volume of 25 μL containing 12 μL of iQ SYBR Gene Surpermix, 10 μL of sterile H_2O, 1 μL of rat caspase-3 sense and antisense primers (sense 5′-CTGGACTGCGGTATTGAGAC-3′ and antisense 5′-CCGGGTGCGGTAGAGTAAGC-3′), and 10 μL of sterile H_2O. In addition, 1 μL of rat β-actin sense and antisense primers (sense 5′-CCGTAAAGACCTCTATGCCAACA-3′, antisense 5′-TAGGAGCCAGGGCAGTAATC-3′) was added as an internal reference to normalize the samples for the RNA amount. The thermal cycling procedure started at 95 °C for 3 min and was followed by 40 amplification cycles (95 °C for 10 s and 62 °C for 50 s). Melting-curve analysis using iQ SYBR Gene Supermix was conducted to identify the dissociation temperature of caspase-3 and β-actin amplicons and the specificity of the FQ-RT-PCR reactions using total RNA from brain tissue samples.

Statistical Analysis. Results are expressed as mean ± SD. Student's unpaired *t* tests and one-way analysis of variance (ANOVA) were used to determine significant differences and for comparison between groups, respectively, with the SPSS 11.5 (SPSS Inc., Chicago, IL) statistical package program. A value of $p < 0.05$ was considered statistically significant.

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