Protective effects of effective ingredients of Danshen (*Radix Salviae Miltiorrhizae*) and Honghua (*Flos Carthami*) compatibility after rat hippocampal neurons induced by hypoxia injury

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Abstract

**OBJECTIVE:** To investigate the effective ingredients of Danshen (*Radix Salviae Miltiorrhizae*) and Honghua (*Flos Carthami*) (Danhong) on protective properties towards neonatal rat hippocampal neurons under hypoxia condition.

**METHODS:** Primary culture of neonatal rat hippocampal neurons was used to model hypoxia damage on the hippocampus. Methyl thiazolyl tetrazolium (MTT) assay and preliminary experiments were conducted to identify the four effective ingredients of Danhong, along with the injection of positive drug control group and nine compatibility groups: the normal group, model group, age level. The cultured cells were randomly divided into 12 groups: the normal group, model group, positive drug control group and nine compatibility groups of the four effective ingredients. Different test methods were applied to determine lactate dehydrogenase (LDH), total superoxide dismutase (T-SOD), malondialdehyde (MDA), total superoxide dismutase (T-SOD), malondialdehyde (MDA), 6-keto prostaglandin F, (6-keto-PGF,.) tromboxane B, (TXB,), mitochondrial membrane potential (MMP), free calcium ions concentration ([Ca$^{2+}$]) and early onset.
cell apoptosis.

RESULTS: Different compatibility groups could inhibit the content of LDH and intracellular calcium overload, increase activity in T-SOD, decrease level of MDA and TXB₂, improve level of 6-keto-PGF₁α and MMP, and prevent the early onset cell apoptosis.

CONCLUSION: The compatibility of four effective ingredients of Danhong had protective effect toward hippocampi hypoxia. The mechanism might be related to inhibit oxidative stress damage and cell apoptosis, resist thrombosis, and reduce the intracellular calcium ion of overload.

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Keywords: Salviae Miltiorrhiza; Carthamus tinctorius; Hippocampus; Neurons; Cell hypoxia; Antioxidants; Thrombosis; Apoptosis

INTRODUCTION

Nowadays, Traditional Chinese Medicine (TCM) and their bioactive ingredients have become more and more popular in Asian and Western countries due to their stable therapeutic effects and weak toxicity in clinical. Danshen (Radix Salviae Miltiorrhizae) and Honghua (Flos Carthami) (Danhong) were the precious TCM widely used in China for thousands of years. They were used as medicine to promote blood circulations and remove blood stasis. Both of them were often used in Chinese traditional medicine to treat cardiovascular and cerebrovascular diseases. Their compatibility was accounted for the compatibility of mutual promotion (Xiangxu in Chinese) in TCM. Salvianic acid A (SAA), protocatechuic aldehyde (PA) and salvianolic acid B (SAB), which were the representative water soluble effective ingredients in Danshen (Radix Salviae Miltiorrhizae), had been proved to dilate blood vessels, improve blood flows and blood coagulation, stabilize mitochondrial membrane potential, antioxidant, anti-inflammatory and other benefits. Hydroxysafflor yellow A (HSYA) was one of the chalcone ketones, which was extracted from Honghua. Meanwhile, HSYA, a highest content in the safflower yellow, had the protective effects of ischemia induced mitochondrial damage in brain cells, enhancement function of the ability of the body to scavenge hydroxyl free radicals, inhibiting effects of peroxidation and neuronal apoptosis, etc. Most importantly, the above effective ingredients (SAA, PA, SAB, HSYA) that were constituted Danhong effective ingredients compatibility, had the ability to rehabilitate brain ischemia. The above mentioned chemical structures were shown in Figure 1.

