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# Study on the Pharmacodynamic Material Basis of Naomaitong for Cerebral Ischemia Based on vitro Cell Model

Ke-ning Li, Xu-lei Fan, Lan-fang Tan, Sheng-wang Liang, Lei Chen\* and Shu-mei Wang\*

Guangdong Pharmaceutical University, Xiaogwei Street, Panyu District, Guangzhou 510006, China

\*Corresponding author: Shu-mei Wang, Guangdong Pharmaceutical University, Xiaogwei Street, Panyu District, Guangzhou 510006, China, Tel: +86-(0)20-3935-2559; E-mail: shmwgdu@126.com; Lei Chen, Guangdong Pharmaceutical University, Xiaogwei Street, Panyu District, Guangzhou 510006, China, Tel: 020-3935-2177; Email:18964873@qq.com

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## Abstract

**Background:** Based on the theoretical basis of the pathological mechanism of cerebral ischemic injury, from the molecular level, using PC12 cells in vitro cell model pharmacodynamics and PK-PD correlation model research, to explain NaomaitongNMTpharmacodynamic material basis for the treatment of cerebral ischemic injury.

**Methods:** Four in vitro cell models of cerebral ischemic injury were established. The side-leakage of lactate dehydrogenase (LDH) and NO content were used as pharmacodynamic indicators to establish the corresponding drug-time curves of NMT and each single-drug; Based on the SVM algorithm, a PK-PD correlation mathematical model was established.

**Results:** The cerebral ischemia model caused by NMT has obvious protective effects compared with each single herb. Each single herb has different protective effects on the four damage mechanisms. The protective effect of Radix Pueraria(RP) was stronger in cerebral ischemic injury induced by Glu, In the cerebral ischemic injury caused by calcium overload mechanism, the protective effect of Ligusticum chuanxiongLCwas greater than that of Panax Ginseng(PG) and RP. The 15 components in NMT contained drug serum had a certain correlation with the cell injury model caused by the four mechanisms. It may be a medicinal material for NMT, and the different components have different correlations for cerebral ischemia damage caused by different mechanisms, and each component also has different efficacy.

**Conclusions:** It reveals the medicinal substance basis of NMT in treating cerebral ischemia, and provides reference for the clinical rational use of NMT for the treatment of cerebral ischemic injury, and provides guidance for the basic research on the substance of traditional Chinese medicine.

**Keywords** Naomaitong; Cerebral ischemia; Vitro cell model; Pharmacodynamic material basis

## Abbreviations

NMT: Naomaitong; LDH: lactate dehydrogenase; RP: Radix Pueraria; LC: Ligusticum Chuanxiong; PG: Panax Ginseng; RR: Radix et Rhizoma Rhei; SVM: Support Vector Machine

## Introduction

Naomaitong is a clinical experience in the treatment of ischemic stroke, consisting of Radix et Rhizoma Rhei(RR), Panax Ginseng(PG), Ligustici Chuanxiong (LC) and Radix Pueraria (RP). The mechanisms of cerebral ischemic injury include excitatory amino acids, calcium overload, excitatory amino acid toxicity, NO, oxygen free radical damage, inflammatory reaction, energy failure and apoptosis, etc [1]. However, the substance basis for the treatment of cerebral ischemic injury is not yet clear, and because of the characteristics of multiple components [2] multiple targets, and multiple pathways of traditional Chinese medicine compound formulas, the difficulties in the basic research of NMT effective substances are increased [3]. Therefore, establishing a scientific research method suitable for the complex system of traditional Chinese medicine and clarifying the material basis of NMT is the key to study the effect of NMT on cerebral ischemia injury [5].

PC12 cells are derived from rat adrenal pheochromocytoma cell lines. Their cell morphology and function are close to neurons. It is currently an internationally recognized model for studying neurobiological, neurochemical and neurological diseases in vitro [6]. It is also the most common cell line for the study of neurotoxicity and is widely used in vitro studies of neurological diseases [7]. The four commonly used in vitro models cover four important steps of the cascade reaction during cerebral ischemia [8]. The model of re-irrigation after hypoxia-glucose deprivation was the initial injury process, and the injury model of glutamate, potassium chloride and hydrogen peroxide were the most serious three-step process leading to cerebral ischemic injury [9]. The model was in line with the theory of cerebral ischemic injury. Has a certain theoretical basis [10].

LDH (lactate dehydrogenase) is a key enzyme in the energy metabolism process in the body and is present in all cells [11]. It is a stable cytoplasmic enzyme. When the cell membrane is damaged, it is quickly released into the cell culture fluid and detected in the cell supernatant [12]. The side leakage of LDH can reflect the extent of cell damage [13]. Cerebral ischemia stimulates nitric oxide synthase (NOS) and induces the production of large amounts of NO [14]. In particular, during reperfusion, it stimulates nerve endings to release large amounts of glutamate and activates the receptor of N-methyl-D-aspartate (NMDA), the  $\text{Ca}^{2+}$  channel on the cell membrane is activated [15], so that a large number of  $\text{Ca}^{2+}$  enters the membrane from outside the cell membrane [16], leading to an increase of the intracellular  $\text{Ca}^{2+}$  concentration, activating NOS activity, increasing the amount of NO production [17], and a large amount of NO may cause neurotoxicity, thereby leading to cells Damage or death, determination of NO content can reflect the extent of nerve cell damage [18].

Support vector machine (SVM) algorithm is a new machine learning method proposed by Vapnik based on statistical learning theory [19]. The algorithm is a convex optimization problem, which accords with the principle of minimizing structural risks and converts the problem of solution to two [20]. The problem of solving the problem is converted into a quadratic optimization problem, so that the SVM converges to the global optimal solution of the problem and prevents over-learning [21]. Its greatest feature is that its generalization ability is relatively strong, that is, a small error from a limited training set sample can still guarantee a small error to an independent test set. Siera et al. [22] established a compound neurotoxicity discriminant model using SVM to investigate the neurotoxic components of Shandou root. As a result, 13 potential neurotoxic components were obtained, of which 4 components have been reported in the literature. Qu et al. [23] established the anti-hypoxic effective site of Xiangdan injection by establishing the SVM prediction model of compound drug efficacy. and the results showed that the efficacy of multiple compatibility groups was superior to that of the whole party.

