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Original Article

Caulis spatholobi promotes ischemic tolerance, inhibits apoptosis and oxidative stress by targeting HSP70 in ischemic stroke

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Abstract: Inflammation is usually correlative with the increase of active oxygen in the tissue, so oxidative stress is one of the key factors for the morbidity of ischemic stroke. *Caulis Spatholobi* (CS) has been used in oriental medicine to treat cancer and blood stasis, but the role of CS extractive for the treatment of ischemic stroke is rarely known. Adult Sprague-Dawley (SD) rats underwent middle cerebral artery occlusion (MCAO), and CS extractive was used to determine if it possesses brain ischemic tolerance activity. Neurological deficit and infarct area were evaluated after CS treatment in MCAO rats, and TUNEL assay used to determine the apoptosis of rats brain cells. The cerebral human microvascular endothelial cell line (hCMEC) was used for *vitro* experiments. *HSP70* gene silencing was performed with passive small interfering RNA in hCMEC cells. Nuclear staining was also performed for ROS production. Cell proliferation was evaluated by MTT assay, the apoptosis was evaluated by flow-cytometric, and western blot analyses were performed in rats brain tissue and hCMEC cells to determine the expression level of target protein. Our findings show that CS extractive can remarkably relieve neurological deficit and infarct size of MCAO rats, suppress the oxidative stress in the brain tissue, restrain the apoptosis of brain cells and promote the reproduction of hCMEC cells. In addition, CS extractive can remarkably inhibit the expression of P53, Caspase 3 and NF-κb 2 in MCAO rats and hCMEC cells, while the gene knockout of HSP70 can remarkably impact the curative effect of CS extractive. Our research findings show that CS can enhance the endurance capacity of cerebral ischemia, suppress cell apoptosis and reduce oxidative stress by targeting HSP70 in ischemic stroke.

Keywords: *Caulis Spatholobi*, HSP70, ischemic stroke, oxidative stress

Introduction

In the developed and developing countries, ischemic stroke has become a serious health problem, because it may result in high mortality and disability, thus bringing about enormous economic burden to the family [1, 2]. Temperate inflammatory reaction after the stroke is beneficial to the patient's recovery, but the excessive inflammatory reaction at the acute stage may cause further damage to the brain tissue [3, 4]. Therefore, it is better to decrease the inflammatory reaction at the acute stage of ischemic stroke, so as to lessen the cerebral injury and accelerate the recovery of cerebral functions [5]. Study shows that cerebral injury caused by oxidative stress and ischemic stroke are closely associated and NADPH oxidase may play a vital role at this stage. More NADPH oxidase after the cerebral injury can induce neuroinflammation and the expression of proinflammatory factors. Besides, NF-κb, as a transcription factor of inflammation, can regulate the expression of NADPH oxidase [6-8].

Caulis Spatholobi (CS; also called Jixueteng) is the dried vine stem of *Spatholobus suberectus Dunn* (Leguminosae). In clinical research, CS features the effect of tumor prevention and anti-inflammation [9-11]. In recent years, researchers have found that the bases of CS extractive consist of such compounds as flavonoid, terpene, sterols, anthraquinones, lactone and volatile oil [12, 13]. In order to probe for the protective action and mechanism of CS in the inflammatory reaction at the acute stage of

ischemic stroke, we extract the main ingredients of CS for *in vivo* and *in vitro* experimental study.

Despite the great interest in regulation of protein degradation in developing therapies against neuronal injury following stroke, the role of aberrant protein clearance in the outcome of ischemic stroke remains far from being understood [14-16]. Heat shock proteins (HSPs) are a set of highly conservative protein in mammals, which can be induced by different physiologic and environmental factors like anti-cancer chemotherapy and help cells survive under the impact of various deadly factors [17, 18]. HSP70 is the family member of DnaK/HSP70, which is a kind of important protein inducible to the stress. Intracellular HSP70 not only mediates chaperone-cytoprotective functions but can also block multiple steps in the apoptosis pathway [19, 20]. This inducible HSP70 can not only combine with abnormally folded proteins, but has been proved to combine with some proteins that can regulate cell apoptosis [21, 22]. Moreover, HSP70 can be initiatively released to the extracellular environment, thus accelerating the congenital and adaptive immunity reactions of cells [23, 24]. In the earlier research, we found that CS could remarkably up-regulate the expression of HSP70 in MCAO rats' brain tissue. Therefore, we suppose that HSP70 takes share in the ischemic endurance of stroke, and design *in vivo* and *in vitro* experiment in this study. Laboratory findings prove that the Chinese medicine CS extractive can restrain cell apoptosis and oxidative stress via the target HSP70 and enhance the ischemic endurance of brain tissue. Eventually this hypothesis has been verified.

