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Full Length Research Paper

The traditional Chinese medicine Huang-Lian-Jie-Du-Tang inhibits hypoxia-induced neuronal apoptosis

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Huang-Lian-Jie-Du-Tang (HLJDT) has been used clinically for cerebral ischemia therapeutics. Here, we aimed to demonstrate whether and how HLJDT regulates neuronal apoptosis under hypoxia/ischemia. Apoptosis analysis was performed *in vitro* in PC12 cells with flow cytometry and *in vivo* in MCAO rats using TUNEL staining. The levels of caspase 9, caspase 3, Bcl-2, Bax and HIF-1α were demonstrated with western blotting. HLJDT remarkably inhibited apoptosis of neurons, both *in vitro* and *in vivo*. Caspase 9, caspase 3m Bcl-2, Bax and HIF-1α play important roles in anti-apoptotic effect of HLJDT on neuronal apoptosis hypoxia and ischemia induced.

Key words: Huang-Lian-Jie-Du-Tang, apoptosis, HIF-1, ischemia.

INTRODUCTION

Huang-Lian-Jie-Du-Tang (HLJDT), a traditional Chinese medicinal prescription widely used in treating clinical stroke, is comprised of four common herbs, *Rhizoma coptidis* (*Coptis chinensis* Franch, Ranunculaceae), *Radix scutellariae* (*Scutellaria baicalensis* Georgi, Labiatae), *Cortex phellodendri (Phellodendron amurense* Rupr, Rutaceae) and *Fructus gardeniae* (*Gardenia jasminoides* Ellis, Rubiaceae), in a 3:2:2:3 proportion. In addition to having favorable effects on gastrointestinal disorders, acute liver injury and cardiovascular diseases, further evidence has indicated that HLJDT could have potential therapeutic effects on cerebral ischemia (Mori et al., 1991; Ohta et al., 1998; Takase et al., 1989; Wu et al., 2004). HLJDT has been used clinically to treat subjects suffering from stroke, sequelae of stroke or vascular dementia (Xu et al., 2000). Furthermore, a study in rats injected with Aβ demonstrated that HLJDT diminished the enhancement of inflammatory cytokines tumor necrosis factor-α, interferon-γ and interleukin-2 (Hwang et al., 2002). Moreover, 845 mg/kg/day HLJDT administered to C57BL/6 mice with transient cerebral ischemia resulted in overexpression of Cu/Zn-SOD, a defense system against oxidative stress that leads to the elimination of the production of reactive oxygen species and a reduction in ischemic neuronal death during ischemia-reperfusion (Kondo et al., 2000). Additionally, the neuronal protection against lipid peroxidation conferred by HLJDT was detailed; it was found to be independent of microsomal drug-metabolizing activity and could not be fully accounted for by its action on microsomal electron transfer. In solution, HLJDT could effectively scavenge OH^* and O^{2*}- anion radicals (Stefek and Benes, 1994). It has been documented that HLJDT reversed both the overactive serotonin neurotransmission and the reduction in muscarinic acetylcholine receptors in gerbils with transient cerebral ischemia (Kabuto et al., 1997). And in rats with the same condition, treatment with HLJDT markedly reduced the area of cerebral infarction and activity of myeloperoxidase, an index of neutrophil infiltration, in ischemic brain tissue (Hwang et al., 2002). Consistent with vasorelaxation in hypertensive rats, HLJDT increased cerebral blood flow in the areas around the margins of ischemic areas (Zheng et al., 2008). Results from proteomics analysis indicated that HLJDT regulated protein expression in the hippocampus of senescence accelerated mice, including the expression of proteins closely associated with energy metabolism, signal transduction, cytoskeletal function and amino acid metabolism (Wang et al., 2007). Moreover, HLJDT has the ability to increase neuron density in the ischemic hippocampal CA1 region. In addition, impairment of

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learning and memory induced by transient cerebral ischemia in mice was prevented by HLJDT via increasing the acetylcholine content in cerebral cortex, hippocampus and striatum (Zhang et al., 2009). To the best of our knowledge, although, favorable effects on anti-inflammation, antioxidation, the regulation of cerebral blood flow and neurotransmitters and the maintenance of hippocampal function might all be responsible for ameliorating the pathophysiological process of cerebral ischemia, the potential effects of HLJDT on neuronal apoptosis, the major pathway of neuronal injury in cerebral ischemia and the mechanisms underlying these effects remained elusive.