Therefore, in this paper, LDH side leakage and NO content of four kinds of damaged cells were used as efficacy indicators to study the drug-time curves of NMT and each single drug in PC12 cells at different time points. Meanwhile combined with previous pharmacokinetics. The data was analyzed by SVM algorithm to establish a PK-PD correlation mathematical model. The correlation between the plasma concentration, time, and efficacy of blood components of brain injury in different injury models was analyzed to elucidate the therapeutic effect of NMT on cerebral ischemia injury.

## Methods

### Material

PC12 cell provided by Shanghai Institute for Biological Cell Research; DMEM, DMEM without sugar, fetal bovine serum, trypsin, penicillin-streptomycin solution (GIBCO); sodium dithionate, potassium chloride, glutamic acid, hydrogen peroxide (Tianjin Zhiyuan Chemical Reagent Co., Ltd.); Healthy

male SD rats, SPF grade, weighing 220-280g, provided by Experimental Animal Center of Guangzhou University of Traditional Chinese Medicine, license: SCXK (Guangdong) 2013-0034. All medicinal materials were purchased from the market of Chinese herbal medicines in Bozhou, Anhui; Lactate dehydrogenase (LDH) test kits and Nitric Oxide (NO) test kits (Nanjing Jiancheng Bioengineering Institute); all reagents were of analytical grade.

### Liquid preparation of NMT and each single herb

According to NMT, a certain amount of each single herb was weighed, plus 10 times of 60% ethanol reflux extraction twice, each time 1 h, filtering, merging the filtrate, by rotary evaporation instrument to concentrate without alcohol taste, add distilled water constant volume 0.5 g/ml of liquids, stored in 4°C until used.

### Preparation of drug-containing serum of NMT and each single herb

30 healthy male SD rats weighing  $280 \pm 20$  g were bred for 1 week. Weighed rats were randomly divided into 5 groups, NMT+PGLCRP, 6 in each group, according to NMT and single herb liquid /12 g/Kg gavage, respectively. 12h before administration, fasting was not allowed, water was taken, and blank serum was taken 1 hour before administration. Eyelids were used 5 min, 15 min, 30 min, 45 min, 1h, 1.5h, 2h, 4h, 6h, 8h, 12h, 24h after administration. After standing in a refrigerator at 4°C for 2 hours, centrifugation was performed at 4000 rpm/min for 10 min. The serum of the same group of rats was centrifuged and mixed. After being inactivated by a 56°C water bath for 30 min, the solution was filtered through a 0.22 $\mu\text{m}$  microporous membrane to sterilizing, stored in -80°C until used.

### Cell culture

PC12 cells were seeded in 25 cm<sup>2</sup> polystyrene flasks with 4.5 g/l glucose in DMEM medium, containing 10% FBS, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$  streptomycin. Cells were incubated at 37°C under a humidified atmosphere of 95% air and 5%  $\text{CO}_2$  [24].

### Preparation of hippocampal specimens

After PND 21, all offspring mice were weighed, and sacrificed via cervical dislocation, and brains were quickly removed, weighed, and stored in a nitrogen canister before use, except those for Golgi-Cox staining. After weighing the body and brains, the coefficients of brain mass to BW were calculated as the ratio of brain (wet weight, mg) to BW (g).

Hippocampi were dissected from brains and frozen at -80°C. Every effort was made to minimize animal suffering.

### Establishment of four models of cerebral ischemic injury

After the cells were in the logarithmic growth phase, the cell density was 105 cells/ml, inoculated in 24 well plates, 1 ml/well

[25], they were divided into blank serum control group, model control group, NMT and each single herb-containing serum group with different blood collection time. The blank serum control group and model control group were added with DMEM medium containing 5% blank serum, and the other groups were respectively added with DMEM culture fluid containing 5% different time points NMT and each single herb-containing serum. After 12 hours of protection of H<sub>2</sub>O<sub>2</sub>, KCL, GLU model and 2 hours of protection of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> model, except for the blank serum control group, the remaining groups respectively were treated with 10 mmol/L Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> sugar-free DMEM medium for 4 hours; 100μmol/L H<sub>2</sub>O<sub>2</sub> solution for 2h; 125mmol/L KCL solution for 2h ; 25mmol/L GLU solution for 2h, and then changed to normal culture medium and placed in a 37°C, 5% CO<sub>2</sub> incubator for 24 hours, take the supernatant of each group for used.

### Biochemical indicators determination

**LDH leak detection:** Take the supernatant of each of the above four models, according to the instructions of the LDH Kit of Nanjing Jiancheng Bioengineering Institute, test the LDH activity in cells and draw the corresponding drug time curve, and measure it in parallel three times [26].

**NO content:** Take the supernatant of each of the above four models, according to the instructions of the NO Kit of Nanjing

Jiancheng Bioengineering Institute, test the NO activity in cells and draw the corresponding drug time curve, and measure it in parallel three times [27].

**Data analysis:** The experimental data were analyzed with Excel software. The experimental results were expressed as mean ± standard deviation ( $\bar{X} \pm S$ ), and the drug-time curves of four kinds of PC12 cell injury models were obtained with the software of Origin 9.0[28].

### Establishment of PK-PD Correlation Mathematical Model

**Data processing of PK and PD:** In the early stage, HPLC-MS was used to analyze the NMT-containing sera at the same time point as the cellular pharmacodynamics experiment, and the drug-time curve of 15 components in NMT-containing serum was measured, and the PKSolver software was used to process the drug-time curve. The plasma concentration parameters of each component are shown in Table 1.

The efficacy data of the NMT-containing serum obtained above was organized by Excel software to obtain the time-dependent change of LDH release and NO content in different injury models of NMT contained serum at different time points. The results are shown in Tables 2 and 3.