Materials and methods

Preparation of CS active constituents

Weigh 200 g CS powder (Guangzhou Traditional Chinese Medicine University, China) and equally put it into four reflux units at 80°C respectively filled with 75% ethyl alcohol, ddH_2O, ethyl acetate and petroleum ether for 3 h. After filtering the solutions, first concentrate CS extractive with the rotatory evaporator, dry them in the vacuum oven and weigh the drying products. DMSO is used as the solvent to dissolve these 4 CS extracts into the solution of 3.5 mg/mL for the use of the follow-up experiment.

Animals and administration

Male Sprague-Dawley (SD) rats (n=50; 8-10 weeks; 200-220 g; Laboratory Animal Center of Southern Medical University of China) were used in this study. All the rats are raised in separate cages. Before the operation, and all the rats are raised for environmental suitability for one week, i.e., ambient illumination for 12 h for daytime/nighttime alternately and normal diet. After 7 days, middle cerebral artery occlusion (MCAO) model was used in ischemic stroke experiments [25]. For ischemic stroke experiments, rats were separated randomly into 5 groups: control group; sham group; IS group; IS plus CS group; CS group. The model of thrombus formation in the middle cerebral artery is based on the activation of the coagulation cascade induced by thrombin injection. IS plus CS group and CS group rats were given by gavage 3 hours after with or without induction of stroke. The experimental protocol was approved by Institutional Animal Care and Use Committee of the Southern Medical University of China. In order to minimize the suffering of mice, all surgery were conducted under sodium pentobarbital anesthesia.

Evaluation of neurological deficit and infarct area

The rats' neurological deficit is evaluated 24 h after MCAO operation. 0 point: the rats can act normally; 1 point: the rats can't stretch out the left paw; 2 points: the rats orbit around the left side; 3 points: the rats tilt to the left; 4 points: the rats are low-spirited and they can't walk by themselves [26]. When the evaluation on neurological deficit is completed, all the rats have to be killed after narcotized with ether. Next, collect the specimens of the rats' brain tissue, some of which are conserved in the freezer at -80°C for follow-up experiment and the rest are sliced into 2mm with the rat brain microtome. Dye these slices with 1% TTC in the darkness at 37°C for 30 minutes. The normal tissue will be dyed into red, while the infarcted tissue is unable to be dyed and remains white. Sip up the dye liquor on the surface of slice with the filter paper, scan and take photo of the slices

and analyze the size of infarcted area with Image J software.

TUNEL assay

Detect the cell apoptosis of the rats' brain tissue according to TUNEL kit (Scirince, Guangzhou, China) and user instructions provided by the reagent supplier. Redye the tissue slices with hematoxylin; select 5 fields of vision at random under 100× microscope; count the number of cells in the field of vision that are positive after dyeing with TUNEL and take the mean value. Dark brown represents apoptotic cells and the blue denotes nuclei. The cell apoptosis rate is the ratio of the cell population that is positive to TUNEL dyeing to the total cells in the field of vision.

Western blot analysis

After unfreezing the specimens, take 50 mg specimen each and treat the cells with the cell lysis buffer; extract the total protein with protein extraction kit (Beyotime, Haimen, China). After the protein is purified, take 50 μg specimen each for SDS-PAGE electrophoresis; transfer the membrane of products with nitrocellulose filter to produce primary antibody and second antibody. After photographic fixing and developing, analyze the optical density of stripes with Quantity One v4.4.0; with reference to GAPDH, the expression quantity of all proteins should be normalized upon Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Cell culture and siRNA transfection

The cerebral human microvascular endothelial cell line (hCMEC) was purchased from CICLR (Beijing, China). Cells were cultured in the DMEM (GIBCO-BRL) and supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma, USA), at 37°C, in 5% CO_2 incubator (Memmert, Germany) for 24 h. For hypoxia experiments, hCMEC cells after treatment with and without CS were cultured under hypoxic conditions (1% O_2, 5% CO_2, and 94% N_2) for 24 h. For knockdown experiments, *HSP70* gene silencing was performed with passive small interfering RNA (siRNA, purchased from Ribobio, China) following the manufacturer's protocol. HCMEC cell lines were transfected with HSP70 siRNA with Lipofectamine 2000 (Invitrogen). After trypsin digestion, cells were collected, counted, and centrifuged at 1000 r/min for 5 min to prepare a $5*10^6$~$5*10^7$ cell suspension and cryopreserved for later use. During dosing, the logarithmic growth phase cells were taken and seeded in 96-well culture plates at a concentration of $4*10^3$ per well. The volume of each well was 200 μl, and the aqueous solutions of CS extract were added respectively. Cells were separated randomly into 4 groups: control; hypoxia group (H); hypoxia plus CS (3.5 mg/ml, HC) group; siRNA plus hypoxia plus CS (3.5 mg/ml, SHC).