Ischemic cerebrovascular disease (CIVD) happened when vertebral artery or carotid artery had deficient blood and oxygen supply resulting in dysfunctions of the brain. This disease had a characteristic of high incidence, lethality, and relapse rates. Numerous studies had reported that the main reasons for brain ischemia were due to secondary injury pathologic mechanisms. All these pathways were related to each other and often occurred together. Therefore, this research was devoted to studying the underlying protective mechanisms of Danhong effective ingredients compatibility on hypoxic hippocampal neurons through investigating formation of free radicals, calcium overload, cell apoptosis and the other ways. This would provide some valid evidences for new ideas for medicine and guidance in clinical medication, as well as experimental basis for clinical treatment of ischemic cerebrovascular disease.

MATERIALS AND METHODS

Animals

Healthy male Sprague-Dawley (SD) neonatal rat (body...
weight 6-8 g) born within 24 h with clean grade was used and provided by Shanghai Sippr-BK laboratory animal Co. Ltd. Animal license number was SCXK (Shanghai) 2014-0001.

**Chemicals and reagents**

HSYA (purity: ≥ 98%) was bought from Tianjing Phyto marker Co., Ltd. (Tianjin, China); SAA (purity: ≥ 98%) and SAB (purity: ≥ 98%) were purchased from Shanghai Tauto Biotech Co., Ltd. (Shanghai, China); PA (purity: ≥ 98%) was obtained from national institute for the control of pharmaceutical and biological products (Beijing, China); positive drugs (Nimodipine and Aspirin) were supplied by Bayer healthcare Co., Ltd. (Leverkusen, Germany); Dimethyl sulfoxide (DMSO) and methyl thiazolyl tetrazolium (MTT) were purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China); Neurobasal-A medium, B27 liquid supplement (50X) and 0.25% trypsin-EDTA (0.2%) mixed digestion solution were bought from America Gibco Co., Ltd. (New York, NY, USA); Poly-L-Lysine (relative molecular mass was 30-70 KD) was obtained from Japan Sigma Koki Co., Ltd. (Saint Louis, MO, USA); Mycoplasma free fetal bovine serum was got from Zhejiang Tianhang Biotechnology Co., Ltd. (Hangzhou, China); D-Hank’s buffer solution was got from Hangzhou Haotian Biotechnology Co., Ltd. (Hangzhou, China); Normal goat serum, readily available type of Neuron Specific Enolase (NSE) rabbit polyclonal antibody (enclosed type) and SABC-POD (rabbit IgG) kit, DAB kit were provided from Wuhan Boster Biological Technology Co., Ltd. (Wuhan, China); Lactate dehydrogenase (LDH) kit, total superoxide dismutase (T-SOD) kit, Malondialdehyde (MDA) kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China); Rat thromboxane B, (TXB.) Enzyme-Linked Immunosorbent Assay (ELISA) kit, Rat 6 keto prostaglandin F1α (6-keto-PGF1α) kit were supplied by Shanghai yuanye Bio-Technology Co., Ltd. (Shanghai, China); BCA protein concentration test kit, PIPA lysate (medium), JC-1 kit, Fluo-3/AM (Ca²⁺ fluorescent probe) were obtained from Beyotime Biotechnology Institute (Shanghai, China); FITC Annexin V Apotosis Detection Kit I was got from Becton Dickinson (BD) Company (Franklin Lakes, NJ, USA); Anhydrous Ethanol, concentrated sulfuric acid, potassium dichromate and other reagents were analytical regents which were made in China.

**Identify hippocampus neurons by immunochemistry NSE**

NSE was one of isoenzymes of glycolytic enolase in neuron. It had the highest activity in brain tissue. Identifying hippocampal neuron by NSE immunochemistry staining was stable as well as easy to operate. At the beginning of this method, origin medium was discarded and matured hippocampal neurons were washed with PBS 3 times, fixed with 4% pre-cooling paraformaldehyde for 30 min, and washed 3 times with PBS. Secondly, 0.3% Tritinin-100 was added for 30 min. PBS was added to wash for 5 min with 3 times after the Tritinin-100 was removed. Thirdly, the drop 10% normal goat serum was instilled in culture plates. After 30 min in a 37 °C, we removed the goat serum and instilled NSE primary antibodies. All the plates were placed in a humid box with 4 °C overnight. Fourthly, the plates were rewarmed under the room temperature for 30 min and washed with PBS 3 times for 5 min. Then, we instilled secondary antibodies and put the plates in constant temperature incubator with 37 °C for 30 min. We used SABC method to do coloration,
gradient ethanol to do dehydration, dimethylbenzene to do transparency, and neutral resin to do seal. Finally, the number of positive cells could be observed with inverted microscope.

