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Journal homepage: [www.tiprpress.com](http://www.tiprpress.com) E-mail: [chm@tiprpress.com](mailto:chm@tiprpress.com)**Original article****Effect of Chinese Patent Medicine Naodesheng against Repeated Transient Global Cerebral Ischemia in Mice**Chun-hua Hao<sup>1</sup>, Wen-gong Xi<sup>1</sup>, Hai-juan Zheng<sup>2</sup>, Wei-ting Wang<sup>1\*</sup>, Zhuan-you Zhao<sup>1\*</sup>

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**ABSTRACT**

**Objective** To investigate the therapeutic effect and possible mechanisms of Chinese patent medicine Naodesheng (NDS) on repeated transient global cerebral ischemia (GCI) in mice. **Methods** The repeated transient GCI mice were induced by bilateral carotid arteries ligation, and were randomly divided into model group, Sham group without arteries ligation, NDS groups (1.25 and 2.5 g/kg) and positive control (vinpocetine 3.1 mg/kg, VP) group. After oral administration once daily for successive 7 d, the transient GCI was induced. The degree of neurological deficit, histological changes, and neurons loss in the hippocampus were evaluated. In order to investigate the possible mechanisms, the oxidative stress and inflammatory factor were measured after 24 h of GCI. Comparison among multiple groups was performed with one-way analysis of variance (ANOVA). **Results** NDS could significantly alleviate the neurological function impairment, histological injury, and neurons loss, increase the superoxide dismutase (SOD) activity, decrease the content of malondialdehyde (MDA), and reduce inflammatory factor in the ischemic brain tissue. **Conclusion** NDS could significantly reduce brain injury induced by global ischemia, and its mechanism is closely associated with anti-oxidation and anti-inflammation.

*Key words*

Naodesheng; repeated transient global cerebral ischemia; vascular dementia

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**1. Introduction**

The estimated prevalence of vascular dementia (VaD) varies from 1.2% to 4.2% of individuals over the age of 65 and the incidence is estimated at 6–12 cases per 1000 persons over 70 years old per year (Rohn, 2014). Dementia of VaD has gained much attention for being the second most common type of dementia after Alzheimer's disease (AD) and increased the risk of recurrent stroke, dependent living, and death (Han et al, 2010; Jellinger, 2007; Catindig et al, 2012).

This disease is associated with tangles and plaques in the brain, loss of connections, inflammation, and eventual death of brain cells, all these brain changes lead to memory loss and alterations in thinking and other brain functions (Rizzi et al, 2014; Bandyopadhyay et al, 2014). Previous research has recently reported that VaD could be induced in mice by repeated transient global cerebral ischemia (GCI) with bilateral carotid arteries ligation resulting in significant white matter lesions, learning and memory impairment, and hippocampal neuronal damage (Kim et al, 2008; Grewal et al,

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2013). Thus, the model used in this research is useful for understanding the pathophysiology of chronic cerebrovascular hypoperfusion and for screening drugs with potential therapeutic value for VaD (Gong et al, 2012).

Various acute mechanisms have been proposed for this type of injury, including oxidative stress, inflammatory, apoptosis, and so on.

Naodesheng (NDS), a Chinese patent medicine recorded in *Chinese Pharmacopoeia* (1995) (Chen and Lei, 1995) composed of five medicinal plants including *Notoginseng Radix*, *Chuanxiong Rhizoma*, *Carthami Flos*, *Puerariae Radix*, and *Crataegi Fructus*, has been widely used in the treatment of cardio/cerebrovascular ischemic diseases in China, especially for cerebral arterioclerosis, ischemic stroke, and sequela of ischemic cerebrovascular disease (Zhang, 2007; Sugawara and Chan, 2003). In the present study, the cognitive-enhancing activity of NDS in VaD mice model was investigated using vinpocetine (VP) as the positive control. VP is a cerebral vasodilator that improves brain blood flow, and a cerebral metabolic enhancer by enhancing oxygen and glucose uptake and increasing neuronal ATP production, it has been widely used in many countries for the prevention of cerebrovascular disorders and cognitive impairment, including stroke, senile dementia, and memory disturbances (Bagoly et al, 2007). Now there was no report about the effect of NDS on VaD, this study evaluated the effect of NDS on amelioration of learning and memory by VaD model and observed the mechanism of anti-oxidation and anti-inflammation, and VP used as the positive control.