Cell viability

Cell Proliferation Reagent Kit I (Roche, USA) was used to evaluate cell proliferation of hCMEC. According to the user instructions from the reagent supplier, the cells under experimental treatment are collected and then transferred into the fresh culture medium with 1 mg/ml MTT for 3-hour incubation at 37°C. Shake gently to make sure that the product of formazan could be solved in 0.5 ml Islpropanol. OD value of such product could be measured through the spectrophotometer (Bio-Rad Model 550, Hercules, CA) of 570 nm wavelength, to calculate and analyze the data.

Flow-Cytometric analyses of apoptosis

The cells under experimental treatment are collected after 24-hour incubation and then cleaned with PBS buffer solution for 3 times. The cells are re-suspended with 1 ml binding buffer. Then, the cells are under double stain with FITC and PI for 15 minutes at room temperature. The flow cytometer is used to detect the cell apoptosis. All the cells are divided into living cells, dead cells, early apoptotic cells and later apoptotic cells.

Assays for superoxide dismutase (SOD) and malondialdehyde (MDA)

After the cells are cultivated for 24 hours, the cell culture media are collected. According to the user instructions from the reagent supplier, Total Superoxide Dismutase Assay kit (Beyotime, Beijing, China) is used to test SOD in the culture media. Lipid Peroxidation MDA Assay kit (Beyotime, Beijing, China) is used to test MDA in the culture media. All the tests should be

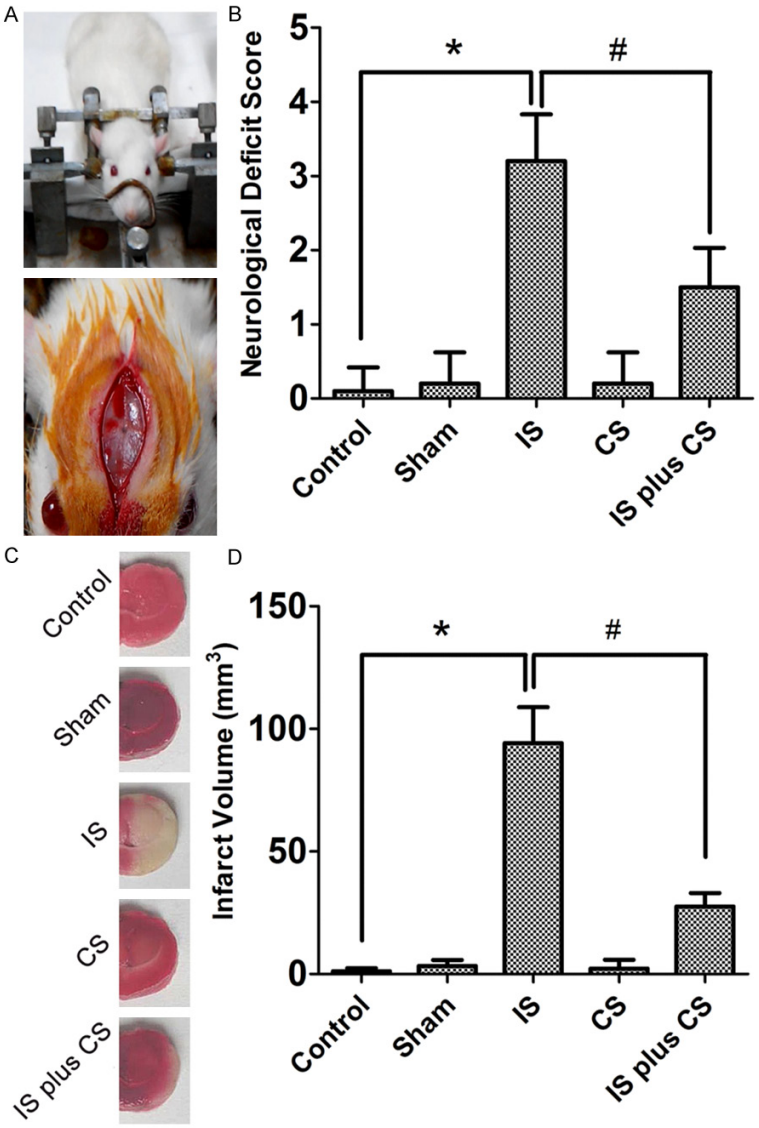

Figure 1. The effect of CS to reduce neuronal damage after MCAO. A: The rats MCAO model was constructed on the scaffold. B: Neurological deficit score in rats after MCAO. C: FJB staining of coronal sections from ischemic rat brains. The white indicates infarction. D: Measurement of infarct volume. Compared with control group, $^{*}P < 0.05$; compared with IS group, $^{\#}P < 0.05$.