**Table 1:** Blood concentration of each ingredient in NMT drug-containing serum at different time points (ng/ml).

Component Group	Chrysophanein	chrysophanol	Aloe-emodin	chrysophanol	rh ein	Emodin	Rg 1	Rb 1	Rb 3	Rc	pue rarin	Dai dze in	3 methoxypue rarin	ligu stili de	Senkyun olide I
0.083	33.49	41.47	41.01	41.41	41.421	53.71	2.5	7.39	3.9	8.33	148.97	531.52	25.68	29.19	44.97
0.25	36.14	96.22	81.99	63.83	74.901	76.623	7.23	15.5	4.89	9.29	725.94	124.699	44.26	173.03	162.48
0.5	27.47	135.07	72.06	45.47	67.679	43.03	8.68	27.28	8.18	12.31	943.94	121.869	28.95	213.47	121.12
0.75	22.73	161.29	53.49	33.67	49.809	30.01	10.63	35.59	10.75	14.31	631.79	891.13	20.18	70.88	129.97
1	23.09	111.98	37.9	26.81	42.009	25.79	13.59	39.99	16.53	18.93	481.78	771.7	17.48	81.02	141.64
1.5	20.39	97.7	32.56	24.19	32.093	15.57	16.63	45.54	22.98	23.71	404.1	628.65	13.62	58.95	88.79
2	15.21	92.85	17.98	20.76	26.669	13.29	20.49	56.32	30.58	29.31	255.25	457.3	9.64	63.46	60.01
4	9.11	79.71	10.83	14.83	14.539	4.2	22.58	65.99	36.36	35.19	300.19	307.8	10.53	30.34	39.94
6	7.08	45.79	8.47	15.04	13.479	5.13	27.3	79.11	57.48	47.12	264.41	314	14.79	35.25	28.98

8	6.399	27.51	7.41	11.66	129.49	6.26	98.31	106.14	110.18	79.62	350.08	285.96	14.87	18.06	4.57
12	5.91	11.64	4.92	13.49	192	11.28	36.6	84.01	36.39	53.42	248.89	344.28	22.88	12.27	4.71
24	3.26	6.23	3.51	14.38	40.65	5.24	16.01	52.57	17.04	30.48	146.7	86.2	3.28	7.35	4.98

**Table 2:** LDH release at different time points.

time group	Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub>	H <sub>2</sub> O <sub>2</sub>	KCl	Glu
0	419.89	133.97	103.14	115.15
0.083	433.06	140.79	106.14	121.84
0.25	464.72	136.7	112.86	126.27
0.5	442.22	115.14	93.57	88.47
0.75	324.44	103.92	70.57	57.06
1	389.44	125.75	79.14	73.12
1.5	418.05	138.05	84.57	71.42
2	410.83	109.55	88.57	77.96
4	431.33	121.72	101.85	84.18
6	467.83	131.03	98.57	88.42
8	471.67	133.68	104.43	98.47
12	472.39	138.7	103.14	105.37
24	471.84	141.68	104	119.04

**Table 3:** NO release at different time points.

Time Group	Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub>	H <sub>2</sub> O <sub>2</sub>	KCl	Glu
0	54.23	61.43	107.68	58.08
0.083	58.68	64.28	116.32	61.5
0.25	61.36	55.79	133.95	67.96
0.5	53.59	37.93	104.11	54.62
0.75	33.36	41.43	83.21	34.15
1	29.45	46.71	78.42	49.81
1.5	40.91	60	89.16	38.69
2	37.77	38.64	97.58	53.19
4	44.32	58.5	107.79	50.57
6	55.45	64.5	119.47	54.62
8	60.23	64.71	128	57.5
12	59.95	64.5	129.37	63.15
24	60.4	64.5	130.63	62.99

**Model establishment:** SVM is a learning method that specializes in finite sample prediction and is suitable for data analysis and relationship description between the complex

active ingredients and biological effects of “non-linear, small samples” [29]. Matlab was used to program and optimize the relevant parameters. A nonlinear correlation analysis model was established for the relationship between the LDH release and the NO content of each blood drug component and the four cell injury models. The contribution of each variable to the drug efficacy was calculated.

The observation table found that the concentration of LDH and the content of NO in different blood levels of different components and in four different cell injury models are quite different [30]. To comprehensively evaluate the correlation between each component and each efficacy index, and the value of each indicator is normalized using the Mapminmax function [31].

$$x(i) = \frac{x_0(i) - \text{Min}(x_0)}{\text{Max}(x_0) - \text{Min}(x_0)}$$

In which  $X_0(i)$  represents the blood concentration or LDH release, NO content in each group. Max/Min indicates the maximum/minor value of blood concentration or LDH release amount and NO content in each group. After this normalization process, it can be ensured that all vector values fall within the range of [0,1]. Using LIBSVM software, the RBF kernel function was selected for SVM calculation [32]. Use the weight factor  $\omega$  to judge the importance of the input variable to the output variable. Its expression is:

$$\omega = \text{model.SVs} \cdot \text{model.sv\_coef}$$

If  $\omega$  is greater than 0, it means that the variable has a positive effect on the output variable; if  $\omega$  is less than 0, it means that the variable has a negative effect on the output variable; if  $\omega$  is equal to 0, it means that the variable has no effect on the output variable [33].

## Results

### LDH drug-time curve of four damage models at different time points

The results are shown in Figures 1-4 below. LDH is an enzyme that exists in the cytoplasm. The release of LDH is positively correlated with the degree of cell damage. The more LDH is released from the cell supernatant, the more cell damage is serious. The LDH side leakage of NMT and each single herb is different, and the drug effect peaks of drug-containing serum at different time points are also different. In the PC12 cell injury model induced by Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, NMT drug-containing serum showed