## 2. Materials and methods

### 2.1 Drugs and reagents

The malondialdehyde (MDA) kit (batch No. 20140521) and superoxide dismutase (SOD) kit (batch No. 20140517) were purchased from Nanjing Jiancheng Bioengineering Institute. Rat interleukin-1 $\beta$  (IL-1 $\beta$ ) Sunny ELISA kit (Lot: 2301B40623, Cat: EK301B2) and Rat IL-6 Sunny ELISA kit (Lot: 230640635, Cat: EK3062) were purchased from Lianke Biotechnology Co., Ltd. Rat tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) Elisa (Lot No. 110618) was purchased from eBioscience.

Naodesheng was from Harbin Huayu Pharmaceutical Group Co., Ltd., (batch No. 20130801); Vinpocetine was from Northeast Pharmaceutical Group Shenyang No. 1 Pharmaceutical Co., Ltd. (batch No. 5131002).

### 2.2 Animals

Male ICR mice (purchased from Vital River Laboratory Animal Technology Co., Ltd.) weighing 20–25 g maintained on a standard laboratory. All animals were maintained in laminar flow cabinets with free access to food and water under specific pathogen-free conditions in facilities approved by the Accreditation of Laboratory Animal Care and in accordance with the Institutional Animal Care and Use

Committee (IACUC) of the Animal Research Committee of Tianjin Institute of Pharmaceutical Research. They had free access to pellet food and water in plastic cages fewer than 12 h light and 12 h dark cycle with room temperature at (22  $\pm$  1)  $^{\circ}$ C and relative humidity of 50%–70%. The experiments complied with current ethical regulations on animal research of the university, and all of the mice used in the experiments received humane care.

### 2.3 Establishment of mice repeated transient ischemia model

The repeated transient GCI model was induced by bilateral carotid arteries ligation (Zhang et al, 2009). Briefly, adult male ICR mice 8–12 weeks' old and weighing 20–25 g were used. During 0.35% pentobarbital sodium anesthesia, a midline ventral incision was made in the throat. Right and left common carotid arteries (CCA) were located and freed from surrounding tissue and vagus nerve, and the bilateral CCA were carefully exposed and occluded with 6-0 suture. GCI was induced by pulling the ends of thread with constant weight. After 10 min of GCI, weight on the thread was removed to allow the reflow of blood through carotid arteries for 5 min, and these operations were repeated for three times. The incision was sutured back in layers. Body temperature of mice was maintained at 37  $^{\circ}$ C during the surgery by electrically heated surgical platform during reperfusion of 24 h by housing the animal cage in appropriate temperature and humidity-controlled room (Rehni and Singh, 2009). The Sham group was performed with the same procedure including anesthesia and all surgical procedures, except the CCA occlusion. After the completion of the surgical procedure, the animals were shifted individually to their home cage and were allowed to recover.

### 2.4 Grouping and treatment

Animals were randomly divided into five groups: one Sham group and four treatment groups with surgeries [model (0.5% Carboxymethyl Cellulose), NDS (1.25 and 2.5 g/kg), and VP (3.1 mg/kg)]. There were eight mice in each group. Test drugs were administered orally for 7 d prior to surgeries, and mice survived for 24 h after ischemia. Selection of the concentration of test drugs was based on clinic recommendation and on previous studies that successfully reported the neuroprotective effects of NDS against focal cerebral ischemia in rats (Zhang, 2007). All drugs were orally given, once daily for 7 d.

### 2.5 Step-down avoidance test

Short-term memory was evaluated by step-down avoidance test according to a previously described method (Kim et al, 2013; Cho et al, 2013) with MSD-multifunction step-down instrument (Institute of Chinese Academy of Medical Sciences Pharmaceutical Research). Mice were positioned on a 7 cm  $\times$  25 cm platform, at a height of 2.5 cm,

and allowed to rest on the platform for 3 min. The platform faced a 42 cm × 25 cm grid of parallel 0.1 cm caliber stainless steel bars, which were spaced 1 cm apart. In the training session, mice were exposed to a 5 min learning course, during which they were permitted to move freely throughout the chamber before being placed on the platform. If the animals stepped down from the platform (error trial), they were exposed to an electric foot shock (40 V, AC). Retention time was assessed after 24 h of the training session and recorded as the learning grade (latency) and error trials within 5 min, which was taken as a measure of memory retention.