In severe hypoxia, excitotoxic mechanisms are generally thought to be involved in neuronal damage (Rothman and Olney, 1986). Both hypoxia and ischemia result in an increased release of glutamate following the activation of N-methyl-D-aspartate receptors, which causes subsequent excitotoxicity that may lead to programmed cell death or apoptosis (Nicholls and Attwell, 1990). Additionally, severe hypoxia causes increased expression of proapoptotic proteins, such as p53, p21, Bax and caspases as well as decreased expression of antiapoptotic proteins, such as Bcl-2 and heat-shock proteins (Banasiak and Haddad, 1998; Bossenmeyer-Pourie et al., 2002; Hu et al., 2000; Wang et al., 2001). Under hypoxic conditions, hypoxia-inducible factor 1 (HIF-1), a transcription factor regulating oxygen homeostasis, binds to canonical DNA sequences, which play vital roles in developmental and physiological processes, such as angiogenesis, glucose transport and cell proliferation/survival (Iyer et al., 1998). HIF-1 is also required for hypoxia-induced apoptosis. HIF-1 can increase the stability of the product of tumor suppressor gene p53, which induces apoptosis by regulating proteins, such as Bax and causes growth arrest mediated by p21 (Boyd, 1994; Chen et al., 2003). Furthermore, HIF-1 is a dependent factor for BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3), which is upregulated by hypoxia and induces apoptosis by binding to and inhibiting the antiapoptotic proteins Bcl-2 and Bcl-xL (Kothari et al., 2003).

In the present study, we aimed to investigate the effects of HLJDT on apoptosis and the potential signaling pathway involved in the protection of hypoxic/ischemic neurons.

MATERIALS AND METHODS

Herbal

Herbal materials for HLJDT preparation were purchased from Bozhou Medicine Company (Anhui, China) and kindly authenticated by Dr Qi-nan Wu, Professor of Pharmacognosy, Nanjing University of Chinese Medicine. The composition of HLJDT and voucher specimen numbers are listed in Table 1. Voucher specimens are deposited in the Museum of Materia Medica, Nanjing University of Chinese Medicine.

Chemicals and reagents

PC12 cells were obtained from the Chinese Academy of Sciences Committee Type Culture Collection (Shanghai, PRC). Cell culture media, phosphate buffer solution (PBS) and fetal bovine serum (FBS) were from Gibco (Tulsa, OK, USA). All cell culture plastic ware (COSTAR$^{®}$) was purchased from Corning (New York, USA). Sprague-Dawley rats were obtained from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, PRC). $CoCl_2$, DMSO, MTT, chloral hydrate, heparin, procaine, paraformaldehyde, sucrose, H_2O_2, methanol, ethylenediaminetetraacetic acid (EDTA), NP-40, Na-deoxycholate, DAB, phenylmethylsulfonl fluoride and protease inhibitor cocktail were obtained from Sigma-Aldrich (St. Louis, MO, USA). Annexin V (AV) and propidium iodide (PI) were from Molecular Probes (Eugene, OR, USA). The LDH kit was purchased from Jiancheng (Nanjing, China). Goat serum was obtained from Gibco (Tulsa, OK, USA). The TUNEL kit was obtained from Promega Corporation (San Leandro, CA, USA). Antibodies for HIF-1α, Bcl-2, Bax, caspase 9, caspase 3 and secondary antibodies were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). The enhanced chemiluminescent detection system was purchased from Applygen (Beijing, PRC).

Preparation of the HLJDT extract

The mixture (100 g) of *R. coptidis*, *R. scutellariae*, *C. phellodendri* and *F. gardeniae* was combined as indicated in Table 1 and decocted twice by refluxing with water (1:10 and then 1:8, w/v) for 1 h. The resulting solution was spray-dried, and 25.20 g product was finally obtained. The content of baicalin in that extract was 4.02%, which was quantitatively determined by HPLC following ultrasound with methanol for 30 min and filtration. The HLJDT extract was suspended in 0.50% carboxymethyl cellulose sodium (CMC-Na) aqueous solution before use.