**Hippocampal neurons hypoxia injury model**
The OGD injury model of rat hippocampal nerve cells was based on the references and improved. 19,20 Primary cultured hippocampal neurons of neonatal rat were cultured for 8 d in vitro. The well differentiated neurons cells were used for experiment. The culture medium was completely removed before oxygen deprivation. We washed the culture plates 3 times with the sugar-free Earle’s solution and put the sugar-free Earle’s solution in the culture plates. All the culture plates were placed in an oxygen-deficient device that was a microwave oven container box (Ethylene, the volume of 2 L). There were two small holes in the top of the left and right sides. We placed the medical three-way pipe end to insert into the holes and seal fixed. Finally, the other end was connected to the silicone tube that the mixture of 5% CO2, 94% N2 and 1% O2 was imported into the device. After 5 min, the inlet and outlet of the hypoxic device were occluded in order to simulate the process of ischemia in vivo.

**Groups and treatment**
According to the cytotoxicity test results from MTT, non-cytotoxic concentration of SAA, PA, HSYA, SAB, nimoipidine and aspirin were 0-200, 0-200, 0-150, 0-200, 0-200, 0-400 μg/mL, respectively. We selected appropriate high, medium, and low concentrations within the non-cytotoxic dose range of each ingredient (Table 1). According to the orthogonal experiment design of L9 (34) (Table 2), 9 groups of liquid culture medium which contained Danhong effective ingredients compatibility were prepared. There were 12 groups as follows: normal group, model group, positive drug control group (nimodipine or aspirin) and nine compatibility groups of the four effective ingredients.

**Determination of the levels of LDH, MDA, T-SOD, 6-keto-PGF1α and TXB2 in neuron cells**
Each group of centrifugal cells was collected and then rinsed with PBS. After that, we added cell lysate into each group of centrifugal cells, shook them for 5 s on the oscillator and then placed them on ice for 5 min. The above operation was performed more than 6 times. Supernatant protein sample from the high-speed centrifuge was collected, placed in the pre-cooled EP tube and stored in the –80 °C refrigerator. The above samples were used for testing the levels of MDA, T-SOD, 6-keto-PGF1α, and TXB2. And the collected cell culture medium of each group was determined the level of LDH.

According to the usage of each kit, the content of LDH was measured by chemical colorimetric method at 450 nm of wavelength.

The level of intracellular MDA was detected by TBA method at 532 nm. The activity of T-SOD was determined by the method of xanthine oxidase at 550 nm. The levels of 6-keto-PGF1α and TXB2 were investigated at 450 nm by ELISA.

**Determination of intracellular mitochondrial membrane potential (MMP)**
It followed from the instruction of kit that cells were managed with CCCP (10 μM) for 20 min which had been diluted 1000 times. After that, MMP was detected by the method of emplaced JC-1. For each hole in six-hole plate, we removed the nutrient solution, washed with PBS once, then added with 1 mL nutrient solution and 1 mL JC-1 liquid stain, thoroughly incorporated. The cells were placed into cell incubator at 37 °C for 20 min. After incubation, we removed the supernatant, utilized JC-1 buffer (1X) to rinse twice, added into 2 mL nutrient solution, and placed the cells under a confocal laser scanning microscopy for observations (excitation wavelength 525 nm, emission wavelength 590 nm). The average intensity of green fluorescence which was observed in six visual fields was calculated Image-Pro Plu 6.0 software. Finally, the data were compared with the average fluorescence intensity.

