OPEM
www.opem.org

Oriental Pharmacy and Experimental Medicine 2005 **5(2)**, 156-159

Experimental research for the protective effect of Naoxingtong-containing serum on rat cerebral microvascular endothelial cells

Zhou Jun[1], Guo Jianyou[1], Guo Jian[2], Li Lanfang[1], Li Canghai[1], Jiang Nan[1], Guo Shuying[1], Huo Hairu[1] and JiangTingliang[1,*]

[1]Tang Center for Herbal Medicine Research, Institute of Chinese Materia Medica, China Academy of TCM, Beijing, 100700; [2]Shaanxi Xianyan Changfeng Pharmaceutics Company, Xianyan 710075, China

SUMMARY

The protective effect of Naoxingtong (NXT) on rat cerebral microvascular endothelial cell (rCMEC) was investigated. rCMEC was injured in vitro by incubating for 4 hours at 100% NO in a hypoxia chamber. After treated with NXT-containing serum, the cellular viability rate (90.3%) was significantly elevated when compared with that of control group and the inhibitive rate of lactic dehydrogenase (LDH) activity (9.2%) was far lower than the control group with dose-dependent effect. The results indicate that NXT can increase viability of rCMEC, and protect cell membrane from injury during hypoxia.

Key words: NXT; Drug-containing serum; CMEC; Lactic dehydrogenase

INTRODUCTION

Pathological process of ischemic cerebrovascular disease includes change of penetration of cell membrane, over-loading of calcium, produce of oxygen free radical and release increase of excitatory amino acids induced by severe lack of oxygen. The pathological change results in cellula swell, appode. Ischemic cerebrovasular disease is fatal disease to hurt human health (Wolfgang, 1999). Unfortunately, as yet these is no effective treatment method. Naoxintong (NXT) is an effective formula of Chinese Materia Medica treating cerebral infarction in clinic (Li, 2000; Wang *et al.*, 2001). The protective effect of NXT-containing serum on rCMEC hypoxia injury was investigated.

*Correspondence: JiangTingliang, Tang Center for Herbal Medicine Research, Institute of Chinese Materia Medica, China Academy of TCM, Beijing, 100700, China. E-mail: tctcmjtl@yahoo.com.cn

MATERIALS AND METHODS

Experimental drugs

NXT capsules were provided by Shanxi Xianyan Changfeng pharmaceutics company, which consists of radix astragali, radix salviade miltiorrhizae, radix paeoniae rubra, flos carthami, frankincense, myrrh, and ramulus cinnamomi. Every capsule contains 0.4 gram drug. 2 capsulels one time and 3 times per day for adult in clinic.

Animals

Male healthy SD rats, weighting 80 to 100 gram, were purchased from Beijing Vital River Laboratory Animal Co., Ltd. Animal certification number was SCXK 2002-0003 (Beijing).

Equipment

96-well costar culture plates and flasks, purchased from Corning Incorpated. BB16 CO_2 incubator was

purchased from Hertus Company, and microplate reader (Model 550) was purchased from Bio-Rad Company. Sorvall centrifuger (T_{21}) was purchased from Du Pont Company.

Reagents and preparations

DMSO and MTT were purchased from Sigma. Fetal calf serum (FCS) was purchased from Hyclone, Penicillin and LDH assay kit were purchased from Beijing BHKT Clinical Reagent Co., Ltd, Lot. 030519. Endothelial cells growth factor (ECGF) Medium 199 was Gibco's product. 1000 ml M199 (dissolved with distilled water) supplemented with heparin sodium (10 U/ml), penicillin and streptomycin (each 100 U/ml), $NaHCO_3$ (2.2 gram) were referred to as stored liquids (filtrate sterilized) and stored at 4°C for further use. Media with 8% FCS was referred to as washing media, media with 25% FCS as culture media containing 150 μg/ml of ECGF. Heparin sodium was provided by Changzhou Xinhua Reactive Material Institute, Lot. 000424. Collagenase was purchased from Gibco BRL, Lot No. 1075068.

rCMEC preparation

rCMEC were cultured as previously described, the new-born SD rats were sacrificed and soaked in 75% alcohol; brains were removed and rinsed in PBS. Cerebral pia matters were stripped; cerebral white matter, cerebellum and great vessels were removed. Then the cerebral cortex was isolated and soaked in fresh washing media. After homogenized, the cells were filtered through 80 mesh sieves. Then the filtered solution was collected and filtered through 200 mesh sieves again. The remnant microvessel cells on sieves were collected, and incubated for 20 minutes at 37°C after 0.1% collagenase was added. Then the filtered solution was centrifuged at 1500 rpm for 5 minutes, the pellet was washed with PBS for 3 times. Culture media was added to the pellet as cell suspension and seeded in culture flask. Then the culture media were changed every 3 days, and rCMEC were confirmed with VIII antigen response (Gordon *et al.*, 1991; Xu *et al.*, 1997).