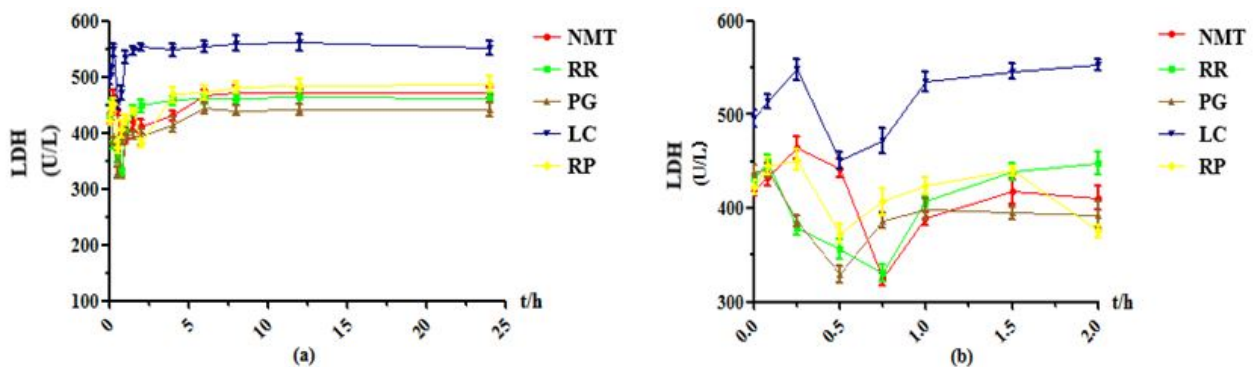
a significant effect peak at 0.75h after absorption into the blood, achieving maximum efficacy. At this time, the side leakage of LDH was lower than 0.25h by 140 U/L, and the protective effect was very obvious. After 2 hours, the protective effect decreased significantly. After 6 hours, the efficacy of drug-containing serum disappeared. RR drug-containing serum reached its maximum efficacy at 0.75h, at this time, the side leakage of LDH in cell supernatant was decreased by about 119U/L compared with 0.083h, which had obvious protective effect, but the protective effect gradually weakened and disappeared after 1h; The side leakage of LDH in the PG-containing serum group increased from 0 to 0.083h, indicating that the 0.083h cell injury reached a maximum, there was an effect peak at 0.5h, and the LDH activity decreased by about 111 U/L compared to 0.083h, and the protective effect was obvious. , And there is a strong protection between 0.25~2h, after 2h the protective effect gradually decreased or even disappeared; The drug-containing serum of LC reached its maximum efficacy at 0.5h with a strong protective effect. At this time, the side leakage of LDH decreased by about 98U/L compared with 0.25h, and there was no protection after 1.5h. The RP drug-containing serum had two effect peaks, 0.5h and 2h, respectively. The leakage of LDH decreased by about 48 U/L compared with 0.25 h. leakage of LDH increased rapidly after 2h, indicating the protection of drug-containing serum quickly declined or even disappeared after 2h.

Under the H<sub>2</sub>O<sub>2</sub> injury model, it was found that NMT drug-containing serum had two effect peaks at 0.75h and 2h, and the 0.5h LDH leakage decreased by 37U/L, which had obvious protective effect, and the drug-containing serum protection gradually weakened or even disappeared after 4h; RR drug-containing serum had two effect peaks at 0.5h and 1.5h. leakage of LDH began to decrease after 0.083h, and LDH activity at 0.5h decreased by 41U/L, reaching the maximum efficacy. the efficacy decreased significantly even disappeared After 4h; In the PG drug -containing serum group, the cell damage was aggravated at 0.083 h, reached the effect peak at 0.75 h, and then the efficacy decreased, and the efficacy disappeared after 1.5 h; In the serum containing LC, the effect peak was reached at 0.5 h, the leakage of LDH was about 35 U/L lower than 0.083 h, and there was a weak protective effect between 0.25h and 1.5 h, and the protective effect was basically disappear 2h later; The

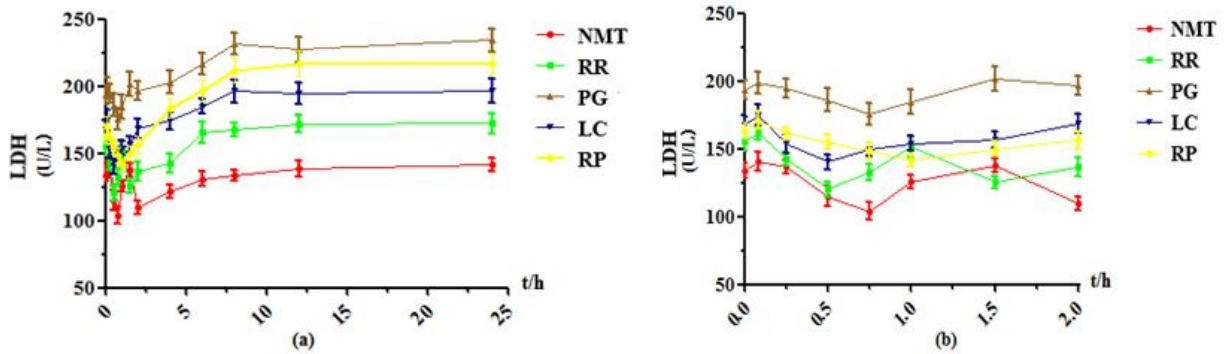
RP drug-containing serum group reached the maximum efficacy at 1h, and the drug-containing serum had no protective effect after 2h.

Under the KCL injury model, an effect peak was observed at 0.75h in the NMT drug -containing serum group, and the LDH leakage was significantly reduced, and the protective effect of the drug-containing serum was gradually reduced after 6h; The RR-containing serum group had one effect peak at 0.75h. the side leakage of LDH decreased by about 40 U/L compared with 0.083 h, and the protective effect was strong. There was no protective effect after 4 h. The PG drug-containing serum group had the greatest efficacy at 0.75h, but the LDH leakage decreased less, and the protective effect was weak. the protective effect disappeared after 2h; The LC drug-containing serum group reached the maximum efficacy at 0.5h, and the protective effect of drug serum gradually decreased or even disappeared after 0.75h; The RP drug-containing serum group reached the maximum efficacy at 0.5h, and the LDH activity decreased less than that of the RR drug-containing serum group. The protective effect was lower than that of the RR drug-containing serum, and the protective effect disappeared after 6h.