**Determination of intracellular free [Ca2+]i concentration in hippocampal neurons**
Cells were mixed with 1 μM of Fluor-3/AM solution, incubated in cell incubator for 15-60 min, washed with HEPES solution for thrice, and finally added 1 mL of HEPES solution to each hole. Cells were placed on the stage of the laser confocal microscope to observe with oculars under ordinary light source. We selected 488 nm of excitation light and 525-530 nm of emission wavelength through control computer. Fluorescence intensity analysis was performed on the required area using Image-Pro Plus 6.0 software.

**Detection of annexin V-FITC apoptosis**
The cultured neurons were digested with 0.25% trypsin, causing the adherent cells to cell suspension. The

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Table 1 Four factors and three levels of four effective ingredients compatibility (μg/mL)

<table>
<thead>
<tr>
<th>Level</th>
<th>SAA</th>
<th>PA</th>
<th>SAB</th>
<th>HSYA</th>
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<td>1</td>
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<td>3</td>
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Notes: SAA: salvianic acid A; PA: protocatechuic aldehyde; SAB: salvianolic acid B; HSYA: hydroxysafflor yellow A.
cell density was adjusted to $1 \times 10^4$ / mL by cell count plate and added into the flow tube. The cells were centrifuged at 1000 rpm for 5 min, discarded the supernatant, added 2 mL of D-Hank’s solution, vibrated the flow tube to make cells mixed, again centrifuged at 1000 rpm for 5 min, discarded the supernatant, and oscillated flow tube to make cells mix. 5 µL of Annexin V-FITC was added and 5 µL of propidium iodide (PI) was placed at room temperature for 30 min. Totally 400 µL of buffer was added and the apoptosis rate was measured by flow cytometry within 1 h, in order to calculate the rate of early apoptosis.

**Statistical data analysis**

For all analyses, the observer was blind to the identity of the samples. First, Shapiro-Wilk tests were performed on each data set to test for distribution normality. Statistical analyses were run with statistical package for the social science 17.0 (SPSS 17.0, International Business Machines corporation, New York, NY, USA). Data containing two experimental groups were analyzed using the Student-Newman-Keuls test. Statistical analyses on data containing more than two experimental groups were performed using two-way analysis of variance test, followed by Bonferroni post hoc analyses, to account for multiple comparisons. Data were presented as mean ± standard error of mean (SEM). $P < 0.05$ was considered statistically significant and $P < 0.01$ was considered extremely significant.

**RESULTS**

**Morphological observation and identification of primary hippocampal neurons**

Newly inoculated cells were translucent round, small in volume and suspended, evenly distributed. After inoculated for 4-8 h, almost all the hippocampal neurons were affixed to the 6-well culture plate walls which were pretreated by poly-lysine. Nutrient solution should be replaced after inoculating for 6 h but no more than 12 h. In the cell suspension, a large number of cell debris was firmly adhered on the culture plate wall. Furthermore, glial cells would split and would be difficult to suppress after 12 h. Inverted microscope was used to observe the different periods of hippocampal neurons. In this research, the representative photographs were manifested in Figure 2. After cultivating for 12 h, neuronal cells were all adherent to culture plate wall and became fusiform shape. A few neurons appeared linear and unbranched. After 24 h, the neurons of double protrusions, three protrusions and multiple protrusions were appeared. But the neurons of double protrusions were major neurons among them. At 2 d, the most of cell bodies became plump and bright. Moreover, glial cells were rare. At 3-4 d, cell protrusions gradually became longer and thicker. Finally, some protrusions were connected together and stag-
gered into a network. At 7-8 d, cell bodies became plump, round, fusiform, or polygon. And nucleolus turned to be visible. The cell bodies protruded a large number of protrusions and connected to each other into a neural network, which was a typical structure characteristic of hippocampal neurons. The cells had been mature at this time.

In this research, cells cultured for 8 d were subjected to detection of immunofluorescence. It was proved that the cultured cells were hippocampal neurons. The staining result was shown that cell bodies of the hippocampal neurons were plump. In addition, the well growing protrusions were become thick and then intertwined into a network. Based on the above information, the status of hippocampal neurons was suitable for further use. The result of specific enolization dyeing of hippocampal neurons was shown in Figure 3.

