



Effects of Danhong injection on hemodynamics and the inflammation-related NF- κ B signaling pathway in patients with acute cerebral infarction

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ABSTRACT. The objective of the current study was to investigate effects of Danhong injection on hemodynamics, inflammatory cytokines, and the NF- κ B pathway in acute cerebral infarction. In total, 246 patients with acute cerebral infarction were divided into control (N = 121) and observation (N = 125) groups based on treatment. The control group underwent conventional treatment, while the observation group was treated with conventional medicine and Danhong injection. Fourteen days later, the curative effect, hemorheology, mRNA, and protein levels of inflammatory cytokines (IL-6, TNF- α , and IL-1 β) in peripheral white blood cells, and changes in the NF- κ B signaling pathway were analyzed. The observation group had a significantly higher curative effect compared to the control group. The hemodynamic indices (high shear viscosity, low shear viscosity, plasma viscosity, hematocrit, platelet aggregation rate, and erythrocyte aggregation index) were significantly improved in both groups, although changes were more remarkable in the observation group. Peripheral white blood cells from patients in the observation

group had significantly lower mRNA and protein levels of inflammatory cytokines IL-6, TNF- α , and IL-1 β after treatment compared to cells from patients in the control group. NF- κ B p65 in the cytoplasm of peripheral blood cells of the observation group increased significantly after treatment compared to that of the control group, while nuclear NF- κ B p65 decreased compared to that in the control group. In conclusion, Danhong injection has a significant curative effect on patients with acute cerebral infarction, lowers inflammation, and improves hemodynamic changes; therefore, it is worth clinical application.

Key words: Danhong injection; Acute cerebral infarction; Inflammation; Hemodynamics

INTRODUCTION

Acute cerebral infarction is a common clinical cardiovascular disease that occurs suddenly and has a high disability and death rate, and as such, is a serious human health concern (Wartenberg, 2012). Increases in blood viscosity, aggregation of blood platelets, and inflammatory cytokines cascades are primary causes of a second thrombus after thrombolysis and patient death (Orlov et al., 2007; Puddu et al., 2010). Therefore, it is important to effectively decrease blood viscosity and reduce inflammatory reactions to improve hypercoagulable states, which in turn reduces the risk of rebleeding after thrombolysis. NF- κ B is expressed in almost all cells because it is an essential transcription factor with an important function in regulating synthesis and expression of inflammatory cytokines (O'Driscoll et al., 2015). Without stimulation, NF- κ B is located in the cytoplasm in an inactive state, whereas upon cellular stimulation, NF- κ B is activated and translocated to the nucleus where it binds to response elements of target genes to initiate gene transcription (O'Driscoll et al., 2012). Moreover, NF- κ B can mediate the release of many inflammatory mediators. Recently, NF- κ B has been found to be activated in patients with acute cerebral infarction, and thus, it is possibly involved in the synthesis of peripheral inflammatory cytokines, which promote the development of the disease (An et al., 2013). Danhong injection is a preparation of traditional Chinese medicine extracts and has been extensively applied as a clinical treatment for cardiovascular and cerebrovascular diseases with good effects (Shi et al., 2010; Zhang and Zhang, 2012). However, reports on the mechanisms of action that achieve its clinical results are rare. The current study systematically analyzes the effects of Danhong injection on acute cerebral infarction and its influence on inflammatory mediators, blood viscosity, and on the NF- κ B pathway with the goal of providing a mechanistic basis for its curative action.

MATERIAL AND METHODS

Study participants

In total, 246 cases of acute cerebral infarction were admitted into the First Affiliated Hospital of Zhengzhou University from June 2010 to December 2012. The inclusion criteria for study participation were as follows: 1) diagnosis of cerebrovascular disease by the standards revised by the fourth National Academic Conference on cerebrovascular disease of the Chinese