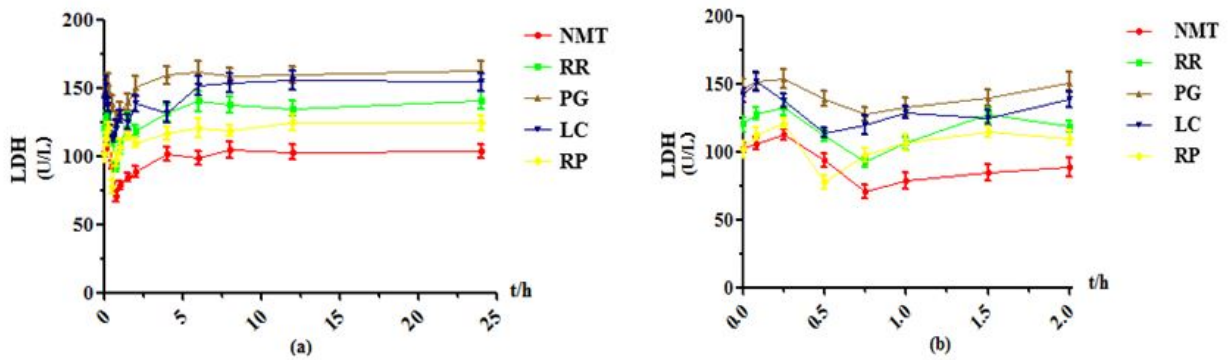
Under the Glu injury model, it was observed that NMT drug-containing serum contained maximal efficacy at 0.75h, LDH leakage decreased 69U/L, and the protective effect decreased gradually after 6h; RR drug-containing serum group showed effect peak at 0.75h, LDH leakage decreased by about 73U/L compared with 0.083h, and the protective effect gradually decreased or even disappeared after 2h; The PG drug-containing serum group reached the maximum efficacy at 0.75h, LDH leakage decreased less, the protective effect was weak, and the efficacy disappeared after 6h; The LC drug-containing serum group reached maximum efficacy at 1h, LDH leakage decreased less and the protective effect was weak, and the efficacy gradually weakened or even disappeared after 1.5h; There was an effect peak at 0.5h in the RP drug-containing sera group, and the side leakage of LDH was about 63 U/L lower than 0.25h. The protective effect lasted for a long time and still had a weak protective effect at 24 h.



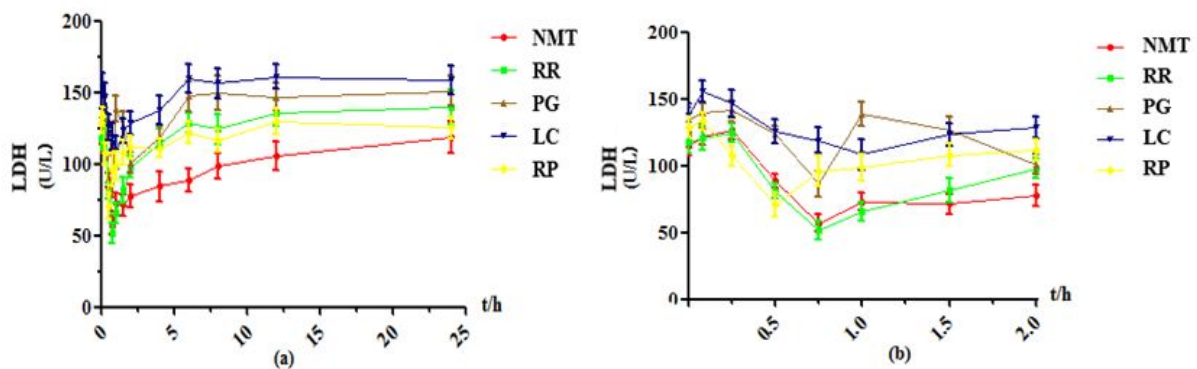
**Figure 1:** Effect of NMT and each herb drug-containing serum on LDH of Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> injury at different time points ( $\bar{x} \pm s, n=6$ ) (a): 0-24h; (b): 0-2h.



**Figure 2:** Effect of NMT and each herb drug-containing serum on LDH of  $H_2O_2$  injury at different time points ( $\bar{x} \pm s, n=6$ ) (a):0-24h; (b):0-2h.



**Figure 3:** Effect of NMT and each herb drug-containing serum on LDH of KCL injury at different time points ( $\bar{x} \pm s, n=6$ ) (a):0-24h; (b):0-2h.



**Figure 4:** Effect of NMT and each herb drug-containing serum on LDH of Glu injury at different time points ( $\bar{x} \pm s, n=6$ ) (a):0-24h; (b):0-2h.

#### NO drug-time curve of four damage models at different time points

As shown in Figures 5-8, under the  $Na_2S_2O_4$  injury model, NMT drug-containing serum achieved maximum efficacy at 1h,

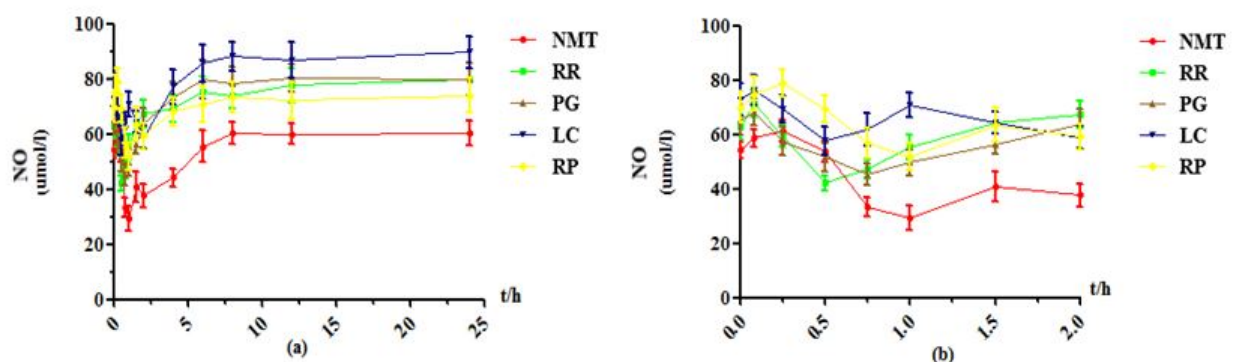
and the NO content was reduced by about 32 $\mu$ mol/L compared with 0.025h. The protective effect was very significant. After 8 hours, the drug efficacy of the drug-containing serum tended to be stable, and the duration was longer, and there was still a weak protective effect at 24 h. RR drug-containing serum