![Figure 3 Specific enolization dyeing of hippocampal neurons (× 100)](image)

Immunohistochemistry NSE dyeing were observed hippocampal neurons by invert contrasted fluorescent microscope. The staining result was shown that cell bodies of the hippocampal neurons were plump and the purity of hippocampal neurons was more than 90%.

Moreover, the purity of hippocampal neurons was more than 90%.

**Effect of hypoxia for the morphology of hippocampal neuron cells**

After hypoxia injury, there were significant changes in the morphology and survival rate of hippocampal neuronal cells. The phenomena of protrusion shortening, halo disappearing, refractivity decreasing and granular substances increasing were taken place. Eventually, cell bodies were swollen, deformed and collapsed completely (Figure 4).

![Figure 4 Effect of hypoxia for the morphology of hippocampal neuron cells](image)

After glucose deprivation and hypoxia injury, primary hippocampal neurons were not be dyed. After that, its observation and identification were checked by invert contrasted fluorescent microscope. A: normal group (× 100); B: model group (× 100). Compared with normal group, The phenomena of protrusion shortening, halo disappearing, refractivity decreasing and granular substances increasing were taken place in model group. Eventually, cell bodies were swollen, deformed and collapsed completely.

Free radicals and lipid peroxides could destroy the structure of nerve cells, so that the damage degree was aggravated.

After the oxygen-Glucose deprivation of hippocampal neurons, a series of cascade reaction will be happened in rat hippocampal neurons that included abnormal thrombosis index. Thromboxane B2 and 6-Keto-PGF1α were stable metabolites of thromboxane A2 and prostacyclin, respectively. They could represent the activity of thromboxanes and prostacyclin in vivo. However, thromboxane A2 and prostacyclin were contradictory. Thromboxane A2 was known to be a strong vasoconstrictor substance and had the function of promoting platelet aggregation. But prostacyclin had the effects of vasodilator and anti-platelet aggregation that could antagonize the action of thromboxane. The above two indexes were in dynamic equilibrium in normal people. Once dysequilibrium, thrombus would be formed. It was hard to detect thromboxane A2 and prostacyclin due to their unstable nature. Therefore, thromboxane B2 and 6-Keto-PGF1α were detected to know the functional status of thromboxane and prostacyclin in clinic.

The consequences of the levels of LDH, MDA, T-SOD and 6-keto-PGF1α, TXB, were shown in Figures 5 and 6, respectively. In contrast with the normal group, the releases of LDH were extremely elevated in the model group (P < 0.01). It was indicated that the cell membrane of hippocampal neurons was damaged. In other words, permeability of cell membrane was increased which led to increase the amount of LDH leakage. Other compatibility groups, except group 4, had significant difference in comparison with the model group (P < 0.01). And compared with the model group, the level of LDH was declined markedly in the nimodipine group (P < 0.01). It was revealed that the compatibility groups had therapeutic effects on LDH leakage. In comparison with the normal group, the activity of T-SOD was sharply descended in the model group (P < 0.01). This was indicated that the activity of intra-
cellular T-SOD had been greatly declined after in the anoxic hippocampal neurons. The above results led to generate large amount of oxygen free radical, which could be destroyed the balance of oxygen free radical. Compared with the model group, in the levels of T-SOD, groups 6 and 7 had no significant difference \((P > 0.05)\); the other groups, except group 5 \((P < 0.05)\), were increased distinctly \((P < 0.01)\); the nimodipine group also was rised apparently \((P < 0.01)\). It was verified that the compatibility groups had helpful effects on antioxidant.

In contrast with the normal group, the model group extremely elevated the levels of MDA in the anoxic hippocampal neurons \((P < 0.01)\). It was demonstrated that a large number of oxygen free radicals after hypoxia were reacted with lipids, proteins and nucleic acids. Compared with the model group, in the levels of MDA, groups 5 and 6 had no significant difference \((P > 0.05)\); the other groups, except groups 4 and 9 \((P < 0.05)\), were reduced dramatically \((P < 0.01)\); the nimodipine group was also dropped significantly \((P < 0.01)\). It demonstrated that the compatibility groups had beneficial effects on anti-lipid peroxidation.