### 2.6 Histological observation and analysis of neuronal loss in hippocampus

After 24 h of bilateral CCA occlusion, the brain tissues of mice were fixed by 4% paraformaldehyde solution and embedded in paraffin followed by the preparation of 5 μm-thick coronal sections with a Leika VT 1000S Vibratome (Germany). The sections were stained with hematoxylin and eosin and examined with a light microscope at × 20 magnification in the CA1 region of hippocampus. The number of surviving hippocampal neurons was counted in hippocampal CA1 region.

### 2.7 Estimation of SOD activity and MDA content in ischemic brain tissue

MDA (an index of lipid peroxidation) and SOD (the markers of oxidative stress) were measured according to the manuscripts of the kits by Thermo Varioskan Flash multifunctional ELISA (Thermo Fisher Scientific Company, America). In brief, after experimental treatments, hippocampal tissues were dissected and washed for three

times with phosphate-buffered saline and homogenized in 20 mmol/L phosphate buffer (pH 7.4) containing 0.5 mmol/L butylated hydroxytoluene to prevent sample oxidation. Lysates were centrifuged at 1000 g for 10 min, and 200 μL aliquots of the supernatants were used according to the instructions of the manufacturer. A standard curve was used to determine the absolute concentration. Values were standardized to micrograms of protein for each sample.

### 2.8 Estimation of inflammatory cytokines in serum of GCI mice

Samples of the serum supernatants from the GCI mice were obtained, and the inflammatory factors TNF-α, IL-1β, and IL-6 were quantified with an ELISA kit (Dakewe, Beijing, China) according to the manufacturer's protocol.

### 2.9 Statistical analysis

All data were expressed as  $\bar{x} \pm s$ , and they were analyzed by Comparison among multiple groups was performed with One-way analysis of variance (ANOVA) using statistical software (SPSS v.18, USA).  $P < 0.05$  was considered statistically significant.

## 3. Results

### 3.1 Effect of NDS on step-down test

The step-down latency and error trials of model group were significantly ( $P < 0.05, 0.001$ ) longer and more than these of the Sham group. This effect was significantly ( $P < 0.05, 0.001$ ) reversed by NDS (2.5 g/kg) and VP (3.1 mg/kg), as shown in Table 1.

Table 1 Effect of NDS on step-down test of repeated transient GCI mice ( $\bar{x} \pm s, n = 8$ )

Groups	Doses	Error trials / times		Latency / s	
		Learning	Memory	Learning	Memory
Sham	–	3.88 ± 0.99	2.50 ± 0.76	16 ± 4	35 ± 5
model	–	5.50 ± 1.20 <sup>△</sup>	4.75 ± 1.16 <sup>△△△</sup>	17 ± 4	17 ± 3 <sup>△△△</sup>
NDS	1.25 g/kg	4.75 ± 1.49	4.00 ± 1.41	17 ± 5	22 ± 9
	2.50 g/kg	4.25 ± 0.89*	3.88 ± 0.99	16 ± 5	30 ± 7***
VP	3.10 mg/kg	4.25 ± 1.04*	2.63 ± 0.52***	13 ± 5	32 ± 6***

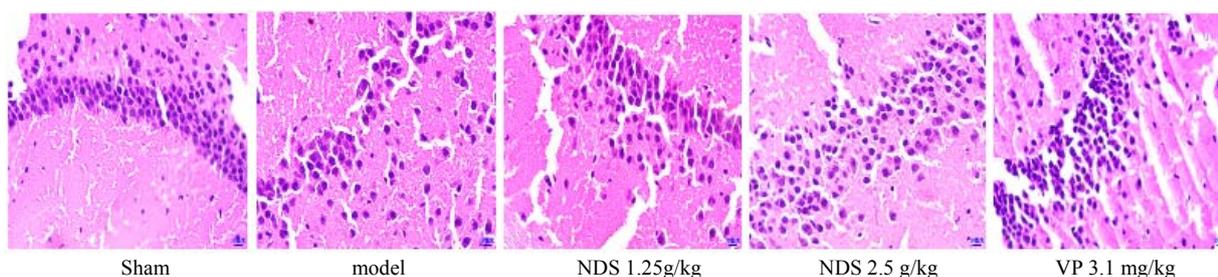
<sup>△</sup> $P < 0.05$  <sup>△△</sup> $P < 0.01$  <sup>△△△</sup> $P < 0.001$  vs Sham group; \* $P < 0.05$  \*\*\* $P < 0.001$  vs model group; same as below