Medical Association (Kang et al., 2009); 2) patient age between 28 and 75 years; 3) disease occurrence within 72 h; 4) no cerebral hemorrhage as detected with cranial computed tomography (CT) or nuclear magnetic resonance imaging (NMRI); 5) neurologic impairment score between 5 and 22 points; and 6) receipt of signed informed consent from all participants. The exclusion criteria included: 1) medical history of cerebral hemorrhage 3 months before hospital admittance; 2) additional diagnosis of myocardial infarction or other cardiovascular and/or cerebrovascular diseases; 3) blood pressure higher than 200/100 mmHg; 4) severe dysfunctions of heart, liver, lung, and/or kidney; 5) use of similar anti-inflammatory drugs or drugs to improve microcirculation; and 6) incomplete clinical data or changed therapeutic plan. The included patients were divided into a control group and an observation group based on the treatment method. There were 121 cases in the control group with the following characteristics: 65 males and 56 females aged between 28 and 75 years with an average age of 60.3 ± 10.4 years; cases with complications that included 67 cases of hypertension, 51 cases of diabetes, and 22 cases of both hypertension and diabetes; average neurologic impairment score (NIHSS) of 10.54 ± 3.4 ; and cases with infarction sites that included 61 cases of infarctions in the basal ganglia, 34 in the brain lobe, 16 in the brainstem, and 10 in the cerebellum. There were 125 cases in the observation group with the following characteristics: 66 males and 59 females aged between 28 and 74 years with an average age of 61.9 ± 11.0 years; cases with complications that included 65 cases of hypertension, 57 cases of diabetes, and 24 cases of both hypertension and diabetes; NIHSS of 10.04 ± 3.9 ; and cases with infarction sites that included 64 cases of infarction in the basal ganglia, 32 in the brain lobe, 18 in the brainstem, and 11 in the cerebellum. Differences in gender, age, complications, NIHSS, and infarction sites between the two groups were not statistically significant ($P > 0.05$). This study was conducted in accordance with the Declaration of Helsinki and with approval from the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. Written informed consent was obtained from all study participants.

Treatments

The control group underwent conventional treatment, including 250 mL normal saline containing 0.5 g citicoline by intravenous drip once per day, 250 mL low molecular dextran by intravenous drip once per day, aspirin, vitamin E, and vitamin C. Patients with increased intracranial pressure were treated with the appropriate dehydration therapy, while patients with diabetes and hypertension were treated with therapies to reduce blood pressure and blood sugar. The observation group was treated with 20 mL Danhong injection (Buchang Pharma Co., Jinan, China) in addition to the treatment administered to the control group, which was dissolved in 250 mL normal saline and delivered by intravenous drip. After 14 days treatment, the curative effects and indices of both groups were analyzed.

Quantitative real-time PCR

The peripheral blood of patients was taken before treatment and 14 days after treatment. After the erythrocytes were lysed, the RNA of white blood cells was extracted with an RNA extraction kit (Kangwei Co., Beijing, China), which was then transcribed to cDNA with a reverse transcription kit (TaKaRa, Dalian, China). Based on gene sequences provided by GenBank, primers for interleukin-6 (IL-6), tumor necrosis factor alpha (TNF- α), and interleukin-1 beta (IL-

1 β) were designed with optimized annealing temperatures, and then quantitative real-time PCR was conducted with SYBR Green mix (Roche, Basel, Switzerland). Data were collected via a quantitative real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). The mRNA expression of IL-6, TNF- α , and IL-1 β of each patient before and after treatment was analyzed and expressed as the mean \pm SD.

ELISA analysis

The detection of levels of inflammatory cytokines (IL-6, TNF- α , and IL-1 β) in the control and observation groups before and after treatment was carried out with commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Inc., Minneapolis, MN, USA) for the specified cytokines according to manufacturer instructions.

Western blot

White blood cells from the peripheral blood of both control and observation groups before and after treatment were extracted with a nuclear and cytoplasm extraction kit, and then centrifuged for 5 min at 12,000 rpm. The supernatants were then collected and added to loading buffer and boiled for 10 min. Later, SDS-PAGE was conducted, and when the bromophenol blue was out of the lower edge of the gel, SDS-PAGE was stopped and the proteins were transferred to nitrocellulose (NC) membranes. The membranes were then blocked for 30 min with 5% skim milk powder (w/v), followed by incubation with an anti-NF- κ B p65 antibody (Abcam, Cambridge, UK) diluted 1:2000 overnight. The next day, the membranes were washed three times with PBST, and then incubated with a goat anti-mouse secondary antibody (Jinqiao Zhongshan, Beijing, China) for 1 h, and the luminescent liquid was used for development. Actin was used as the internal reference for cytoplasmic fractions and lamin B was used as the internal reference for the nuclear fractions. Changes in NF- κ B p65 levels in both groups before and after treatment were analyzed.