Table 1. Neurological deficit score of 10 rats in each group

Gene name	1	2	3	4	5	6	7	8	9	10	Mean ± SEM
Control	0	0	0	1	0	0	0	0	0	0	0.10±0.32
Sham	0	0	0	0	1	0	1	0	0	0	0.20±0.42
IS	3	3	4	3	2	3	4	4	3	3	3.20±0.63*
CS	0	0	1	0	0	1	0	0	0	0	0.20±0.42
IS plus CS	2	1	2	1	1	2	1	1	2	2	1.50±0.53#

Compared with control, $^{*}P < 0.05$; compared with IS group, $^{\#}P < 0.05$.

repeated for 3 times, and then the average value is taken for the test data.

Statistical analysis

Data are expressed as mean ± SEM. The difference among groups was determined by ANOVA analysis and comparison between two groups was analyzed by the Student's t-test using GraphPad software version 5.0 (GraphPad Software, CA). Differences were considered significant at $P < 0.05$.

Results

CS decreased neurological deficit score of MCAO rats

In order to verify that CS could improve the ischemic tolerance of the rat, we adopted the instant middle cerebral artery occlusion surgery to simulate the rat's cerebral arterial thrombosis. The experimental result shows that the rat MCAO model is constructed successfully (**Figure 1A**). As compared with the rats in the sham-operated group, the rats in IS group suffer from poor nervous function. However, as compared with the rats in IS group, the rats in IS+CS group show higher ethology scores under CS pretreatment. This result shows that CS could decrease the scores of MCAO rat neurological deficit (**Table 1**; **Figure 1B**, $P < 0.05$).

CS decreased infact area of MCAO rats

In order to further verify that CS could reduce the brain damage caused by the rat ischaemia, we test the infarct size of MCAO rat brain tissue. As shown in **Figure 1C**, **1D**, we are unable to detect the ischemic lesions of rats in sham-operated group, while for the rats in IS group, infarct size is obviously increased. As compared

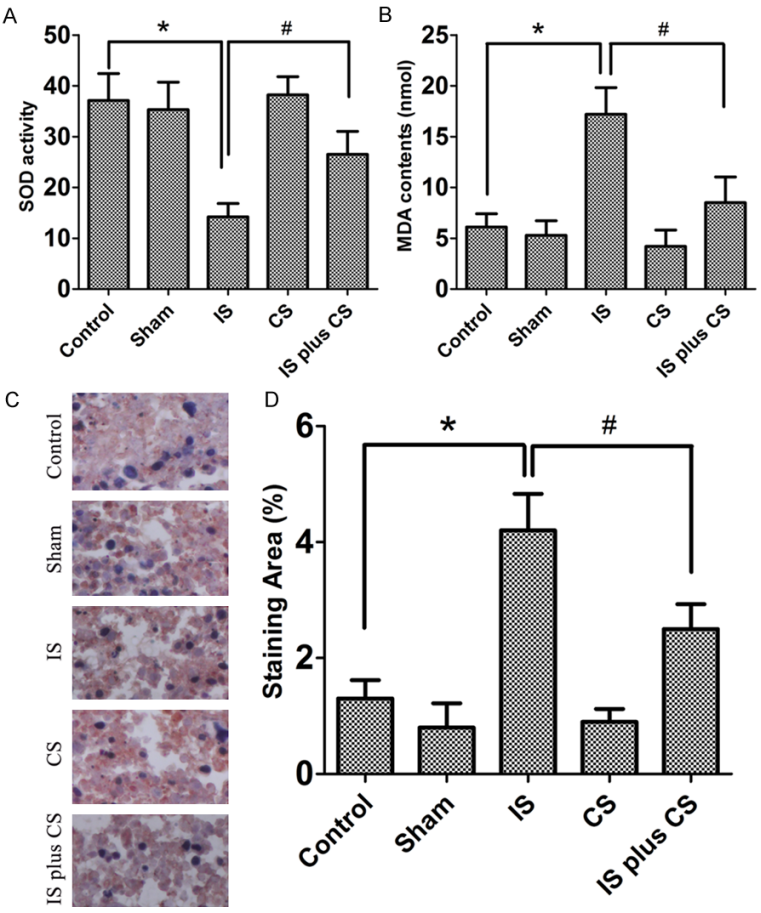

Figure 2. Assessment of oxidative states using assays of SOD and MDA, as well as apoptosis of brain cells in rats by the TUNEL assay. A: SOD activity in rats treated with IS exhibited a significant decrease compared with controls, whereas CS plus IS groups appeared to reverse that trend. B: MDA contents in rats treated with IS were significantly higher than those of controls, and MDA contents in CS plus IS groups were partly inhibited by CS addition. C, D: IS groups rats showed a significant increase in apoptosis rate compared with those of control groups, and CS can reverse the trend of apoptosis induced by IS. Dark brown represents apoptotic cells and the blue denotes nuclei. Compared with control group, $^{*}P < 0.05$; compared with IS group, $^{\#}P < 0.05$.

with the rats in IS group, the infarct size of rats in IS+CS group is obviously decreased after CS pre-treatment (**Figure 1C**, **1D**, $P < 0.05$). These results suggest that CS can reduce ischemia-caused brain injury in SD rats.