Animals and MCAO

Adult male Sprague-Dawley rats (270 to 300 g) were maintained in well-controlled temperature (22 ± 2°C) and humidity (55 ± 5%) and a 12 h/12 h light-dark cycle, and had *ad libitum* access to water and standard rodent chow. MCAO was performed using the intraluminal suture occlusion method as described previously (Leonardo et al., 2010). Briefly, rats were anesthetized with chloral hydrate (350 mg/kg), and the right common carotid artery and ECA were exposed via a ventral midline skin incision. A 3 to 0 nylon monofilament suture with a tip rounded by heating and coated with silicone (0.30 to 0.32 mm in diameter) was advanced from the ECA into the internal carotid artery. The suture was inserted about 18 to 20 mm from the carotid bifurcation to occlude the right MCA. After 2 h of MCAO, the suture was withdrawn to archive reperfusion. HLJDT (2.5 g extract/kg) was administrated intragastrically to rats when MCAO occurred. The sham-operated rats underwent the same procedure, but the suture was inserted only 10 mm and was withdrawn 1 min later. The rectal temperature of the rats was monitored and maintained at 37 ± 0.5°C throughout the surgery. Rats were allowed to freely access food and water after recovery from anesthesia. After 4 h of reperfusion, rats were anesthetized again and transcardially perfused, first with preperfusing solution (0.01 mg/ml heparin and 1 mg/ml procaine) and subsequently with 4% paraformaldehyde in 0.1 M PBS. The brains intended for immunohistochemical and TUNEL procedures were removed and postfixed overnight in 4% paraformaldehyde and cryoprotected with 30% sucrose (w/v) in 0.1 M PBS at 4°C for 48 h. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication

Table 1. Recipe for HLJDT formulation, voucher specimen numbers.

Component	Voucher specimen number	Part used	Ratio used
R. coptidis	20081106	Rhizome	3
R. scutellariae	20081105	Root	2
C. phellodendri	20081107	Cortex	2
F. gardeniae	20081108	Fruit	3

No. 85-23, revised 1996).

Cells and treatment

Rat pheochromocytoma PC12 cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 25 mmol/L glucose, supplemented with 10% (FBS, 100 µg/ml streptomycin and 100 U/ml penicillin, in a humidified atmosphere of 95% air/5% CO_2 at 37°C. The HLJDT extract was dissolved in DMSO (final concentration 0.1%). Cells were pretreated with various concentrations of the HLJDT extract in FBS-free and lower glucose medium (5 mmol/L) 24 h prior to the hypoxia induced by $CoCl_2$ (375 µmol/L) for another 24 h. A laser confocal microscope (ZEISS LSM710, German) was utilized to observe apoptosis of PC12 cells with double staining of annexin V and PI. Experiments were performed 1 to 2 days after plating in 96-well plates or 100 mm dishes.

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

Cell viability was determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). First, PC12 cells were placed on 96-well plates. After treatment with HLJDT for 48 h and $CoCl_2$ for the last 24 h, MTT (0.5 mg/ml) dissolved in PBS was added to the medium at a final concentration of 0.1 mg/ml and then incubated at 37°C for 4 h in an incubator. The reduction product of MTT, blue formazan crystals, was solubilized in DMSO, and optical density (OD) was spectrophotometrically measured at 570 nm with a reference of 620 nm using a SPECTRAmax spectrophotometer (MD, Sunnyvale, CA, USA).

Lactate dehydrogenase (LDH) measurement

The release of LDH into the medium was measured to evaluate cell membrane damage at these experimental conditions using an LDH detection kit according to the manufacturer's protocols. 100 µl of cell-free culture medium was collected and incubated with the reaction mixture, and LDH activity was determined by a spectrophotometric assay. The OD of the solution was then measured at 340 nm using a SpectraMax spectrophotometer.

Flow cytometry analysis of apoptotic cells

Cells were cultured as described earlier and then were collected 24 h post hypoxia. After resuspension in annexin-binding buffer, cells were stained with annexin V-FITC conjugate and PI according to the manufacturer's recommendation in the annexin V-PI staining kit. Annexin V-FITC labels apoptotic cells by binding phosphatidyl serine exposed on the outer leaflet of the plasma membrane. PI is impermeable to live cells and to cells undergoing early phase apoptosis, but stains dead cells with red fluorescence by binding to nucleic acids. After staining, the cells were analyzed with a flow cytometer (Becton Dickinson FACScalibur, Franklin Lakes, NJ, USA) and CellQuest Pro software (Becton Dickinson).

TUNEL assay

TUNEL staining was employed to determine the levels of nuclear DNA fragmentation during apoptosis using the *in situ* cell death detection kit following the manufacturer's instructions. Briefly, the prepared brain sections from MCAO rats were first incubated with proteinase K, followed by a 5 min incubation in equilibration buffer, and then incubated at 37°C for 60 min in solution with biotinylated dUTP and terminal deoxynucleotidyl transferase. Color was developed with DAB.