### 3.2 Effect of NDS on pathohistologic changes and numbers of neurons in hippocampal CA1 region

The neuroprotective effect of NDS against injury of ischemic hippocampal CA1 pyramidal neuron was determined with HE staining. The normal cells showed round and pale stained nuclei. Some neurons showed the features of ischemic morphological change including shrunken cell bodies, triangulated pyknotic nuclei, and eosinophilic cytoplasm. All of those features declared that the transient

GCI induced severe cell death.

Numbers of neurons in the defined area were counted by an observer who was blinded to the experimental condition. The administration of NDS obviously limited the neuronal degeneration and reduced neurons loss compared with the model group. NDS (2.5 g/kg) and VP increased numbers of neurons by 19.5% ( $P < 0.05$ ) and 23.5% ( $P < 0.05$ ). The results indicated that NDS (2.5 g/kg) could be capable of protecting neurons against injury induced by ischemia as the same as VP, as shown in Figure 1 and Table 2.



**Figure 1** Effect of NDS on numbers of neurons in hippocampal CA1 region of repeated transient global ischemia mice

**Table 2** Effect of NDS on numbers of neurons in hippocampal CA1 region of repeated transient GCI mice ( $\bar{x} \pm s, n = 8$ )

Group	Dose	Neurons / numbers
Sham	–	110.4 ± 23.3
model	–	73.3 ± 10.2 <sup>△△△</sup>
NDS	1.25 g/kg	85.1 ± 14.9
	2.50 g/kg	87.6 ± 15.4 <sup>*</sup>
VP	3.10 mg/kg	90.5 ± 17.6 <sup>*</sup>

### 3.3 Effect of NDS on SOD activity and MDA content

To investigate potential effects of NDS on the endogenous anti-oxidant system in the hippocampus, the levels of enzymatic activity of SOD and content of MDA were measured. The SOD activity and MDA content were more significantly decreased and increased in ischemic brain tissue of the model group compared with those in the Sham group ( $P < 0.01$ ), while NDS at doses of 2.5 g/kg increased the levels of SOD by 8.3% ( $P < 0.05$ ) and decreased the MDA content by 11.6% ( $P < 0.05$ ) compared with

the model group ( $P < 0.05$ ). VP also had the same effects by 8.6% ( $P < 0.05$ ) and 10.3% ( $P < 0.05$ ), as shown in Table 3.

### 3.4 Effect of NDS on inflammatory cytokines in serum

To evaluate the mechanism of NDS on inflammatory cytokines in repeated transient GCI mice, the levels of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in serum were measured by assay kits. All of them increased in the model groups compared with those of Sham group ( $P < 0.05$ ). Treatment with NDS (1.25 and 2.5g/kg) can dose-dependently decrease levels of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in serum, with respect to the model group. TNF- $\alpha$  decreased by 25.4% ( $P < 0.05$ ) and 30.0% ( $P < 0.01$ ); IL-1 $\beta$  decreased by 19.3% ( $P > 0.05$ ) and 24.4% ( $P < 0.05$ ); IL-6 decreased by 7.5% ( $P > 0.05$ ) and 15.6% ( $P < 0.05$ ), respectively. VP also decreased TNF- $\alpha$  by 27.2% ( $P < 0.05$ ). So there might be a certain relationship between the protective role of NDS and serum inflammatory cytokines, as shown in Table 4.