CS reversed the oxidative-stress reaction in MCAO rats

As compared with the rats in IS group, the rats in IS+CS group have an obviously lower oxidant MDA level for the brain tissue, while SOD activity of antioxidant is markedly increased (**Figure 2A**, **2B**, $P < 0.05$). Both of these indices did not show a significant difference between control and IS plus CS groups. These experimental results show that CS could reduce MCAO rat oxidative stress.

Inhibition of apoptosis by CS in MCAO rats

TUNEL experimental result shows that the apoptosis rate of brain tissue cells for the rats in IS group is obviously higher than that for the rats in the normal control group. However, after CS pre-treatment, the apoptosis rate of brain tissue cells for the rats in IS + CS group is substantially lower than that of the rats in IS group (**Figure 2C**, **2D**, $P < 0.01$). This result shows that CS could restrain the apoptosis of MCAO rat brain cells.

The expression of P53, Caspase3 and NF-κ-B2 was inhibited by CS addition in MCAO rats

Western blot experiment shows that expression level of P53, Caspase 3 and NF-κ-B2 protein in the brain tissue of rats in MCAO model group is substantially increased as compared with that for the rats in the normal control group. However, expression level of HSP 70 protein is obviously lowered. After CS pre-treatment, expression level of HSP 70, P53, Caspase 3 and NF-κ-B2 protein for MCAO rats is obviously changed as compared with that for the rats in MCAO model group (**Figure 3A**, **3B**, $P < 0.05$). These results show that HSP 70, P53, Caspase 3 and NF-κ-B2 protein may participate in the ischemic tolerance of MCAO rats.

Knockdown of HSP70 counteracted the effect of CS in suppressing apoptosis

In order to study the relationship between HSP70 expression pattern and CS treatment in ischemic tolerance, HSP70 gene silencing was