showed an effect peak at 0.5h. At this time, the NO content was decreased by about 29  $\mu\text{mol/L}$  compared with 0.083h. The protective effect was obvious, and the efficacy after 6h disappeared. In the PG drug-containing serum group, the effect peak was reached at 0.75h, and the decrease of NO content was little. After 4h, there was almost no protective effect and tended to be stable. LC drug-containing serum has two effect peaks at 0.5h and 2h, with weak protective effect and almost no protection after 4h. In the RP drug-containing serum group, the effect peak appeared at 1 h, the protective effect was obvious, and the duration was longer. There was still a weak protective effect at 24 h.

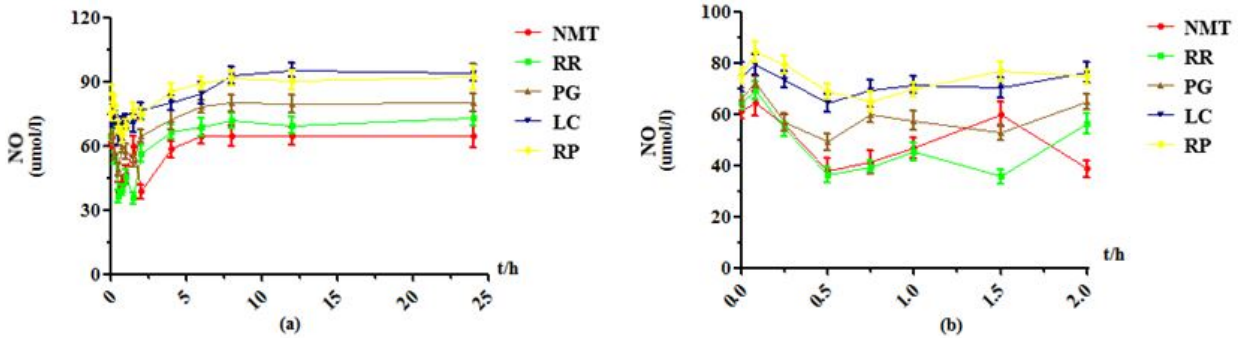
Under the  $\text{H}_2\text{O}_2$  injury model, NMT drug-containing serum showed two effect peaks at 0.5h and 2h, and the drug effect was obvious. After 2h, the protective effect of drug-containing serum rapidly decreased and tended to be stable. RR drug-containing serum achieved maximum efficacy at 0.5h and 1.5h, and the content of NO decreased by 33  $\mu\text{mol/L}$  at 0.5h compared with 0.083h. The protective effect was very obvious, and the protective effect was weakened or even disappeared after 4h. PG drug-containing serum group reached the maximum efficacy at 0.5h, when the NO content decreased less than 0.083h about 23  $\mu\text{mol/L}$ , and the drug-containing serum after 4h had no protective effect. The effect peak of LC drug-containing serum appeared at 0.5h, and the decrease of NO content was less than that of other drug-containing serum groups. The protective effect was weak, and the protective effect disappeared after 4h. The effect peak of RP drug-containing serum appeared at 0.75h, and the content of NO decreased by about 20  $\mu\text{mol/L}$  compared with 0.083h. There was no protective effect after 4h.

Under the KCl injury model, NMT drug-containing serum reached its maximum efficacy at 1 hour, its protection was very obvious, and its duration was longer, and it still had weak protection effect at 24 hours. RR drug-containing serum showed an effect peak at 0.75h, when the NO content was decreased by about 57  $\mu\text{mol/L}$  compared with 0.083h, there was obvious protective effect, gradually weakened and stabilized after 1.5h, and there was still a weak protective effect at 24h; The effect peak of PG drug-containing serum appeared at 0.75h, the efficacy reached the maximum, NO content decreased less, and the protective effect disappeared after 8h; LC drug-containing serum reached the effect peak at 0.25 h, and the NO content decreased significantly. After 1.5 h, the protective effect gradually decreased and stabilized; RP drug-containing serum reached its maximum efficacy at 0.5 h, but the NO content decreased less, and the protection gradually weakened after 6 h.

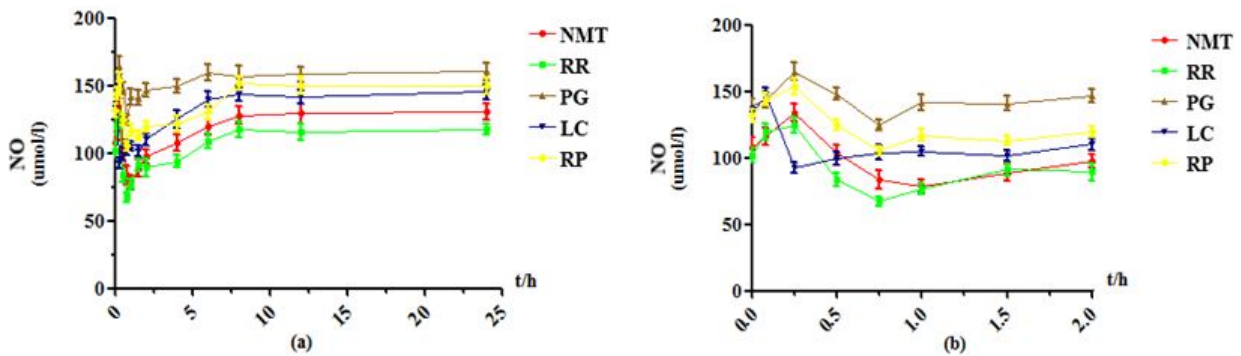
Under the Glu injury model, the NMT drug-containing serum had obvious effect peaks at 0.75h and 1.5h, which had a significant protective effect and a long duration. It still had a weak protective effect at 24 h. RR drug-containing serum had a very obvious effect peak at 0.75h, when the NO content was decreased by about 31 $\mu\text{mol/L}$  compared with 0.083h, the protective effect disappeared after 6h; The PG drug-containing serum reached its maximum efficacy at 0.75h, but the decrease in NO content was less than that of protective effect, and there was no protective effect after 4h. The drug-containing serum of LC had effect peaks at 0.5h, and the NO content decreased greatly. The efficacy disappeared after 6h. RP drug-containing serum had a weaker effect peak at 1h, and the efficacy disappeared after 6h.