Analysis of hemodynamic indices of the two groups before and after treatment

The hemodynamic indices included high shear viscosity, low shear viscosity, plasma viscosity, hematocrit, platelet aggregation rate, and erythrocyte aggregation index. Each index was analyzed with the hemorheology detector.

Assessment of clinical effects

The neurologic impairment score of the National Institutes of Health (NIHSS) was used to assess patient nerve functions, and curative effects of Danhong injection were evaluated based on NIHSS scores and the degree of disease and disability as previously described (Leira et al., 2008). Patients with NIHSS scores reduced by 90%-100% with a degree of disability of 0 were considered cured; NIHSS scores reduced by 45-90% and a degree of disability of 1-3 were considered significantly improved; NIHSS scores reduced by 18-45% were considered improved; decreases in NIHSS scores of less than 17% were considered as no change. Total efficiency = (cured + significantly improved + improved cases) / total cases * 100.

Statistical analysis

All data were analyzed with the SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). Measured data are reported as means \pm SD, and statistical differences were evaluated by the Student *t*-test, whereas the comparison of numeration data was evaluated by the chi-square test. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Comparison of the curative effects between Danhong injection and control groups

As shown in Table 1, the total efficiency rate of the control group was 71.07%, whereas the total efficiency rate of the observation group was 92.00% demonstrating that the Danhong injection (observation) group had a markedly higher total efficiency than that of the control group, and the difference was statistically significant ($P < 0.05$). No adverse reactions were observed in either group during treatment.

Table 1. Comparison of curative effects between control and observation groups.

Group	Cases	Basically cured	Significantly improved	Improved	No changes	Total efficiency rate (%)
Control group	121	25	27	34	35	71.07
Observation group	125	53	37	25	10	92.00

Comparison of mRNA levels of inflammatory cytokines in peripheral white blood cells between Danhong injection and control groups before and after treatment

As shown in Figure 1, quantitative real-time PCR was used to detect changes in mRNA expression of inflammatory cytokines including IL-1 β , IL-6, and TNF- α in peripheral white blood cells. The results show that inflammatory cytokine mRNA was highly expressed in both groups and there were no statistically significant differences ($P > 0.05$). After treatment, mRNA was down-regulated ($P < 0.05$), and the decrease was more pronounced in the Danhong injection (observation) group than in the control group ($P < 0.05$).

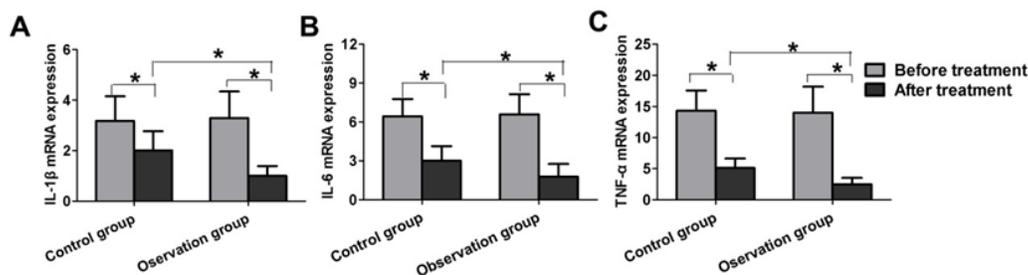


Figure 1. Comparison of mRNA levels of inflammatory cytokines in peripheral white blood cells of both groups before and after treatment. * $P < 0.05$.

Comparison of inflammatory cytokine protein levels between Danhong injection and control groups before and after treatment

As shown in Table 2, no statistically significant differences were observed in the levels of inflammatory cytokines (IL-1 β , IL-6, and TNF- α) between the observation and groups before and after treatment ($P > 0.05$). After treatment, the levels of the inflammatory cytokines (IL-1 β , IL-6, and TNF- α) of both groups were significantly lower than those before treatment ($P < 0.05$). Additionally, the Danhong injection (observation) group had significantly lower levels of these inflammatory cytokines after treatment compared to the control group ($P < 0.05$).

Table 2. Comparison of inflammatory cytokine protein levels between control and observation groups before and after treatment.

Cytokine		Control group	Observation group
IL-1 β (μ g/dL)	Before treatment	618.17 \pm 54.41	610.26 \pm 45.56
	After treatment	525.10 \pm 50.11*	217.43 \pm 48.80**
IL-6 (μ g/dL)	Before treatment	277.31 \pm 55.40	273.45 \pm 59.37
	After treatment	205.77 \pm 46.07*	148.12 \pm 45.31**
TNF- α (μ g/dL)	Before treatment	241.14 \pm 37.44	235.75 \pm 37.37
	After treatment	147.44 \pm 38.67*	98.13 \pm 33.40**

Compared with that before treatment, * $P < 0.05$; compared with that of control group after treatment, ** $P < 0.05$.