**Table 3** Effects of NDS on SOD activity and MDA content in repeated transient global ischemia mice ( $\bar{x} \pm s, n = 8$ )

Groups	Doses	SOD / (U·mg <sup>-1</sup> prot)	MDA / (nmol·mg <sup>-1</sup> prot)
Sham	–	711 ± 68	4.00 ± 0.45
model	–	615 ± 51 <sup>△△</sup> (0.039)	4.74 ± 0.31 <sup>△△</sup> (0.039)
NDS	1.25 g/kg	621 ± 114	4.43 ± 0.32
	2.50 g/kg	666 ± 41 <sup>*</sup>	4.19 ± 0.53 <sup>*</sup>
VP	3.10 mg/kg	668 ± 47 <sup>*</sup>	4.25 ± 0.38 <sup>*</sup>

**Table 4** Effects of NDS on inflammatory cytokines in repeated transient global ischemia mice ( $\bar{x} \pm s, n = 8$ )

Groups	Doses	TNF- $\alpha$ / (pg·mL <sup>-1</sup> )	IL-1 $\beta$ / (pg·mL <sup>-1</sup> )	IL-6 / (pg·mL <sup>-1</sup> )
Sham	–	262.9 ± 43	360.5 ± 132.8	207.1 ± 33.0
model	–	362.9 ± 88.7 <sup>△</sup>	531.1 ± 105.8 <sup>△</sup>	259.8 ± 44.4 <sup>△</sup>
NDS	1.25 g/kg	270.9 ± 56.2 <sup>*</sup>	428.8 ± 164.7	240.4 ± 29.2
	2.50 g/kg	253.9 ± 38.5 <sup>**</sup>	401.3 ± 133.7 <sup>*</sup>	219.4 ± 28.1 <sup>*</sup>
VP	3.10 mg/kg	264.2 ± 54.1 <sup>*</sup>	408.7 ± 99.3	223.7 ± 5.3

## 4. Discussion

This study evaluated the NDS on the amelioration of learning and memory effect by VaD model and observed the mechanism. In our experiments, step-down test was carried out to define the ability of learning and memory. The step-down latency and error trials of the model group were significantly ( $P < 0.05, 0.001$ ) longer and more than those of the Sham controls, which was deemed that mice VaD model was

successfully induced. This effect was significantly ( $P < 0.05, 0.001$ ) reversed by NDS (2.5 g/kg) and VP (3.1 mg/kg). Our pathohistologic experiment also found that there was neuronal loss in hippocampus and some neurons showing the features of ischemic morphological change including shrunken cell bodies, triangulated pyknotic nuclei, and eosinophilic cytoplasm in the model group. However, the administration of NDS (2.5 g/kg) could be capable of protecting neurons against ischemia injury as the same as VP, which was the same as reported by Zhang et

al (2009), just as NDS (1.075 g/kg) could significantly reduce brain injury induced by ischemia in male SD rat. In conclusion, our results verified that NDS was involved in the protective effects of VaD mice.

Nevertheless, the mechanisms underlying these effects remain incompletely defined. It has now been well established that oxidative stress has an important role in the pathophysiology of neurodegenerative disease and stroke (Clemens, 2000), and that excessive production of both ROS and/or RNS occurs during ischemia-reperfusion of neural tissues. Our results indicated that NDS at the dose of 2.5 g/kg increased the SOD activity and decreased the MDA content. Recent research has revealed that inflammation plays an important role in secondary brain insult following cerebral ischemia (Iadecola and Anrather, 2011). During GCI neurons in CA1 and CA2 regions of the hippocampus, which are selectively vulnerable, undergo inflammatory and apoptosis after 3–7 d (Magnoni et al, 2004). Pro-inflammatory cytokines such as IL-1, tumor necrosis factor (TNF), and IL-6 are the principal mediators of the inflammatory reaction. Our experiments found that the treatment with NDS (1.25 and 2.5 g/kg) can dose-dependently decrease the levels of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 in serum, with respect to the model group. These results indicated that NDS might improve repeated transient GCI by anti-oxidation and anti-inflammation. However, our results just only provide a basis for investigations into the role of NDS on VaD in mice, further more studies should be done by increasing sample size and testing the effects of which in other therapeutic mechanism.

## 5. Conclusion

In summary, a mice model of repeated transient GCI has been successfully established. The NDS has therapeutic benefit by improving the ability of learning and memory, lessens histological changes and neurons death, which might through a pathway mediated by anti-oxidation and anti-inflammation. That may provide a therapeutic drug against the VaD.