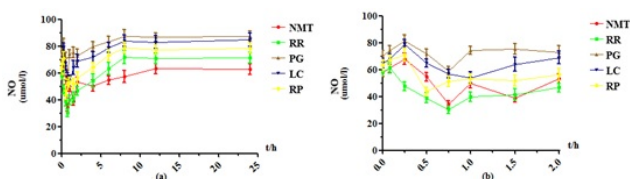
**Figure 5:** Effect of NMT and each herb drug-containing serum on NO of  $\text{Na}_2\text{S}_2\text{O}_4$  injury at different time points ( $\bar{x} \pm s, n=6$ ) (a): 0-24h; (b):0-2h.



**Figure 6:** Effect of NMT and each herb drug-containing serum on NO of H<sub>2</sub>O<sub>2</sub> injury at different time points ( $\bar{x}\pm s, n=6$ ) (a):0-24h; (b):0-2h.



**Figure 7:** Effect of NMT and each herb drug-containing serum on NO of KCl injury at different time points ( $\bar{x}\pm s, n=6$ ) (a):0-24h; (b):0-2h.



**Figure 8:** Effect of NMT and each herb drug-containing serum on NO of Glu injury at different time points ( $\bar{x}\pm s, n=6$ ) (a):0-24h; (b):0-2h.

**Weight coefficient of each component using LDH as indicator**

For the four different damage models, each component has different weight coefficients with LDH release as indicators,

showing different positive and negative correlations, as shown in Table 4 below. For the Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> model group, except for Chrysophanein, Rb1, and Senkyunolide I, there was a positive correlation between the other components and the efficacy of LDH release. H<sub>2</sub>O<sub>2</sub> induced oxygen free radical damage model, rheochrysidin, Senkyunolide I, chrysophanol, emodin, 3'-methoxy puerarin, Rc, Rg1, Rb1 with pharmacodynamics of LDH release as indicators have a positive correlation and the positive correlation decreases in turn. In the KCl-induced calcium overload model, chrysophanol, emodin, 3'-methoxy puerarin, ligustilide, Aloe-emodin, rhein, emodin, Rc, Rb3 with pharmacodynamics of LDH release as indicators have a positive correlation and the positive correlation decreases in turn. For Glu-induced glutamate toxicity damage model, rheochrysidin, Chrysophanein, 3'-methoxy puerarin, chrysophanol, emodin, rhein, Rg1, ligustilide, Rc with pharmacodynamics of LDH release as indicators have a positive correlation.

**Table 4:** Weighting coefficient w (LDH) for each component and four damage models.

Component Group	Chrysophanein	chrysophanol	Aloe-emodin	chrysophanol	rheinchrysidin	Emodin	Rg1	Rb1	Rb3	Rc	puerarin	Daidzein	3-methoxy puerarin	ligustilide	Senkyunolide I



Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub>	32.7	-99.85	30.07	59.17	15 72	21. 14	8. 65 4	39 33	26 17	4 9. 6 2	25. 59	21.47	58.99	45.6 8	-30.94
H <sub>2</sub> O <sub>2</sub>	51.91	-186.2	-1.665	38.8	-1 4. 66	35. 53	5. 08 1	2. 93	-6. 90 7	2 9. 1 1	-43. 57	-10.18	31.41	-29. 29	45.28
KCl	63.43	-93.46	25.56	71.07	8. 35 1	22. 13	-1. 46 7	-1 6. 32	1. 66 8	3. 6 9 6	-11. 67	-3.536	62.15	41.6 3	-47.53
Glu	47.31	-100.3	-12.47	42.83	16 72	24. 85	16 41	-9. 73 5	-4 0. 67	6. 9 0 3	-37. 6	-15.7	30.34	7.64 2	3.845

### Weight coefficient of each component using NO as indicator

For the four different damage models, each component has different weight coefficients with NO release as indicators, showing different positive and negative correlations, as shown in Table 5 below. For the Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> model group, expect for Chrysophanein, Senkyunolide I, there was a positive correlation between the other components and the efficacy of the NO content index. The oxygen free radical damage model caused by H<sub>2</sub>O<sub>2</sub>, expect for Chrysophanein, Rg1, Rb1, puerarin, 3'-methoxy

puerarin, and ligustilide, the other components with pharmacodynamics of NO contents as indicators have a positive correlation. In the KCl-induced calcium overload model, except for Chrysophanein, puerarin, daidzein, and ligustilide, the other components with pharmacodynamics of NO contents as indicators have a positive correlation. For the Glu-induced glutamate toxicity injury model, emodin, 3'-methoxy puerarin, chrysophanol, rhein, ligustilide, Rg1, Rb1 with pharmacodynamics of NO contents as indicators have a positive correlation and the positive correlation decreases in turn.

