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Effects of Naoxintong-containing serum on NO and CGRP in rat cerebral microvascular endothelial cells

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SUMMARY

Effects of Naoxintong (NXT, a formula of Chinese Materia Medica)-containing serum on Nitrogen monoxide (NO) and calcitonin gene related peptide (CGRP) in rat cerebral microvascular endothelial cells (rCMEC) was investigated. rCMEC was injured *in vitro* by incubating for 4 hours at 100% NO in a hypoxia chamber. The results indicated that NXT could antagonize the reduction of NO and CGRP secreted by rCMEC during hypoxia, the effect of which was dose-dependent. After treated with NXT-containing serum at dosage of 5.0 - 3.0 and 5.0 - 1.1 g/kg/U respectively, the amount of NO and CGRP secreted by rCMEC were remarkably increased during hypoxia *in vitro*.

Key words: Naoxintong; Drug-containing serum; Cerebral microvascular endothelial cell; Nitrogen monoxide; Calcitonin gene related peptide

INTRODUCTION

In addition to its protective effect on the vascular smooth muscle, vascular endothelial cells can secrete several factors to keep normal tension of smooth muscle and ensure the circulation and provision of blood. The function of smooth muscles will be in disorder when vascular endothelial cells are injured by multi-factor, and this may be one of factors causing cardiovascular diseases. Naoxintong (NXT) is a formula of Chinese Materia Medica treating coronary heart disease and angina in clinic (Li, 2000; Wang *et al.*, 2001). From the previous experiment, we knew that the NXT-containing serum could protect the rat cerebral microvascular endothelial cell (rCMEC) from injury in hypoxia *in vitro*. To explore the mechanism of protective effect of NXT-containing serum on rCMEC, the amount of Nitrogen monoxide (NO) and calcitonin gene related peptide (CGRP) secreted by rCMEC in hypoxia were measured.

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MATERIALS AND METHODS

Experimental drugs

NXT capsules were provided by Shaanxi Xianyang 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 crude drug. 2 capsules one time and 3 times per day for adults in clinic.

Experimental 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. 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. NO assay kits were provided by Nanjing Jianchen Bioengineering Institute, Lot. 030605; CGRP assay kits were purchased from Dong Ya Institute of Immunological Technology in Beijing, Lot. 2003-7-25.

rCMEC preparation

rCMEC were cultured as previously described (Gordon *et al.*, 1991; Xu *et al.*, 1997), the new-born SD rats were sacrificed and soaked in 75% alcohol; brains were removed and rinsed in PBS. Cerebral white 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 III antigen response.

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. 1 h 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 = 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, 5% CO_2 incubator.

Measurement of NO and CGRP

1×10^5 cell/ml of rCMEC were added to 96-well (200 μl/well) culture plates and incubated for 30 h in incubator with 95% O_2 and 5% CO_2 at 37°C.

4 doses of NXT-containing serum (dilution with control serum) were added to 8 wells in the culture plates (25 μl/well), respectively. The control serum was taken from another 6 rats orally given distilled water. 8 wells added with control serum were referred to as control group, another 8 as hypoxia group. Then all rCMEC cultured for 16 h again, the culture plate (without cover) were placed in humidifier, airtight chamber for 3 h except for the control group, which was gassed with nitrogen at flow-rate of 1.5 kmf/cm^3 for 30 min. Then rCMEC 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 NO and CGRP. The amount of NO (μmol/L) was detected according to the standard material; and the amount of CGRP (pg/ml) was measured by radioimmunity assay.

Statistical analysis

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

RESULTS

Effect of NXT-containing serum on NO secreted by rCMEC

As is shown in Table 1, the amount of NO secreted by rCMEC was significantly decreased during hypoxia, which was about 74.3% that in control group. Treatment with NXT-containing serum encountered the drop of No in dose-dependent manner (Regression equation: Y = 12.77x + 15.35, R = 0.80). The result showed significant difference between the hypoxia group and NXT-containing serum group from the dose of 3 to 5 g/kg/U.