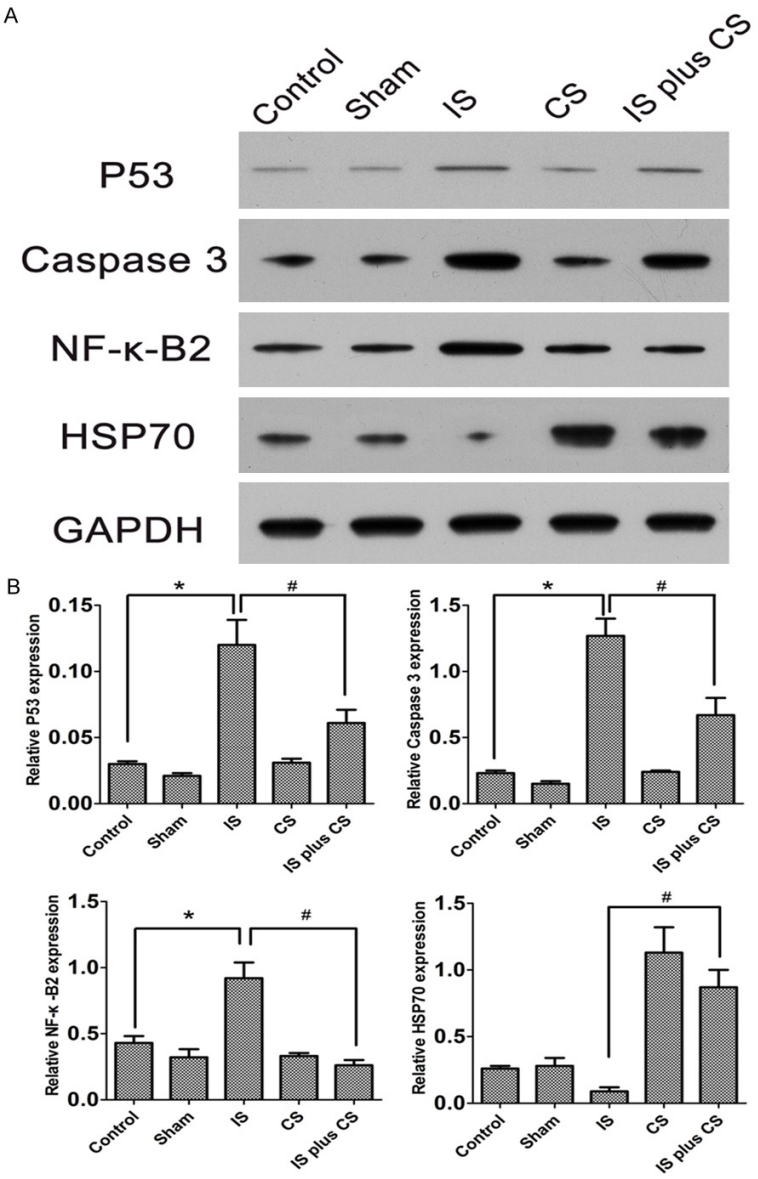

Figure 3. The expression of HSP70, P53, Caspase3 and NF-κ-B2 in IS rats brain tissue. Compared with control group, $^{*}P < 0.05$; compared with IS group, $^{\#}P < 0.05$. Experiments were performed in triplicate.

performed with passive small interfering RNA in hCMEC cells. Compared with the control, the expression of HSP70 in hCMEC cells transfected with HSP70 siRNA was suppressed significantly (**Figure 4A**, **4B**, $^{*}P < 0.05$). This result indicated that the hCMEC cells with HSP70 gene silencing was constructed successfully. And then, we examined effect of HSP70 siRNA on cell apoptosis after CS treatment. Compared with the hypoxic group, hCMEC cells incubated in hypoxic and CS addition conditions for 24 h had a decrease in apoptosis. Reversal of apoptosis was detected when cells were transfected with HSP70 siRNA compared with the hypoxia plus CS group (**Figure 4C**, **4D**, $P < 0.05$). This result is consistent with the expected.

Knockdown of HSP70 reversed cell proliferation in vitro

MTT experiment shows that as compared with the cells in normal control group, the cell viability of hCMEC under hypoxia treatment is obviously lowered. However, when hCMEC cells under hypoxia treatment are treated with CS, the cell viability could be substantially improved (**Figure 5A**). It is also interesting that the influence of CS could be obviously neutralized after the gene knockout of hCMEC cells through HSP 70. Based on the above experimental results, we conclude that CS could stimulate the growth of hCMEC cells after hypoxia treatment. This process may be mediated through HSP 70.

Knockdown of HSP70 reversed the expression of P53, Caspase3 and NF-κ-B2 after hypoxia and CS treatment in vitro

Western blot is used to test the influence of CS and HSP70 to expression of P53, Caspase 3 and NF-κ-B2 of hCMEC cells. The experimental result shows that CS could remarkably restrain the expression of P53, Caspase 3 and NF-κ-B2 protein of hCMEC cells after hypoxia treatment. However, such trend could be neutralized by gene knockout of Hsp 70 (**Figure 5B**, **5C**). These findings further support our assumption: CS targets Hsp 70 to restrain the cell apoptosis and oxidative stress, so as to promote ischemic tolerance.