Western blot analysis

PC12 cells were lysed in RIPA buffer (500 mmol/L Tris-HCl pH 7.4, 1 mmol/L EDTA, 150 mmol/L NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mmol/L phenylmethylsulfonl fluoride and protease inhibitor cocktail) and then centrifuged at 12,000 rpm for 10 min at 4°C. Protein blots obtained with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were subsequently electrotransferred to luminescence membranes. After electroblotting, proteins were incubated with primary antibodies, such as HIF-1α, Bcl-2, Bax, caspase 9 and caspase 3 overnight at 4°C, followed by secondary antibody detection for 1 h at room temperature. Bound antibodies were visualized using an enhanced chemiluminescent detection system. The densitometric analysis was carried out using Gel-pro analyzer and was expressed as arbitrary units.

Statistical analysis

Data are expressed as mean ± SEM, and are evaluated for statistical significance via one-way analysis of variance (ANOVA), followed by Newman Keuls post hoc analysis conducted for pairwise multiple comparisons as significance was reached by ANOVA. $P < 0.05$ was considered to be statistically significant.

RESULTS

The effect of HLJDT on PC12 cell viability under hypoxia

Before determining the effect of HLJDT on PC12 cell viability in the context of $CoCl_2$-induced hypoxia, the toxicity of HLJDT was assayed. Some enhancement of cell viability rather than cell toxicity was observed for concentrations from 0.1 to 0.0001 mg/ml (Figure 1 A). Furthermore, pretreatment with the aforementioned

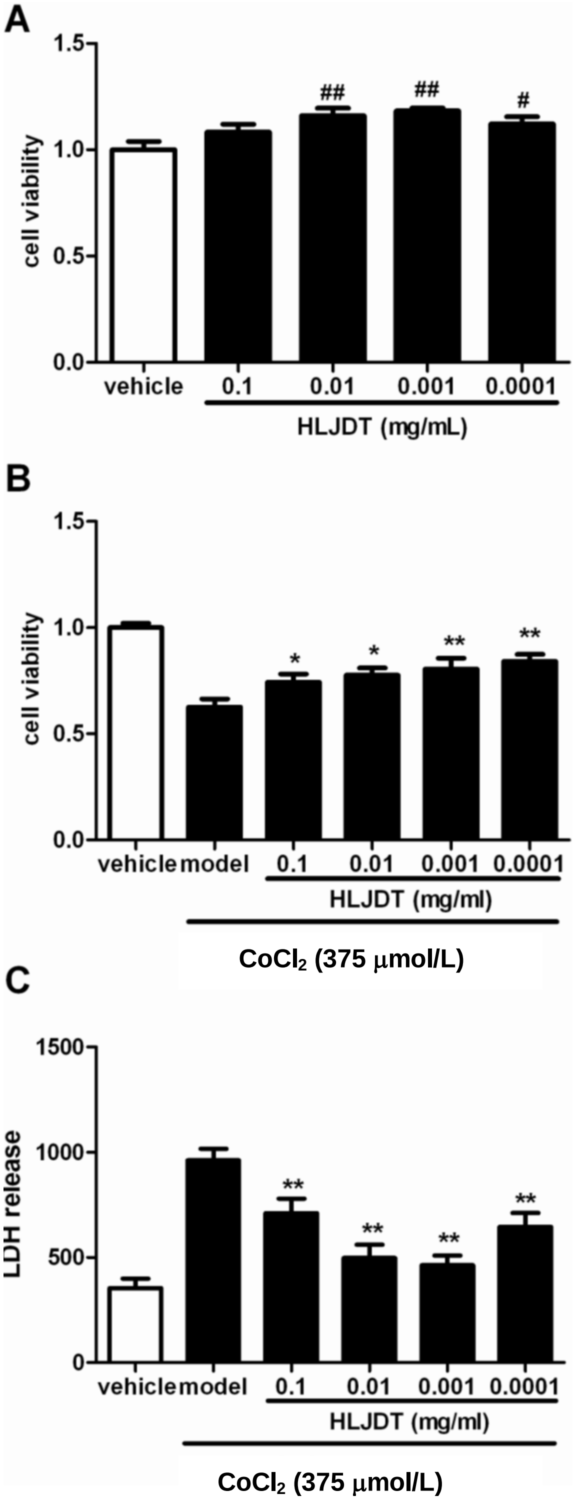

Figure 1. The effect of HLJDT on PC12 cell viability under hypoxia. (A) The cytotoxicity of HLJDT on PC12 cells evaluated by MTT. (B) HLJDT increased cell viability at concentrations of 0.1 to 0.0001 mg/ml under hypoxia induced by CoCl$_2$ (375 μmol/L) as assayed by MTT. (C) HLJDT prevented LDH release from hypoxic PC1$_2$ cells damaged by CoCl$_2$ (375 μmol/L) (n = 8). Data are expressed as mean ± SEM. $^{\#}P<0.05$, $^{\#\#}P < 0.01$, versus vehicle; $^{*}P < 0.05$, $^{**}P < 0.01$, versus model.