Changes in NF- κ B p65 levels in peripheral white blood cells of Danhong injection and control groups before and after treatment

As shown in Figure 2, NF- κ B p65 levels were low in the cytoplasm and high in the nuclei of white blood cells from patients of both groups before treatment. This was expected as because of the inflammatory reaction, the NF- κ B p65 protein is translocated from the cytoplasm to the nucleus, which initiates the expression of inflammatory cytokines. Conversely, nuclear levels of NF- κ B p65 were significantly decreased in both groups after treatment, but they were higher in the control group than in the Danhong injection (observation) group. Similarly, NF- κ B p65 levels in the cytoplasm were increased significantly in both groups after treatment, but were higher in the Danhong injection (observation) group than in the control group ($P < 0.05$).

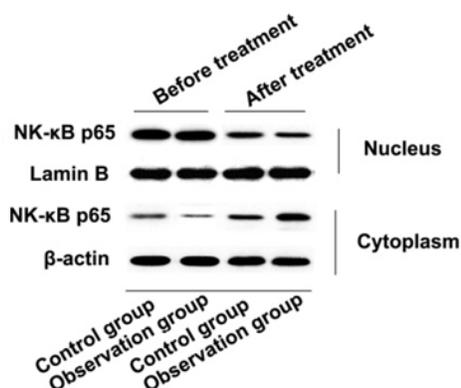


Figure 2. Comparison of NF- κ B p65 in the peripheral white blood cells of both groups before and after treatment.

Comparison of hemodynamic indices between Danhong injection and control groups before and after treatment

As shown in Table 3, before treatment, no statistically significant difference was found in the comparison of high shear viscosity, low shear viscosity, plasma viscosity, hematocrit, platelet aggregation rate, and erythrocyte aggregation index between the two groups ($P > 0.05$). After treatment, the values of the hemodynamic indices were markedly decreased in both groups compared to those before treatment, and the differences were statistically significant ($P < 0.05$). The Danhong injection (observation) group had more notable reductions in the values of the hemodynamic indices than those of the control group, and these differences were statistically significant ($P < 0.05$).

Table 3. Comparison of hemorheology indices between the control and observation groups before and after treatment.

Hemorheology index	Control group		Observation group	
	Before treatment	After treatment	Before treatment	After treatment
High shear viscosity	5.88 ± 1.13	5.06 ± 0.89*	5.83 ± 1.21	4.32 ± 0.59**
Low shear viscosity	12.45 ± 1.45	9.97 ± 1.02*	12.69 ± 1.75	7.48 ± 1.43**
Plasma viscosity	2.98 ± 0.58	2.13 ± 0.39*	2.91 ± 0.62	1.68 ± 0.47**
Hematocrit	48.89 ± 5.80	40.74 ± 6.04*	49.99 ± 6.79	34.33 ± 4.67**
Platelet aggregation rate	68.44 ± 5.27	63.18 ± 4.79*	69.94 ± 6.28	55.19 ± 5.89**
Erythrocyte aggregation index	3.61 ± 0.52	3.13 ± 0.47*	3.68 ± 0.63	2.47 ± 0.61**

Compared with that before treatment, * $P < 0.05$; compared with that of control group after treatment, ** $P < 0.05$.