In comparison with the normal group, the levels of 6-keto-PGF\(_{1\alpha}\) was sharply fallen in the model group \((P < 0.01)\). It was shown that there was a great deal of resistance to synthesize the intracellular 6-keto-PGF\(_{1\alpha}\) after hypoxia injury. Compared with the model group, in the levels of 6-keto-PGF\(_{1\alpha}\), groups 1-6, except groups 7, 8 and 9 \((P < 0.05)\), were raised rapidly \((P < 0.01)\);
0.01). In comparison with the normal group, the levels of TXB, was sharply elevated in the model group ($P < 0.01$). Compared with the model group, in the levels of TXB, groups 1-4 and 9 had no significant difference ($P > 0.05$); the other groups, except groups 5 and 6 ($P < 0.05$), were reduced dramatically ($P < 0.01$). When comparing the model group, the aspirin group had extremely significant difference ($P < 0.01$) in levels of 6-keto-PGF, and TXB. The above results demonstrated that the compatibility groups had positive effects on antithrombotic and thrombolytic therapy.

**Influences of Danhong effective ingredients compatibility on hippocampal hypoxia injury in the fluorescence intensity of MMP**

There was a mitochondrial membrane permeability conversion pore at the inner and outer border of the mitochondrial membrane. It would open up when a variety of noxious stimulated, such as hypoxia and increase of intracellular Ca$^{2+}$. A series of problems were generated, for example, the decrease or even disappear of mitochondrial membrane potential, the rupture of the respiratory chain, the decline of oxidative phosphorylation and the reduction of ATP synthesis. Based on the above situations, the cells were dysfunctional till apoptosis at last. The results of the fluorescence intensity of MMP were shown in Figure 7. For most of the cells, MMP would completely disappear after dealing with 10 μM of CCCP for 20 min. Green fluorescence was observed after JC-1 staining. While red fluorescence was observed in the normal cells after JC-1 treatment. In comparison with the normal group, the fluorescence intensity of MMP was sharply fallen in the model group ($P < 0.01$). It was shown that hypoxia could reduce the MMP of hippocampal neurons. Compared with the normal group, in the fluorescence intensity of MMP, each groups, except group 4 ($P > 0.05$), were ascended notably ($P < 0.01$).

**Effects of Danhong effective ingredients compatibility on hippocampal hypoxia injury in cell early apoptosis rates by flow cytometric method**

After the injury of hippocampal neurons, the apoptotic protein and apoptosis inducing factor were released by cells caused by the above phenomena of MMP abnormalities and calcium overload, that triggered the cascade reaction. The apoptosis of the cells was formed in the end. The outcomes of changes of intracellular free [Ca$^{2+}$]i were shown in Figure 8, respectively. In comparison with the normal group, the content of intracellular free [Ca$^{2+}$]i was sharply elevated in the model group ($P < 0.01$). It was revealed that hypoxia could generate the increase of [Ca$^{2+}$]i content. Compared to the model group, in the content of intracellular [Ca$^{2+}$]i, each group, except group 6, was reduced significantly ($P < 0.01$).