**Table 5:** Weighting coefficient w (NO) for each component and four damage models.

Component Group	Chryso phanein	Chrysop hanol	Aloe- emodi n	chryso phanol	R he in	Em odi n	R g1	R b1	R b3	R c	Puer arin	Daidz ein	3 methoxypu erarin	ligu stili de	Senkyu nolide I
Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub>	19.98	-56.39	40.27	51.99	3. 29 9	1.9 13	18 44	-1. 40 4	17 43	10 75	7.173	8.618	9.755	15.2 6	-55.81
H <sub>2</sub> O <sub>2</sub>	39.67	-66.64	10.24	45.8	-1 6. 95	15. 22	-1 2. 7	-1. 52 6	23 98	9. 28 6	-41.9 7	18.81	-42.53	-42	0.127
KCl	34.87	-69.08	17.99	60.48	9. 54 4	42. 18	14 04	21 09	3. 01 3	29 89	-18.2 4	-1.702	31.46	-11. 51	40.07
Glu	47.01	-236.2	-9.902	91.16	49 14	121 .1	18 31	3. 46 8	-7 1. 57	36 09	-76.4 6	-3.685	107.8	30.3	-45.71

## Discussion

Ischemic stroke is a common disease with rapid onset, diverse changes, serious illness and high mortality, which is a great hazard to human health. The prevention of stroke has become a common concern. research on drugs for treating stroke has attracted the attention of the scientific community. Therefore, this article establishes commonly used four types of cerebral ischemic in vitro cell injury models, these four in vitro models cover an important four-step response to the cascade of cerebral ischemia, in which the Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> model is the initial injury process, while H<sub>2</sub>O<sub>2</sub>, KCL, and GLU are the three most serious processes leading to cerebral ischemic injury, consistent with the theory of cerebral ischemic injury. that is, Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>-

induced hypoxia-glucose injury model, H<sub>2</sub>O<sub>2</sub>-induced oxygen free radical injury model, KCL-induced calcium overload model, and Glu-induced amino acid toxicity injury model. Taking PC12 cells as the research object, the release amount of LDH and NO content were used as pharmacodynamic indicators. through compared NMT and each single herb drug-time curves of different indicators of the four damage models found that the protective effect of NMT on cerebral ischemic injury caused by the four mechanisms is better than that of the individual herbs, which is in accordance with the characteristics of compound multiple effects and integrity. The effect of each single herb on the four damage mechanisms is different. For example, for Glu-induced cerebral ischemic injury, RP has a greater protective effect and can be considered to increase the proportion of RP in

the NMT group when treating cerebral ischemic injury caused by Glu. In the cerebral ischemic injury caused by calcium overload mechanism, the protective effect of LC is larger than that of PG and RP group, and it may be considered that in the treatment of cerebral ischemic injury caused by calcium overload mechanism, the proportion of LC in the compound compatibility can be increased to achieve rational administration of cerebral ischemic injury caused by different mechanisms. However, in the future research, it is necessary to further investigate the specific composition ratio of NMT in the treatment of cerebral ischemic injury caused by four different injury mechanisms in order to achieve the purpose of clinical rational drug use. The protective effect of each single herb on the four damage mechanisms is in line with the combination rule of NMT.

Compound has a significant protective effect on cerebral ischemic injury and is superior to each single drug group. Previous studies have confirmed that emodin, rhein, emodin methyl ether, chrysophanol, daidzein, puerarin, 3'-methoxy Puerarin, Re, Rb1, Rg1, Rb3, ligustilide, senkyunolide I, ferulic acid have significant efficacy against cerebral ischemia-reperfusion injury. therefore, according to the previous pharmacokinetic data combined with the pharmacodynamic indicators of this article, based on the SVM algorithm, a PK-PD correlation mathematical model was established to study its pharmacodynamic material basis for the treatment of cerebral ischemic injury. It was found that the drug-containing serum contain schrysothanein, chrysophanol, aloemodin, chrysophanol, rhein, emodin, Rg1, Rb1, Rb3, Rc, puerarin, daidzein, 3'-methoxypuerarin, ligustilide and senkyunolide I have positive correlations in four cell models of cerebral ischemia-reperfusion injury, which may be cerebral the main components of the pharmacodynamic effects are exerted, and the positive correlations of the models of cerebral ischemic injury caused by different mechanisms are different. This 15 components in NMT contained drug serum had a certain correlation with the cell injury model induced by the four mechanisms. It may be a medicinal material of NMT, and the different components are responsible for the cerebral ischemic injury caused by different mechanisms. The correlation of the models is also different, indicating that for different mechanisms of cerebral ischemic injury, the efficacy of each component is also different. For  $\text{Na}_2\text{S}_2\text{O}_4$  injury model, the positive and negative correlations of the effects of LDH release and NO content were basically the same for all components. They were basically chrysophanol, Aloe-emodin, rheochrysidin, Rb1, Rc, and 3'-methoxy puerarin and ligustilide is greater, suggesting that these components may play a major role in the treatment of brain thrombosis caused by cerebral hypoxia-ischemia. For  $\text{H}_2\text{O}_2$ -induced oxygen free radical damage models, the contribution of rheochrysidin, chrysophanol, Rc, and 3'-methoxy puerarin is basically higher, which suggested that these components may play a major role in the treatment of cerebral ischemia caused by oxygen radical damage. In the KCl-induced calcium overload model, the weighting coefficients of chrysophanol, rheochrysidin, Senkyunolide I, 3'-methoxy puerarin, Rg1, Rb1, and Rc are relatively large, indicating that these components may be NMT treatment of major components of cerebral ischemia induced by calcium overload. The amino acid toxicity damage model

induced by Glu is basically the contribution of emodin, chrysophanol, 3'-methoxy puerarin, rheochrysidin, rhein, Rg1, and ligustilide. The composition may play a major role in the efficacy of NMT in the treatment of cerebral ischemic injury caused by amino acid toxicity. This reveals the pharmacodynamics basis of NMT in treating cerebral ischemia, and provides reference for the clinical rational use of NMT for the treatment of cerebral ischemic injury, and provides guidance for the basic research on the pharmacodynamics of Chinese herbal compound. This also initially reveals the effective material basis of NMT, and also provides reference and basis for the rational use of NMT in the treatment of cerebral ischemic injury. However, the specific pharmacodynamic mechanisms of the four damage mechanisms of the individual monomer components against cerebral ischemic injury remain to be further studied.

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Ke-ning Li and xu-lei Fan conceived and designed the experiments. Lan-fang Tan conducted the experiments and draft the paper. Ke-ning Li, lan-fang Tan, lei Chen and shu-mei Wang conducted part experiments and analyzed the data. All authors revised the paper and approved the final manuscript.

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## Competing and conflicting Interests

The authors declare that they have no competing and conflicting interests.

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