concentrations of HLJDT for 24 h remarkably reversed the decrease in cell viability caused by CoCl$_2$ (375 μmol/L) for another 24 h (Figure 1B). The elevated LDH release from membrane-damaged PC12 cells was strongly alleviated by pretreatment with HLJDT (Figure 1 C). These results suggest that HLJDT can encourage PC12 cell survival even with a hypoxic insult, and that promotion of both proliferation and recovery from damage is responsible for the protective pharmacological effect.

The effect of HLJDT on PC12 cell apoptosis under hypoxia

To determine whether HLJDT influenced the apoptosis of PC12 cells induced by CoCl$_2$, cells treated as indicated in Figure 2 were double stained with PI and AV, and were analyzed using flow cytometry. Compared to PC12 cells treated with CoCl$_2$ alone, apoptosis was significantly inhibited in cells treated with both CoCl$_2$ and HLJDT (Figure 2A). The apoptosis ratio was also decreased as expected in the HLJTD-treated group (Figure 2B). These results establish a link between the effects of HLJDT on inhibiting apoptosis and maintaining cell viability under hypoxia, and one could speculate that the action of HLJDT in preventing apoptosis may partly contribute to its therapeutic effect on cerebral ischemia.

The effect of HLJDT on apoptosis in the penumbra of MCAO rats

To further characterize the effect of HLJDT on apoptosis in cerebral tissue with ischemia, we performed TUNEL staining in the penumbra of tissue from MCAO rats. As indicated in the Figure 3, numerous TUNEL-positive cells, showing strong staining of nuclei or nuclear fragments, were observed in the penumbra near the area of infarction in rats with MCAO. In contrast, minor staining of cells was observed in the penumbra of MCAO rats in the presence of HLJDT (2.5 g extract/kg) (Figure 3). Taken together with results obtained using PC12 cells *in vitro*, we speculate that, for HLJDT, the prevention of apoptosis in the penumbra plays a pivotal role in reducing the development of cell death after ischemia.

The effect of HLJDT on caspase 9, caspase 3 and Bcl-2/Bax in PC12 cells under hypoxia

To ascertain the relevance to apoptosis and the exact mechanisms by which HLJTD inhibited apoptosis, we next measured the levels of apoptosis-related caspase 9 and its cleavage product caspase 3 in PC12 cells by western blotting. As anticipated, HLJDT decreased the elevation of levels of both caspase 9 and caspase 3 induced by hypoxia (Figure 4A and B). On the other hand.