**DISCUSSION**

Hippocampus, which was the most sensitive area for hypoxia in brain tissue, was an important structure of central nervous systems. Hence, hippocampus was always the important objective for the researches of stroke. There were much neurons and less non-neurons distributed in hippocampus. It was easy to take samples due to its unambiguous and straightforward outline. For this reason, hypoxia-injured model of hippocampal neurons *in vitro* was an ideal experimental
Once suffering cerebral ischemia, free radical level in vivo was increased significantly. At the same time, the scavenging system of free radical had been damaged that led to reduce the scavenging abilities. In the mechanism of cerebral ischemia, free radical damage was one of the important mechanisms and key factors, in which the harm of ·OH was considered to be the most mischief. The unsaturated fatty acids in biofilm were susceptible to be attacked by free radicals. This lipid peroxidation was regarded as the indicator of cerebral ischemic injury. The consequence of this indicator destroyed the biological membrane, caused increase the permeability of membrane, and released a large number of LDH to the outside of the cells. Finally, cells would become edema and its microcirculation was destroyed. The levels of LDH in cell cultured medium could reflect the permeability and completeness of the cell membrane. MDA was known as the metabolite that was generated through the lipid peroxidation between biological membrane unsaturated fatty acids and oxygen free radical. The change of concentration of MDA indirectly reflected the concentration of oxyradical in tissue and the degree of cell damage. T-SOD was a free radical scavenger and an enzyme which could catalyze the decomposition of H_2O_2. The way of cell protection was that super anionic free radicals were removed by the disproportionation reaction. The vitality of T-SOD could reflect the change of free radical in vivo. Besides, it could protect the structure of the cell membrane as well as prevent the function of cell membrane from peroxide interference and damage. In this research, we found that each groups of Danhong effective ingredients compatibility had the beneficial effects of different extent after hippocampal hypoxia injury in the levels of LDH, MDA, T-SOD. It suggested that Danhong effective ingredients compatibility had the therapeutic effect to antioxidation reaction. Especially,
And TXA was one of the strongest biological products through the catalysis of platelet thromboxane synthase. which had the strong abilities of platelet aggregation maintaining normal homeostatic function. The dynamic equilibrium between thromboxane A₂ (TXA₂) and prostacyclin (PGI₂) was the foundation for the phenomena of platelet adhesion, aggregation and release, as well as inhibiting coagulation activities. The increase of lipid peroxidation could not only cause the occurrences of cell dissolution, platelet membrane injury that promoted the aggregation of platelet, but inhibit the activity of PGI₂ synthase in arterial walls, which reduced the cell dissolution, platelet membrane injury that promotes the aggregation of platelet.

Figure 9 Effects of Danhong effective ingredients compatibility on hippocampal hypoxia injury in cell early apoptosis rates A: normal group; B: control group; C: nimodipine group; D-L: groups 1-9; M: cell early apoptosis rates in each group. The four quadrants: the upper left quadrant was the mechanical death cells; the lower left quadrant was the normal cells; the upper right quadrant was the later apoptotic cells; The lower right quadrant was the early apoptotic cells. The data are expressed as the mean ± standard error of mean, n = 6. *P < 0.01 vs normal group; †P < 0.01 vs model group.

group 3 of LDH, group 8 of T-SOD and MDA were closer to the normal group after treatment. These outcomes might be related to the pharmacological effects of PA and HSYA. In other words, PA and HSYA had played a greater pharmacological role in antioxidant aspects. The dynamic equilibrium between thromboxane A₂ (TXA₂) and prostacyclin (PGI₂) was the foundation for maintaining normal homeostatic function. TXA₂, which had the strong abilities of platelet aggregation and vasoconstriction to stypticity, was generated through the catalysis of platelet thromboxane synthase. And TXA₂ was one of the strongest biological products for shrinking blood vessels and platelet aggregation. PGI₂ was mainly produced in endothelial cells of blood vessels. As a relatively strong platelet aggregation inhibitors, it could be used for inhibiting the phenomena of platelet adhesion, aggregation and release, as well as inhibiting coagulation activities. The increase of lipid peroxidation could not only cause the occurrences of cell dissolution, platelet membrane injury that promoted the aggregation of platelet, but inhibit the activity of PGI₂ synthase in arterial walls, which reduced the production of PGI₂. Based on the above process, the ratio of TXA₂ and PGI₂ was disordered, which caused the injury of organism. Due to the instability of TXA₂ and...
PGI₂, TXB, and 6-keto-PGF₁₀, that was the metabolites of TXA₂ and PGI₂, were preferred as the indices to determine the concentration of TXA₂ and PGI₂. In this study, we found that each groups of Danhong effective ingredients compatibility had the positive effects of different extent after hippocampal hypoxia injury in the levels of TXB₂ and 6-keto-PGF₁₀. It implied that Danhong effective ingredients compatibility had the helpful effect on the treatment of antithrombotic thrombolysis. Specially, group 4 of 6-keto-PGF₁₀, group 7 of TXB₂, were more closer to the normal group after treatment. These consequences might be related to the pharmacological effects of SSA, SAB and HSYA. Namely, SSA, SAB and HSYA had played a greater pharmacological role in antithrombotic thrombolysis aspects.