Preparation of NXT-containing serum

12 healthy male rats weighting 250 ± 10 g were orally given with the NXT (dissolved with distilled water, 40 g/kg) and twice a day for 5 days. 1h after the last dose, rats were anesthetized with diethyl ether, blood was taken through abdominal aorta and stored at 0°C for 1 h. After blood was centrifuged, all NXT-containing serum was collected, mixed, sterilized and stored at -20°C. The control serum was taken from another 6 rats orally given distilled water (Yin *et al.*, 2000).

Expression of dose of NXT-containing serum

Dose of NXT-containing serum (g/kg/U) = dose (g/kg) of NXT orally given to animal/dilution times (U) of NXT-containing serum in culture system *in vitro*.

Hypoxia conditions

rCMEC were cultured in 48-well culture plates (the covers were removed) and placed in humidified, airtight chamber for 3 h, which was gassed with nitrogen at flow-rate of 1.5 kmf/cm^3 for 0.5 h. Then cells were continual cultured in incubator with 95% O_2 and 5% CO_2 at 37°C.

Protective effect of NXT-containing serum on injured rCMEC under hypoxia

1×10^5/ml of rCMEC were cultured in 48-well culture plates (500 μl/well) in incubator with 95% O_2 and 5% CO_2 at 37°C. After cultured for almost 30 h, 63, 38, 23, 14 and 8 μl of NXT-containing serum were added to 8 wells, respectively. Using the control serum bring the final volume of each well to 563 μl, which was identical to 5.0, 3.0, 1.8, 1.1 and 0.65 g/kg/U of NXT-containing serum per well, respectively. 8 wells added with control serum were referred to as control group, another 8 as hypoxia group. Then all rCMEC were cultured for 16 h, and the culture plate was placed in humidified,

airtight chamber which was gassed with 100% NO at 37°C except for the control group. Then cells were incubated for 2 h in incubator with 95% O_2 and 5% CO_2 at 37°C, and the culture media were collected for analyzing the amount of LDH by chromatometry assay at a wavelength of 490 nm. M199 culture media free from serum (200 μl/well) and 5 mg/ml of MTT (20 μl/well) were added to the wells which remain cells and incubated for 4h in incubator with 95% O_2 and 5% CO_2 at 37°C. Then the supernatant was removed and DMSO (200 μl/well) was added for detecting the absorbance of the converted dye at a wavelength of 490 nm on a Bio-Rad microplate reader. The changes of absorbance of the converted dye are parallel to the changes of the cells activity and cell number (Jiang *et al.*, 2000).

Statistical analysis

Results are presented as mean ± SD ($\bar{x} \pm S$). Differences between groups were analyzed with unpaired Student's *t* test.

RESULTS

Protective effect of NXT-containing serum on injured rCMEC during hypoxia

The MTT method was used to measure the viability rate of rCMEC. As is showed in Table 1, the viability rate of hypoxia group (38.6%) was decreased significantly. And the protective rate of NXT-containing serum treated groups were increased significantly, which was above 90% at all experimental doses.

Effect of NXT-containing serum on the release of LDH in rCMEC

As is showed in Table 2, LDH in extracellular fluid was increased under hypoxia. Treatment with NXT-containing serum encountered the increase of LDH in dose-dependent manner (regression equator: $y = 4.05 + 1.58$, $r = 0.999$, $P < 0.01$). NXT-containing serum was significantly decreased the release of LDH when the dose was above 1.8 g/kg/U.

DISCUSSINON

The protective effect on the injured rCMEC is not only involved in preventing cerebral vascular accident, but also implicated in stopping the deterioration of brain vascular. To investigate the mechanism of NXT effect on cerebral vascular accident, the rCMEC under hypoxia conditions were cultured, the activity of endothelial cells and the amount of LDH were detected among every group.