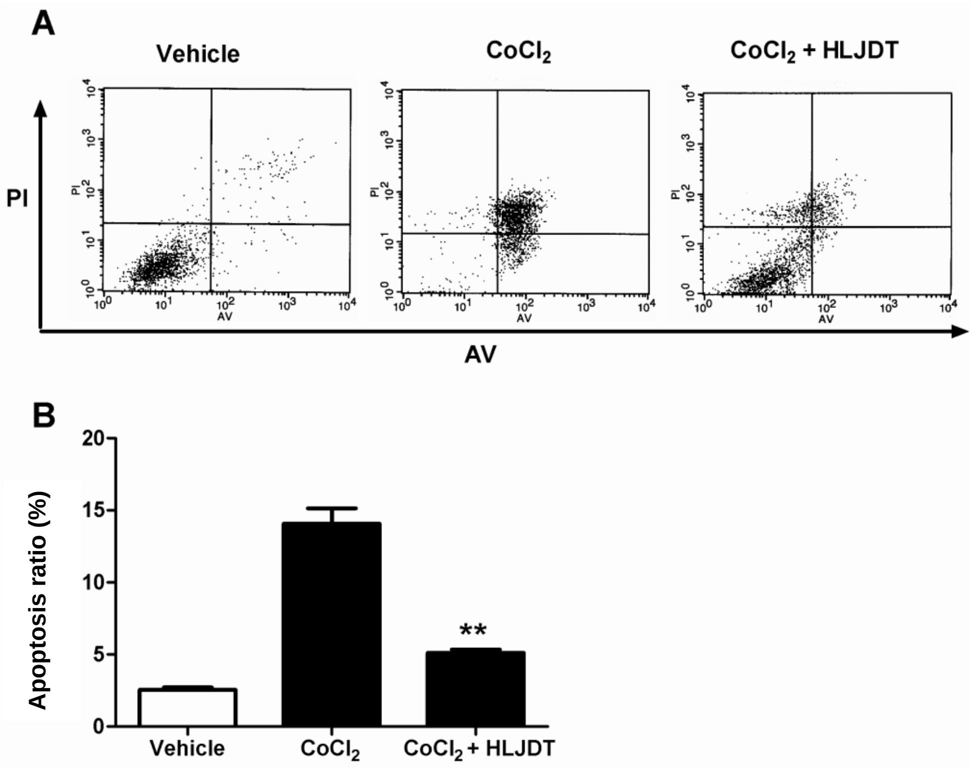

Figure 2. The effect of HLJDT on PC12 cell apoptosis under hypoxia. (A) PC12 cells treated with vehicle, $CoCl_2$ or $CoCl_2$ + HLJDT were double stained with PI and AV and analyzed by flow cytometry. (B) The apoptosis ratio of PC12 cells according to flow cytometry analysis. n = 4. Data are expressed as mean ± SEM. ** $P < 0.01$, versus $CoCl_2$.

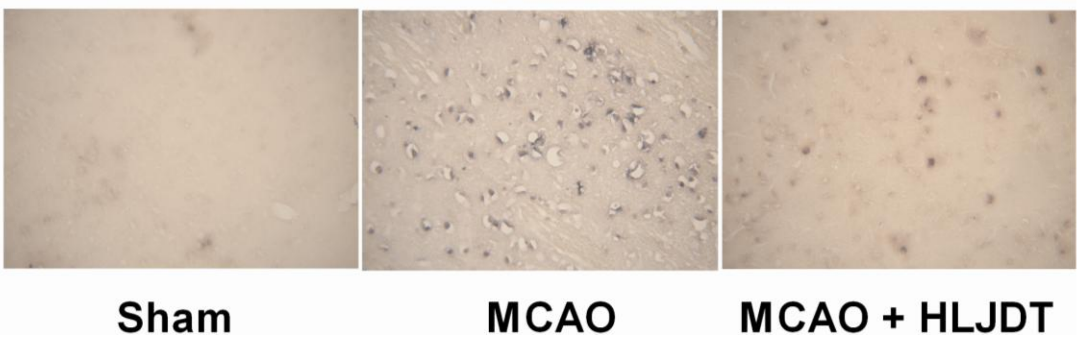

Figure 3. The effect of HLJDT on apoptosis in the penumbra of MCAO rats. Apoptosis was evaluated using TUNEL staining. HLJDT (2.5 g extract/kg) was introduced immediately following MCAO (magnification 400×) (n = 6).

Bcl-2 and Bax, which are involved in the extrinsic apoptosis pathway regulating caspase 9 and caspase 3, were shown to be responsible for the potential therapeutic effects of HLJDT on $CoCl_2$-treated PC12 cells. The benefits of HLJDT were demonstrated by the increase in the ratio of Bcl-2 to Bax (Figure 4C). These particularly pronounced regulations, which prevent the pathological fluctuation of apoptosis-associated proteins, further corroborate the antiapoptotic effect of HLJDT and reveal a possible mechanism of action.