In the process of cell apoptosis, there were three main signal transduction pathways that were mitochondrial pathway, death receptor pathway and endoplasmic reticulum pathway. All the pathways were closely related to Ca²⁺. Normally, Ca²⁺ in cytoplasm was kept in a low level. The concentration of Ca²⁺ in extracellular, mitochondria and endoplasmic reticulum was much higher than the cytoplasm. Therefore, the slight release of Ca²⁺ would dramatically increase the content of [Ca²⁺]. The change of content of [Ca²⁺]i induced to open the permeability transition pore (PTP) which allowed the molecules that Relative molecular mass was more than 1500 to pass. It made a balance between ions and respiratory chain substrates in the mitochondrial stroma and cytoplasm. After that, it led to dissolve the decoupling of mitochondrial electron transport chain and oxidative phosphoric acid, decline the membrane potential, decrease the composite of ATP and the content of reduced glutathione, as well as increase the content of intracellular reactive oxygen, the phenomena of expansion of the mitochondrial matrix, reduction of the outer membrane folds and the superficial area, et al. Then, the cytomembrane was easy to rupture, and pro-apoptotic proteins were released in large quantities. The above situation eventually led to apoptosis. In this experimentation, we concluded that each group of Danhong effective ingredients compatibility could improve the mitochondrial membrane potential, reduce the content of intracellular [Ca²⁺]i and the rate of cell apoptosis in different extent after hippocampal hypoxia injury. It declared that Danhong effective ingredients compatibility could inhibit intracellular calcium overload and early stage of apoptosis. Particularly, group 7 of MMP and intracellular free [Ca²⁺]i, group 9 of cell early apoptosis rate were more closer to the normal group after treatment. These consequences might be related to the pharmacological effects of SSA, PA and SAB. That was to say, SSA, PA and SAB had played a greater pharmacological role in inhibiting intracellular calcium overload and early cell apoptosis.

Calcium, an important second messenger, was participated in releasing the neurotransmitters and transmitting the signals. After cerebral ischemia-reperfusion injury, a large number of electrons overflowed from mitochondria due to the influx of Ca²⁺. Free radicals and lipid peroxides, such as MDA, were generated by the reaction of electron breathing chains with oxygen. The enzyme activity of scavenging free radicals, such as SOD, had reduced. A large amount of free radical was accumulated under above situation that could further cause the disorder of the entry and exit of the cell membrane to Ca²⁺. Finally, it was formed a vicious circle, such as massive accumulation of free radicals and the serious overloading of Ca²⁺. A large excess of free radicals, lipid peroxides and high concentration of Ca²⁺, could damage the membrane structure of neurons. At last, the cells became dysfunctional till apoptosis.

In conclusion, Danhong effective ingredients compatibility, especially in groups 3, 4, 7, 8 and 9, could reduce the measure of LDH, MDA, and TXB₂, and increase the activity of T-SOD and levels of 6-keto-PGF₁₀, MMP, respectively. The underlying mechanism may be attributed to the inhibition of intracellular calcium overload and early cell apoptosis. Although the exact mechanism of the effects of Danhong effective ingredients compatibility remained to be further explored, our data pointed out the direction for the upcoming mechanism research for Danhong compatibility on stroke patients. Simultaneously, it provided theoretical and experimental proofs to improve Traditional Chinese Medicine preparation and efficacy.

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