Effect of NXT-containing serum on CGRP secreted from rCMEC

As is shown in the Table 2, the amount of CGRP secreted from rCMEC was significantly decreased during hypoxia, which was about 44.4% that in control group. The amount of CGRP was increased after treated with NXT-containing serum, which shows dose-dependent effect (Regression equation: Y = 12.25x + 61.52, R = 0.87). Compared with control group, the amount of CGRP in NXT-containing serum group was significantly increased at the experimental dose.

Table 1. Effect of NXT-containing serum on NO secreted by rCMEC ($\bar{x} \pm S$)

Groups	Dose of serum (g/kg/U)	NO (μmol/L)	Increase rate[a] (%)
Control group	-	21.01 ± 9.20	
Hypoxia group	-	15.60 ± 6.57	
Treated with NXT	5.0	27.40 ± 5.94*	75.6
Treated with NXT	3.0	23.83 ± 6.86*	52.9
Treated with NXT	1.8	25.04 ± 14.01	60.5
Treated with NXT	1.1	17.41 ± 4.20	11.6

(n = 6/group). *P < 0.05 when compared hypoxia group. [a]Increase rate = (NO content of treatment groups - NO content of hypoxia group)/NO content of hypoxia group × 100%

Table 2. Effect of NXT-containing serum on CGRP secreted from rCMEC ($\bar{x} \pm S$)

Groups	Dose of serum (g/kg/U)	CGRP (pg/ml)	Increase rate[a] (%)
Control group	-	71.74 ± 13.45*	
Hypoxia group	-	31.87 ± 15.02	
Treated with NXT	5.0	73.57 ± 19.73**	130.9
Treated with NXT	3.0	57.58 ± 14.94*	80.7
Treated with NXT	1.8	59.24 ± 16.24*	85.9
Treated with NXT	1.1	58.03 ± 15.15*	82.1

(n = 6/group). *P < 0.05, **P < 0.01 when compared hypoxia group. [a]Increase rate = (CGRP content of treatment groups - CGRP content of hypoxia group)/CGRP content of hypoxia group × 100%

DISCUSSION

The relationship between NO and heart and cerebral vascular disease is concerned intensively

(Hirate, 2003). It has been suggested that NO is transformed from arginine by a group of isoenzymes known as eNOS and iNOS, then it diffuses into neighbor tissues and cells. NO can not only suppress the contraction of vascular smooth muscle through participating in endothelial cell-dependent vascular relaxation response, but also prohibit multiplication and movement of monocyte, adhesion of blood platelet, oxidation of LDL and synthesis of cytokine. Accordingly, it can protect neuron and treat hypertension, arteriosclerosis. By reducing the amount of NO, the ability of endothelial cells dependent vascular relaxation is depressed and perfusion volume is reduced under hypoxia conditions, which quicken the injury progress during hypoxia and ischemia. The experiment demonstrated that NXT-containing serum antagonisted the reduction of NO in hypoxia, restored or enhanced the ability of rCMEC to secrete NO. Further research was needed to test whether it increase the activity of eNOS or iNOS.

CGRP is a regulatory protein existed in nerve system, heart vascular and lung tissue. It can expand blood vascular and depress blood pressure, increase blood flow of important organ, suppress the oxidation of lipid and antagonize the effect of ET, protect endothelial cell and suppress multiplication of smooth muscle (Han, 2001). It will also improve the condition of patients with cerebral arteriosclerosis and head block. The result indicates that the amount of CGRP secreted by rCMEC was decreased during hypoxia. After treated with NXT-containing serum, the amount of CGRP was increased. It could be concluded that NXT plays an important role in restoring the function of CGRP.

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.

Han GM. (2001) The significance of assay the ET, CGRP on illness of cerebral vasculars in clinical. *J. Radioimmunol.* **14**, 141.

Hirate Y. (2003) Nitro oxide in hypertension. *J. Clin. Exp. Med.* **204**, 607-610.

Li Q. (2000) Bu Chang NXT Treating 31 cases of vascular dementia. *Lishizhen Med. Mater. Med. Res.* **11**, 535-536.

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.

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

Yin CH, Li LF, Jin YH, Yun GD, Fu 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.