DISCUSSION

Recent research suggests that the substantial release of serum inflammatory cytokines and hemodynamic changes may be primary reasons for the high disability and death rates in patients with acute cerebral infarction. During the development of cerebral arteriosclerosis and infarction, damage to endothelial cells, oxidation of low-density lipoproteins, and migration of mononuclear macrophages may lead to significant secretion of TNF- α by these macrophages and by nerve cells. Additionally, research has shown that TNF- α levels in sera of patients with cerebral infarction are positively correlated to infarction size. This indicates that high TNF- α levels lead to larger infarction size (Scholz et al., 2013). IL-6, another inflammatory cytokine secreted by vascular endothelial cells and macrophages, is also highly expressed in the serum of patients with acute cerebral infarction and is positively correlated to cerebral infarction size and degree of neurologic impairment (Xu et al., 2005; Choi et al., 2014). IL-1 β is a major inflammatory-promoting cytokine, and it has been demonstrated that serum levels of IL-1 β in patients with cerebral infarction are significantly increased, which is possibly associated with the development of cerebral infarction (Fernández-Cadenas et al., 2012). In the current study, the analysis of serum levels of inflammatory cytokines including IL-6, TNF- α and IL-1 β in patients with cerebral infarction revealed higher than normal expression levels of these cytokines, which is consistent with previous reports. Apart from inflammation in acute cerebral infarction development, dramatic increases in blood cell numbers, increased hematocrit, and reduced denaturation ability cause blood viscosity to increase and blood velocity to decrease causing blocked blood flow and insufficient support to brain tissues. Meanwhile, because of damaged vascular endothelium, it is easier for platelets to aggregate, and finally a thrombus is formed (Xu et al., 2005). Accordingly, it is effective to lower serum inflammation and to change hemorheology in the treatment of acute cerebral infarction.

Danhong injection is the extract of Danshen (the dried root of *Salvia miltiorrhiza* Bge.), Honghua (*Carthamus tinctorius* L.), and other traditional Chinese medicines and is capable of promoting blood circulation, removing blood stasis, and clearing and activating channels and collaterals. Its major components are tanshinone, salvianolic acid, and carthamin. Tanshinone and salvianolic acid can reduce blood viscosity, increase blood flow and erythrocyte deformation, inhibit thrombosis, scavenge free radicals, and improve microcirculation. Carthamin can dilate blood vessels, reduce platelet aggregation, and inhibit thrombosis (Fernández-Cadenas et al., 2012). Additionally, it has been reported that Danhong injection inhibits the expression of inflammatory cytokines and the resultant inflammatory cascade and reduces damage to organs and cells (Wang et al., 2013). Owing to these features, we analyzed the influence of Danhong injection on inflammation and hemodynamic changes in patients with acute cerebral infarction, providing a basis for its use as a treatment method.

The results herein show that Danhong injection in addition to conventional treatment markedly lowers neurologic impairment attributed to acute cerebral infarction and increases the total efficiency of clinical treatment from 71.07% (conventional treatment alone) to 92.00%. The levels of serum inflammatory cytokines such as IL-1 β , IL-6, and TNF- α were significantly reduced by Danhong injection compared to those in the control group, which suggests that Danhong injection can effectively reduce the inflammatory cascade induced by cytokines in brain tissues, which could avoid cranial nerve cell damage and decrease disability and death rates. We further investigated the mechanism of action of Danhong injection and found that the level of NF- κ B p65 protein in the nuclei of peripheral white blood cells was significantly reduced in the Danhong injection group compared to the control group. NF- κ B is a highly conserved transcription factor in eukaryotic cells, functioning importantly in immune reactions and cell development (Hoesel and Schmid, 2013; Wang et al., 2014). Under normal physiological conditions, two subunits of NF- κ B exist in the cytoplasm, and when cells receive external stimuli, the p65 and p50 subunits are rapidly translocated into the nucleus where they bind to DNA response elements, which activates the transcription of downstream genes and inflammatory cytokines, which are the major initiators of this signaling pathway (Engelmann et al., 2014). The significant reduction of NF- κ B p65 in the nucleus demonstrates that Danhong injection has an effect on the NF- κ B signaling pathway, which may further decrease the levels of inflammatory cytokines. Furthermore, the hemodynamic indices including high shear viscosity, low shear viscosity, plasma viscosity, hematocrit, platelet aggregation rate, and erythrocyte aggregation index were significantly improved in patients after treatment with Danhong injection compared to those in controls, which indicates that Danhong injection can improve blood microcirculation. Taken together, the data herein suggest that Danhong injection may significantly lower disability and death rates associated with cerebral infarction. In conclusion, Danhong injection has a good effect on patients with acute cerebral infarction and is worth clinical application.

Conflicts of interest

The authors declare no conflict of interest.

REFERENCES

- An SA, Kim J, Kim OJ, Kim JK, et al. (2013). Limited clinical value of multiple blood markers in the diagnosis of ischemic stroke. *Clin. Biochem.* 46: 710-715.