## References

- Bagoly E, Fehér G, Szapáry L, 2007. The role of vinpocetine in the treatment of cerebrovascular diseases based in human studies. *Orv Hetil* 148(29): 1353-1358.
- Bandyopadhyay TK, Biswas A, Roy A, Guin DS, Gangopadhyay G, Sarkhel S, Ghoshal MK, Senapati AK, 2014. Neuropsychiatric profiles in patients with Alzheimer's disease and vascular dementia. *Ann Indian Acad Neurol* 17(3): 325-330.
- Catindig JA, Venketasubramanian N, Ikram MK, Chen C, 2012. Epidemiology of dementia in Asia: Insights on prevalence, trends and novel risk factors. *J Neurol Sci* 321(1-2): 11-16.
- Pharmacopoeia Committee of P. R. China, 2010. *Pharmacopoeia of People's Republic of China*. China Medical Science and Technology Press, Beijing.
- Cho HS, Shin MS, Song W, Jun TW, Lim BV, Kim YP, Kim CJ, 2013. Tread-mill exercise alleviates short-term memory impairment in 6-hydroxydopamine-induced Parkinson's rats. *J Exerc Rehabil* 9(3): 354-361.
- Clemens JA, 2000. Cerebral ischemia: Gene activation, neuronal injury, and the protective role of antioxidants. *Free Radic Biol Med* 28(10): 1526-1531.
- Gong X, Ma M, Fan X, Li M, Liu Q, Liu X, Xu G, 2012. Down-regulation of IGF-1/IGF-1R in hippocampus of rats with vascular dementia. *Neurosci Lett* 513(1): 20-24.
- Grewal AK, Jaggi AS, Rana AC, Singh N, 2013. Effect of neurosteroid modulation on global ischaemia-reperfusion-induced cerebral injury in mice. *Korean J Physiol Pharmacol* 17(6): 485-491.
- Han HS, Jang JH, Jang JH, Choi JS, Kim YJ, Lee C, Lim SH, Lee HK, Lee J, 2010. Water extract of *Triticum aestivum* L. and its components demonstrate protective effect in a model of vascular dementia. *J Med Food* 13(3): 572-578.
- Iadecola C, Anrather J, 2011. The immunology of stroke: From mechanisms to translation. *Nat Med* 17(7): 796-808.
- Jellinger KA, 2007. The enigma of vascular cognitive disorder and vascular dementia. *Acta Neuropathol* 113(4): 349-388.
- Kim SE, Ko IG, Park CY, Shin MS, Kim CJ, Jee YS, 2013. Treadmill and wheel exercise alleviate lipopolysaccharide-induced short-term memory impairment by enhancing neuronal maturation in rats. *Mol Med Rep* 7(1): 31-36.
- Kim SK, Cho KO, Kim SY, 2008. White matter damage and hippocampal neurodegeneration induced by permanent bilateral occlusion of common carotid artery in the rat: Comparison between Wistar and Sprague-Dawley strain. *Korean J Physiol Pharmacol* 12(3): 89-94.
- Magnoni S, Baker A, George SJ, Duncan WC, Kerr LE, McCulloch J, Horsburgh K, 2004. Differential alterations in the expression and activity of matrix metalloproteinases 2 and 9 after transient cerebral ischemia in mice. *Neurobiol Dis* 17(2):188-197.
- Rehni AK, Singh N, 2007. Role of phosphoinositide 3-kinase in ischemic postconditioning-induced attenuation of cerebral ischemia-evoked behavioral deficits in mice. *Pharmacol Rep* 59(2): 192-198.
- Rizzi L, Rosset I, Roriz-Cruz M, 2014. Global epidemiology of dementia: Alzheimer's and vascular types. *Biomed Res Int* 908915, doi: 10.1155/2014/908915.
- Rohn TT, 2014. Is apolipoprotein E4 an important risk factor for vascular dementia? *Int J Clin Exp Pathol* 7(7): 3504-3511.
- Sugawara T, Chan PH, 2003. Reactive oxygen radicals and pathogenesis of neuronal death after cerebral ischemia. *Antioxid Redox Signal* 5(5): 597-607.
- Zhang Li, Cheng XR, Chen RY, Zhu XM, Du GH, 2009. Protective effect of effective composite of Chinese medicine prescription NDS against focal cerebral ischemia in rats. *Chin J Integr Med* 15(5): 377-383.
- Zhang YS, 2007. Clinical observation of NDS on 66 cases with cerebral infarction. *J Practl Med* 23(7): 3107.