NXT capsule is a crude pharmaceutics made with Chinese medicines. If it is added to cell culture system directly, the scientific result of

Table 1. Protective effect of NXT-containing serum on injured rCMEC during hypoxia (MTT, $\bar{x} \pm S$)

Groups	Dose of serum (g/kg/U)	O.D.	Viability rate[a] (%)	Protective rate[b] (%)
Control group	-	0.453 ± 0.031		
Hypoxia group	-	0.175 ± 0.013 △△	38.6	
Treated with NXT	5.0	0.379 ± 0.024**	83.7	116.6
Treated with NXT	3.0	0.386 ± 0.016**	85.2	120.6
Treated with NXT	1.8	0.380 ± 0.019**	83.9	117.1
Treated with NXT	1.1	0.381 ± 0.021**	84.1	117.7
Treated with NXT	0.7	0.333 ± 0.078**	73.5	90.3

(n = 6/group). △△$P < 0.01$ when compared with control group; **$P < 0.01$ when compared hypoxia group.

[a] Viability rate (%) = (OD value of control group- OD value of hypoxia groups (or treatment group))/ OD value of control group × 100%

[b] Protective rate (%) = (viability rate of treatment groups- viability rate of hypoxia groups)/viability rate of hypoxia group × 100%

Table 2. Effect of NXT-containing serum on the release of LDH in rCMEC ($\bar{x} \pm S$)

Group	Dose of serum (g/kg/U)	LDH (U/L)	Inhibitive rate[a] (%)
Normal group	-	264.1 ± 17.3	
Hypoxia group	-	285.7 ± 16.8	
Treated with NXT	5.0	223.6 ± 22.1**	21.7
Treated with NXT	3.0	245.8 ± 15.3**	13.9
Treated with NXT	1.8	259.4 ± 4.1*	9.2
Treated with NXT	1.1	269.3 ± 15.3	5.7

** $P < 0.01$, * $P < 0.05$ when compared with normal group.
[a] Inhibitive rate (%) = (LDH content of hypoxia group - LDH content of treatment group)/LDH content of hypoxia group)×100%

experiments will be interfered by the PH, osmotic pressure, and non-specific confusion by non-activity component. In our experiment, rats were orally given with drug, the serum were collected and added in culture media to observe the effect of NXT, which could increase the creditability of experimental results; in addition, it could predict the effective dose *in vivo*.

At this experiment, microvascular endothelial cells are heavily injured when cultured in sufficient nitrogen fume conditions, and the viability rate was below 40%. After treated with NXT-containing serum, the protective rate was above 90% (0.65 g/kg/U). These findings show NXT-containing serum strongly protects microvascular endothelial cells from injury in hypoxia. LDH is the important enzyme at non-oxygen metabolism, catalyses reaction between pyruvic acid and lactic acid. It exists in cytolymph and will leak out the membrane when membrane is damaged. So the increase of the activity of LDH indicates the damage of membrane. In our experiment, the activity of LDH was increased in hypoxia, which indicated membrane was lightly damaged. And NXT-containing serum was significantly decreased the release of LDH. In summary, NXT can treat cerebral infarction through protecting microvascular endothelial cells from injury.

REFERENCES

Gordon EL, Danielsson PE, Nguyen TS. (1991) A comparison of primary cultures of rat cerebral microvascular endothelial cells to rat aortic endothelial cells. *In Vitro Cell Dev. Biol.* **27**, 312-326.

Li Q. (2000) Bu Chang NXT Treating 31 cases of vascular dementia. *Lishizhen Medicine and Material Medica Research.* **11**, 535-536.

Jiang ZS, Xia CF, Tian QP, Fu MG, Wang XH, Pang YZ, Tang CS, Liu NK. (2000) Effect of batroxobin against dog heart ischemia/reperfusion injury. *Acta Pharmacol. Sin.* **21**, 70-74.

Wang HQ, Liu LQ, Sun HY. (2001) Clinical observation of Bu Chang NXT in treating ischmic cerebrovascular disease. *Mod. J. Int. Chinese Trad. West. Med.* **10**, 720.

Wolfgang W. (1999) Cerebral Ischemia Molecular and Cellular Pathophysiology. Humana Press, Totowa, New Jersey, 3-3.

Xu YG, Liu Y. (1997) Culture and morpholocical observation of rat cerebral microvascular endothelial cell. *J. Micro. Tech.* **2,** 63-65.

Yin CH, Li LF, Jin YH, Yun GD, Tu YJ, Jiang TL. (2000) Inhibitive effect of DGBXT-containing serum of mouse with hepatic injury on production of hepatic LPO. *Chinese J. Exp. Trad. Med. Formulae* **6**, 32-34.