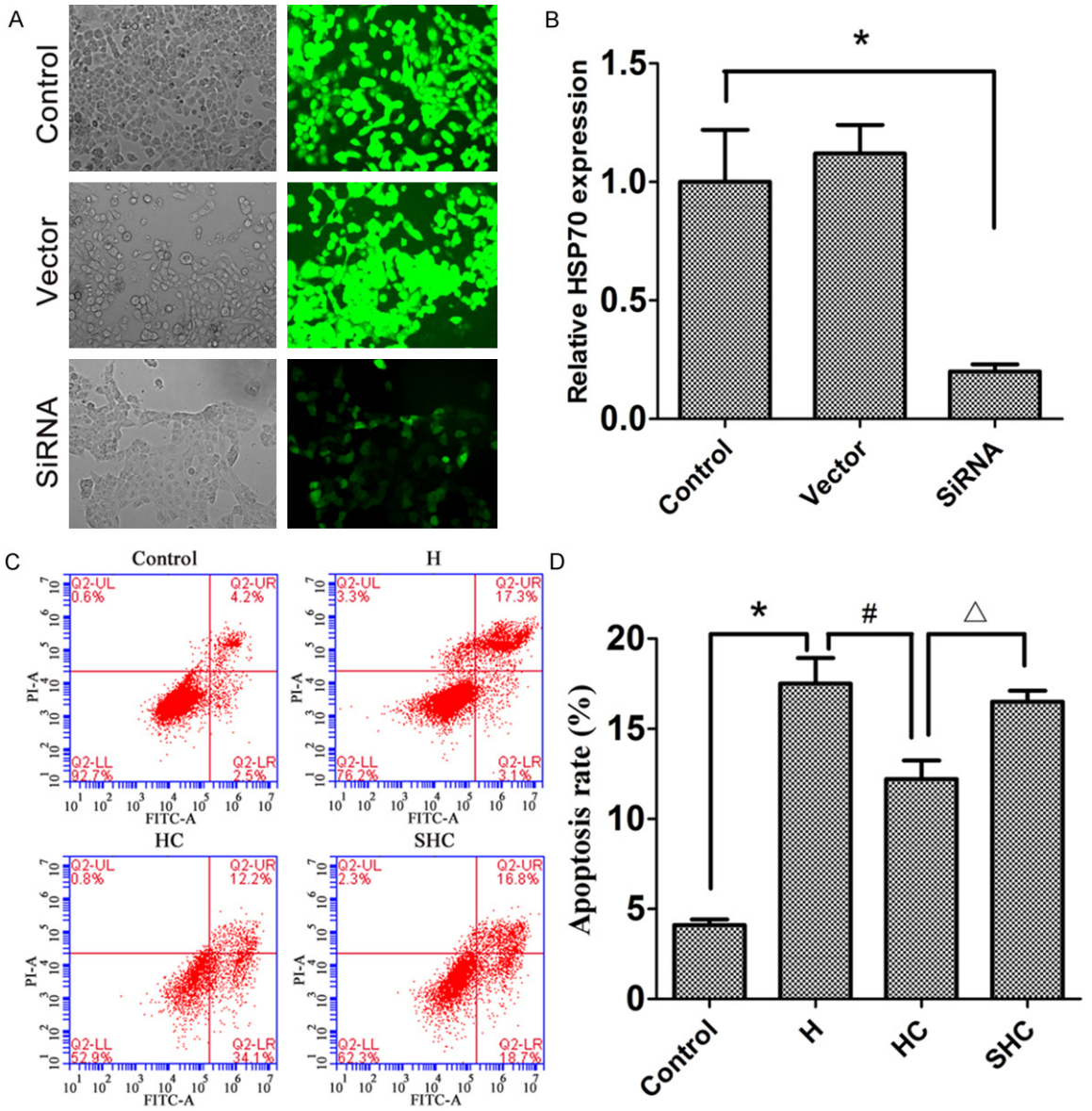

Figure 4. The hCMEC cells line with HSP70 gene silencing was constructed and the apoptosis of hCMEC cells was detected by flow cytometry. A, B: The expression of HSP70 was evaluated using fluorescence microscopy in hCMEC cells line after HSP70 gene silencing. C, D: Apoptosis results of hCMEC cells treated under hypoxic conditions, CS and HSP70 gene silencing. H, hypoxic condition; HC, hypoxic condition plus CS; SHC, hypoxic condition plus CS after HSP70 gene silencing. Compared with control group, $^{*}P < 0.05$; compared with H group, $^{\#}P < 0.05$; compared with HC group, $^{\Delta}P < 0.05$. Experiments were performed in triplicate.

Discussion

The active ingredients of CS could induce the mammal's cardiovascular protection, anti-inflammatory and oxidation resistance. In certain physiopathologic and biological process, CS extractive could restrain the production of ROS and adjust cell apoptosis. As the antioxidant in this research, CS extractive could produce the effect of anti-apoptosis. This has been verified. Though main ingredients of CS include flavonoid, terpene, sterols, anthraquinones lactone and volatile oil [27, 28], CS extractive is still not fully known in terms of the main mechanism of anti-inflammatory and antioxidation. Therefore, in this research, in order to discuss the modulating effect of CS extractive on the cell apoptosis in cerebral arterial thrombosis