- Chen X, Zhan X, Chen M, Lei H, et al. (2012). The prognostic value of combined NT-pro-BNP levels and NIHSS scores in patients with acute ischemic stroke. *Intern. Med.* 51: 2887-2892.
- Choi YH, Kim GY and Lee HH (2014). Anti-inflammatory effects of cordycepin in lipopolysaccharide-stimulated RAW 264.7 macrophages through Toll-like receptor 4-mediated suppression of mitogen-activated protein kinases and NF- κ B signaling pathways. *Drug Des. Devel. Ther.* 8: 1941-1953.
- Engelmann C, Weih F and Haenold R (2014). Role of nuclear factor kappa B in central nervous system regeneration. *Neural Regen. Res.* 9: 707-711.
- Fernández-Cadenas I, Del Río-Espínola A, Giralte D, Domingues-Montanari S, et al. (2012). IL1B and VWF variants are associated with fibrinolytic early recanalization in patients with ischemic stroke. *Stroke* 43: 2659-2665.
- Hoesel B and Schmid JA (2013). The complexity of NF- κ B signaling in inflammation and cancer. *Mol. Cancer* 12: 86.
- Kang DW, Yoo SH, Chun S, Kwon KY, et al. (2009). Inflammatory and hemostatic biomarkers associated with early recurrent ischemic lesions in acute ischemic stroke. *Stroke* 40: 1653-1658.
- Leira EC, Adams HP Jr, Rosenthal GE and Torner JC (2008). Baseline NIH stroke scale responses estimate the probability of each particular stroke subtype. *Cerebrovasc. Dis.* 26: 573-577.
- O'Driscoll CM, Lima MP, Kaufmann WE and Bressler JP (2015). Methyl CpG binding protein 2 deficiency enhances expression of inflammatory cytokines by sustaining NF- κ B signaling in myeloid derived cells. *J. Neuroimmunol.* 283: 23-29.
- Orlov SV, Domashenko MA, Kostyreva MV, Shabalina AA, et al. (2007). Hemorheology and hemostasis in patients suffering from ischemic cerebral stroke and metabolic syndrome. *Klin. Med.* 85: 40-44.
- Puddu P, Puddu GM, Cravero E, Muscari S, et al. (2010). The involvement of circulating microparticles in inflammation, coagulation and cardiovascular diseases. *Can. J. Cardiol.* 26: 140-145.
- Scholz CC, Cavadas MA, Tambuwala MM, Hams E, et al. (2013). Regulation of IL-1 β -induced NF- κ B by hydroxylases links key hypoxic and inflammatory signaling pathways. *Proc. Natl. Acad. Sci. U. S. A.* 110: 18490-18495.
- Sergeeva SP, Erofeeva LM and Gul'tiaev MM (2011). IL-1beta, IL-10, INF-gamma, TNF-alpha, S100beta, AMA-M2 and cell immune response in stroke. *Patol. Fiziol. Eksp. Ter.* 1: 41-45.
- Shi WG, Sun XY, Qu L and Wang GQ (2010). Effects of danhong injection on cardiac inflammatory reaction and coronary artery blood flow after selective percutaneous coronary artery intervention. *Chin. J. TCM WM Crit. Care* 17: 288-291.
- Wang D, Fan G, Wang Y, Liu H, et al. (2013). Vascular reactivity screen of Chinese medicine danhong injection identifies Danshensu as a NO-independent but PGI2-mediated relaxation factor. *J. Cardiovasc. Pharmacol.* 62: 457-465.
- Wang S, Guo H, Wang X, Chai L, et al. (2014). Pretreatment with Danhong injection protects the brain against ischemia-reperfusion injury. *Neural Regen. Res.* 9: 1453-1459.
- Wartenberg KE (2012). Malignant middle cerebral artery infarction. *Curr. Opin. Crit. Care* 18: 152-163.
- Whiteley W, Jackson C, Lewis S, Lowe G, et al. (2009). Inflammatory markers and poor outcome after stroke: a prospective cohort study and systematic review of interleukin-6. *PLoS Med.* 6: e1000145.
- Xu C, Shen G, Chen C, Gélinas C, et al. (2005). Suppression of NF-kappaB and NF-kappaB-regulated gene expression by sulforaphane and PEITC through IkappaBalpha, IKK pathway in human prostate cancer PC-3 cells. *Oncogene* 24: 4486-4495.
- Zhang YX and Zhang Q (2012). Effects of danhong injection on protect myocardium of acute coronary syndrome patients after selective percutaneous coronary artery intervention. *Chin. J. Exp. Trad. Med. Formulae* 18: 308-311.