The effect of HLJDT on HIF-1α in PC12 cells under hypoxia

The aforementioned observation raised the intriguing

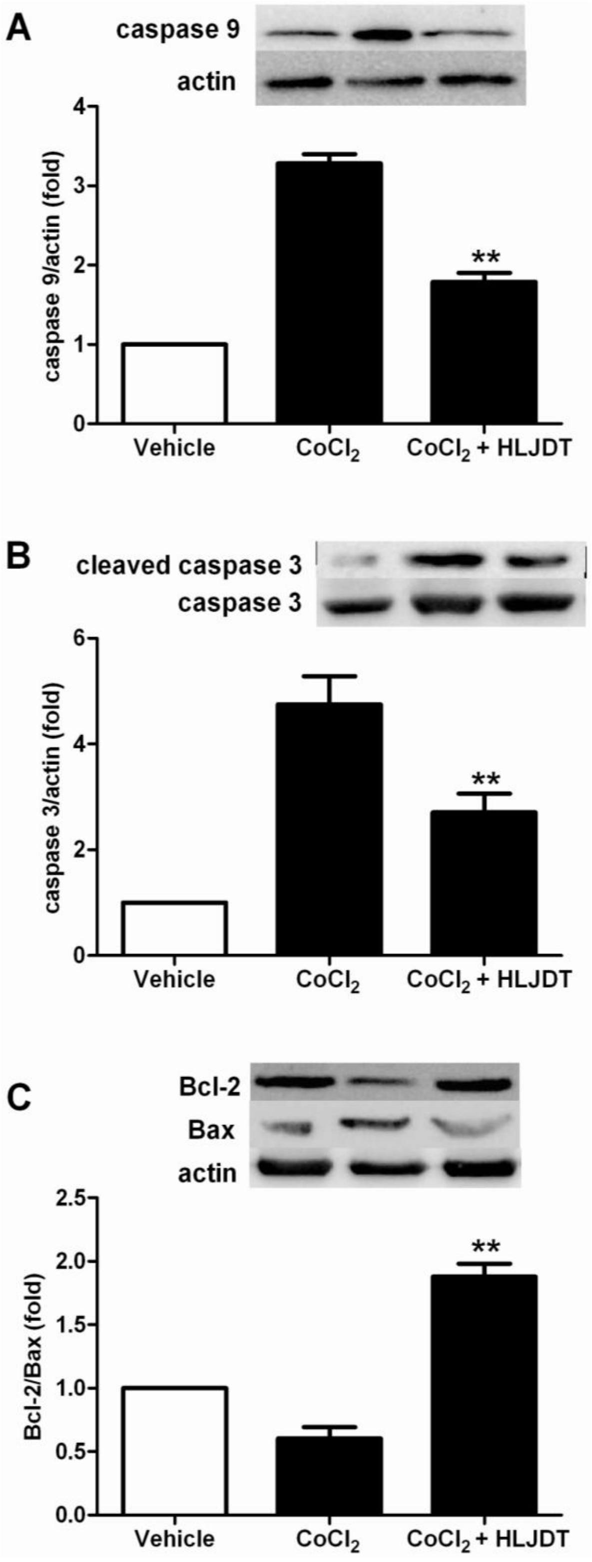

Figure 4. The effect of HLJDT on caspase 9, caspase 3 and Bcl-2/Bax in PC12 cells under hypoxia. Western blotting was performed to assay the levels of caspase 9 (A), caspase 3 (B) and the ratio of Bcl-2/Bax (C) in PC12 cells treated with vehicle, $CoCl_2$ or $CoCl_2$ + HLJDT (n = 3). Data are expressed as mean ± SEM. **P < 0.01 versus $CoCl_2$.

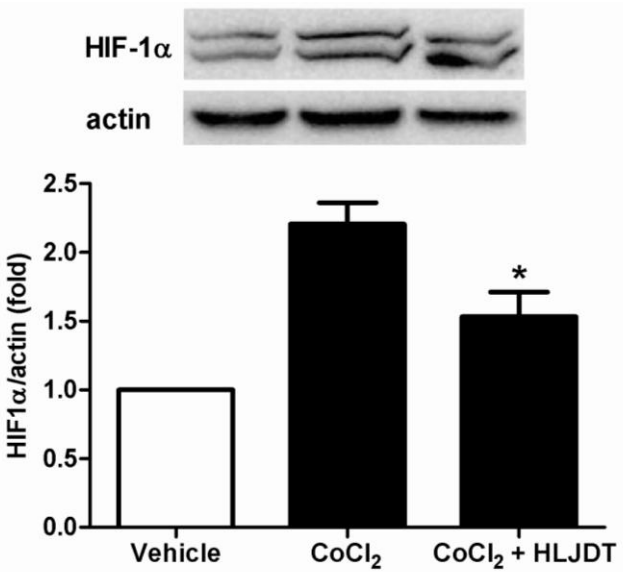

Figure 5. The effect of HLJDT on HIF-1α in PC12 cells under hypoxia. HIF-1α levels in PC12 cells treated with vehicle, $CoCl_2$ or $CoCl_2$ + HLJDT were detected with Western blotting (n = 3). Data are expressed as mean ± SEM. * P < 0.05 versus $CoCl_2$.

possibility that HIF-1, a transcription factor that mediates cellular apoptosis under pathological conditions, such as hypoxia or ischemia, may play a role in delivering the antiapoptotic effect of HLJDT. We tested this prediction with immunoblotting by analyzing the amount of HIF-1α, the primary subunit of HIF-1, in hypoxic PC12 cells in the absence or presence of HLJDT, while HIF-1α was markedly increased in cells treated with $CoCl_2$ alone, HIF-1α abundance clearly decreased in hypoxic PC12 cells exposed to HLJDT. These data allow us to speculate that HLJDT realizes its antiapoptotic effect in cerebral ischemia via lowering hypoxia-induced HIF-1α (Figure 5).