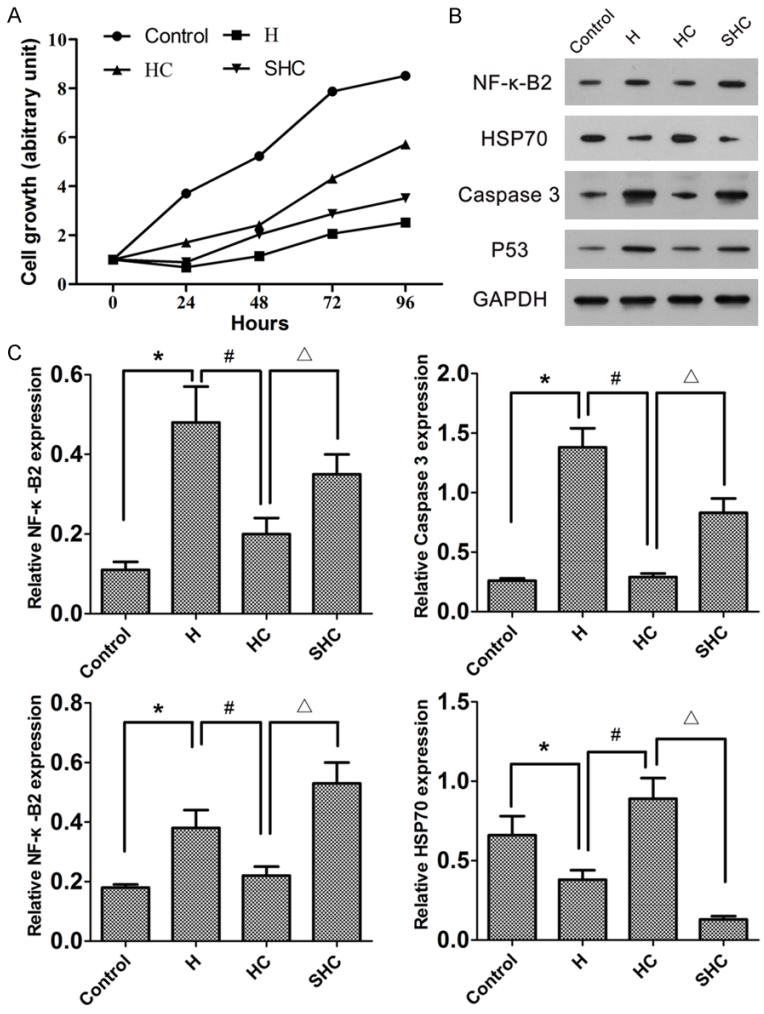

Figure 5. The cell proliferation and the expression of HSP70, P53, Caspase3 and NF-κ-B2with HSP70 gene silencing in hCMEC cells. A: The MTT assays were performed to determine the proliferation in hCMEC cells. B, C: The expression of HSP70, P53, Caspase3 and NF-κ-B2 in hCMEC cells. Experiments were performed in triplicate. H, hypoxic condition; HC, hypoxic condition plus CS; SHC, hypoxic condition plus CS after HSP70 gene silencing. Compared with control group, $^{*}P < 0.05$; compared with H group, $^{\#}P < 0.05$; compared with HC group, $^{\Delta}P < 0.05$. Experiments were performed in triplicate.

and mechanism thereof, the rat model for middle cerebral artery occlusion and cerebral microvascular endothelial cell line are used in the experiment. We score the neurological deficit and analyze the infarct size of MCAO rat as well as adopt TUNEL dying analysis to test the brain tissue cell apoptosis of MCAO rat. The analytical result shows that CS could substantially improve the neurological deficit scores and decrease the brain tissue infarct size of MCAO rat as well as restrain the brain tissue cell apoptosis of MCAO rat. The relationship between oxidative stress and cell apoptosis has been reported by many researchers [29-31]. So, *in vitro* testing is conducted in this research for oxidative stress and cell apoptosis of hCMEC cells. The result shows that CS could restrain the stress damage and cell apoptosis of MCAO rat. The overproduction of reactive oxygen could accelerate DNA damage and regulatory protein apoptosis, so as to cause cell trauma [32]. Certainly, SOD and MDA may be unable to offer the complete evidence for oxidative stress. However, the level of cell reactive oxygen may be reflected by these two indicators to some extent.

What is interesting is that CS can reverse the trend of levels of the oxidant MDA and antioxidant SOD. This is an indication that there are some important mechanism of CS involve in anti-oxidative stress. Thus, to develop potential therapy targeting ischemic stroke, we studied the roles of heat shock protein 70 (HSP70) in ischemic tolerance. HSP70 is expressed at low levels in normal brain but is induced in all cells following ischemia [33], HSP70 is massively induced in response to misfolded proteins following cerebral ischemia in all cell types, but is induced mainly in neurons in the ischemic penumbra [34]. Over expression of HSP70 via transgenes and viruses or systemic administration of HSP70 fusion proteins that allow it to cross the blood brain barrier protect brain against ischemia in most reported studies [35]. In this study, hCMEC cells line with HSP70 gene silencing was used to evaluate the effect of HSP70 involve in ischemic stroke. More interestingly, we found that knockdown of HSP70 can counteract the effect of CS in suppressing apoptosis and reverse cell proliferation after hypoxia treatment *in vitro*. Unfortunately, due to the high fatality rate, we failed

to get HSP70-knockout animals for this study, and we cannot simulate the process of ischemic tolerance with HSP70 knockout *in vivo* for the moment. The new approach will be explored to construct the SD rats with HSP70 knockout in future studies.

In summary, our studies show that CS extract can promote ischemic tolerance, inhibit apoptosis and oxidative stress by targeting heat shock protein 70 (HSP70) in ischemic stroke, which have laid us a theoretical basis for further understanding the mechanism of *Caulis Spatholobi* in ischemic stroke treatment.

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Disclosure of conflict of interest

None.

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