DISCUSSION

In the present study, we demonstrate that HLJDT has promising beneficial effects on the inhibition of neuronal apoptosis under hypoxia or ischemia, thus contributing to cerebral protection. The impact of HLJDT on apoptosis is mediated through reducing HIF-1α and regulating various apoptosis-related signaling molecules, such as p53, Bcl-2, Bax, caspase 9 and caspase 3. Apoptosis is the primary mode of cell death in penumbra tissue, the most potentially salvageable target for acute stroke therapeutics (Fisher, 2006). Preservation of penumbra tissue is an important therapeutic approach in deleterious acute stroke events. There are several mechanisms that have served as pharmacological targets. Besides glutamate antagonists and Ca^{2+} channel blockers, antiapoptotic strategies have also been shown to preserve

preserve penumbra tissue (Paciaroni et al., 2009). For example, caspase inhibitors have been able to promote neuronal protection in stroke animal models (Zivin, 2007). Here, we revealed the antiapoptotic effects of HLJDT on PC12 cells under hypoxia induced by $CoCl_2$ *in vitro* and on the penumbra tissue of rats under ischemia induced by MCAO *in vivo*. These results associated with neuronal protection are consistent with reports about HLJDT in transient cerebral ischemia, which have shown that HLJDT can work through various mechanisms including antiinflammation, antioxidation and regulation of neurotransmitters (Wang et al., 1997; Wu et al., 2010). Although, the exact relationship between antiapoptosis and those mechanisms is unclear, it is still reasonable to postulate that antiapoptotic effects similar to antiinflammation, antioxidation, regulation of neurotransmitters and elevation of blood flow are responsible for the beneficial therapeutic effect of HLJDT on stroke.

It is well known that HIF-1 is a pivotal transcription factor in regulating oxygen homeostasis. The role of HIF-1 in the response of the central nervous system to hypoxia was reported to be due to the duration and types of pathological stimuli (Vangeison et al., 2008). In the context of mild hypoxia, increased HIF-1 promotes expression of adaptive genes, such as vascular endothelial growth factor, erythropoietin and glucose transporter 1, which are documented to enhance neuronal protection (Ferriero, 2005). Furthermore, HIF-1 promotes cell survival through enhancing oxygen and nutrient availability, which is achieved by enhanced angiogenesis and increased glycolytic metabolism (Bergeron et al., 2000). Once hypoxia/ischemia becomes severe and persistent, the prosurvival role of HIF-1 shifts to prodeath. HIF-1 enacts this response partly through interacting with p53 and activating p53-dependent cellular apoptosis. Additionally, increased BNIP3 is regulated by HIF-1 following cerebral ischemia (Renton, 2003). Upon specific knockout of HIF-1 *in vivo*, much deteriorated tissue was observed after focal cerebral ischemia (Helton et al., 2005). The causative relationship between HIF-1 and caspase 3 was additionally indicated by evidence that HIF-1 could bind an element of the caspase 3 promoter, which was strongly augmented by ischemia (Van Hoecke et al., 2007). Although, $CoCl_2$ is commonly used as a preconditioning stimulus that induces the prosurvival response of HIF-1 (Bergeron et al., 2000), the hypoxia induced by $CoCl_2$ (375 μmol/L) in the present study, combined with lower glucose, resulted in the prodeath response: apoptosis with elevated HIF-1. Together with the decreased ratio of Bcl-2/Bax, increased caspase 9 and caspase 3 levels, one could postulate that HIF-1 initiates cell apoptosis under oxygen and glucose deprivation via regulating the function of Bcl-2/Bax, caspase 9 and caspase 3. HLJDT decreased the apoptosis induced by $CoCl_2$ and lower glucose through reducing HIF- 1 and its downstream signaling molecules.

In conclusion, although, the exact mechanism for the regulation of HIF-1α by HLJDT still requires further investigation, our study provides an explanation for the relationship between the antiapoptotic effects and neuronal protection conferred by HLJDT, which is valuable for understanding the mechanism of action of HLJDT in stroke therapeutics.

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Abbreviations: BNIP3, BCL2/adenovirus E1B 19 kDa interacting protein 3; **DAB,** 3, 3-diamino-benzidine tetrachloride; **ECA,** external carotid artery; **FITC,** fluorescein isothiocyanate; **HIF-1,** hypoxia-inducible factor 1; **HLJDT,** Huang-Lian-Jie-Du-Tang; **LDH,** lactate dehydrogenase; **MCA,** middle cerebral artery; **MCAO,** middle cerebral artery occlusion; **MTT,** 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; **OD,** optical density; **TUNEL,** terminal transferase-mediated dUTP nick end-labeling.

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