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Influence of bushenhuoxue on primary visual cortex' BDNF damage in rat model of chronic elevated intraocular pressure

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Foundation item: Key Projects of Sichuan Provincial Education Office, China (No. 10ZA096)

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Received: 2012-08-20 Accepted: 2013-03-10

补肾活血中药对大鼠慢性高眼压模型初级视皮质 BDNF 损害的影响

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基金项目:中国四川省教育厅 2010 年重点项目(No. 10ZA096)

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摘要

目的:观察补肾活血中药对大鼠慢性高眼压(elevated intraocular pressure,EIOP)模型初级视皮质(primary visual cortex, PVC)脑源性神经营养因子(brain-derived neurotrophic factor,BDNF)的干预作用,并对其作用机理进行初步探讨。

方法:采用烙闭上巩膜静脉法,烙闭大鼠 3 支上巩膜静脉,制作大鼠慢性 EIOP 模型,随机分为 3 组:模型组,给药组,空白组。连续灌胃 8wk,并于 8wk 末处死大鼠,观察其对 EIOP 大鼠眼压、PVC 的 BDNF 表达及神经元细胞超微结构的影响。

结果:本实验采用的烙闭上巩膜静脉的造模方法使大鼠眼压明显升高($P<0.01$),与造模前比较差异有显著统计学意义($P<0.01$);PVC 病理切片半定量分析表明,模型组 BDNF 总面积 82438±2597.39(S/μm^2)、平均光密度 1155.9±123.14、积分光密度 12915±673.28,与空白组(总面积 132370±7588.47S/μm^2,平均光密度 5365±379.65,积分光密度 35102±2648.5)比较均有显著统计学意义(均为 $P<0.05$),模型组 BDNF 总面积与给药组(108980±9126.77S/μm^2)比较有统计学意义($P<0.05$),给药组平均光密度(3220.4±413.67)与模型组比较有统计学意义($P<0.05$),给药组积分光密度(23821±3431.68)与模型组比较,差异有统计学意义($P<0.05$)。

结论:补肾活血中药通过增强 BDNF 的表达、改善神经元细胞超微结构而促进 EIOP 大鼠初级视皮质损伤的修复。

关键词:青光眼;大鼠慢性高眼压模型;补肾活血法;初级视皮质;尼氏体;神经元细胞超微结构

引用:李翔,郭红建,文晓霞,谢钊,谢学军,路雪婧,王毅,张静. 补肾活血中药对大鼠慢性高眼压模型初级视皮质 BDNF 损害的影响. 国际眼科杂志 2013;13(4):647-651

Abstract

• AIM: To observe the effect of traditional Chinese medicine (TCM) of bushenhuoxue on primary visual cortex (PVC) brain-derived neurotrophic factor (BDNF) in rat model of chronic elevated intraocular pressure (EIOP), and explore the mechanism of it initially.

• METHODS: The rat model of chronic EIOP was established by unilaterally cauterizing 3 episcleral veins, then 30 rats were divided into 3 groups randomly: control group, model group, and treatment group. After given drugs or normal saline for 8 weeks, the rats were put to death. The effect of intraocular pressure (IOP), expression of BDNF and ultrastructure of neuron cell in the PVC was observed.

• RESULTS: Unilaterally cauterizing episcleral veins increased IOP of the rat model obviously, there was significant difference compared with pre-operation ($P<0.01$). Semi-quantitative pathological analysis on PVC showed that BDNF of total area in the model group was (82438±2597.39)S/μm^2, mean optical density was (1155.9±123.14), integrated optical density was (12915±673.28), compared with the control group {total area was (132370±7588.47)S/μm^2, mean optical density was (5365±379.65), integrated optical density was (35102±2648.5)}, there were statistical differences (all $P<0.05$), there was statistical difference in BDNF of total area between model group and treatment group {(108980±9126.77)S/μm^2, $P<0.05$}, significant difference in mean optical density between the model group and treatment group (3220.4±413.67, $P<0.05$), statistical difference in integrated optical

density between the model group and treatment group (23821±3431.68, $P<0.05$).

• CONCLUSION: TCM of bushenhuoxue can repair the PVC damage in the rat model of chronic EIOP by enhancing expression of BDNF, improving ultrastructure of neuron cell.

• KEYWORDS: glaucoma; rat model of chronic elevated intraocular pressure; TCM of bushenhuoxue; primary visual cortex; nissl bodies; ultrastructure of neuron cell

DOI:10.3980/j.issn.1672-5123.2013.04.01

Citation: Li X, Guo HJ, Wen XX, Xie Z, Xie XJ, Lu XJ, Wang Y, Zhang J. Influence of bushenhuoxue on primary visual cortex' BDNF damage in rat model of chronic elevated intraocular pressure. *Guoji Yanke Zazhi* (*Int Eye Sci*) 2013;13(4):647-651

INTRODUCTION

Glaucoma is a kind of eye disease caused by optic atrophy and visual - field defect, the pathologic increased pressure is one of major risk factors[1]. Previously, to control the IOP in a safe level (target-level) was the only credible evaluation, but it cannot stop the visual function getting worse and worse. Studies of domestic and aboard demonstrated that[2,3] even if controlled the IOP, the visual defect of the patient(about 40%-50%) will also occurred and developed chronically. Recently, someone suggest that glaucoma is a neurodegenerative disease of the whole visual system which can damage the visual center. Therefore, the visual dysfunction of the glaucoma damaged extensive from RGCs to the senior visual center[4,5] and the injury on each neuron had complex interaction. It caused us to rethink the occurrence and development mechanism of glaucomatous optic function damage from the optic path. So that it can provide new ideas for clinical therapy of the glaucoma. The primary visual cortex is the center of human visual system. It has been proved that if the visual cortex damaged, the visual function damaged too[6]. But it was short of studying on the intervention of traditional Chinese medicine. This experiment used by unilaterally cauterizing 3 episcleral vessels to induce the SD rat model of chronic, moderate EIOP. Qijudihuangwan and Fufangdanshenpian were used as intervention medicine. The effect of IOP, expression of BDNF and ultrastructure of neuron cell on PVC was observed in order to explore the glaucomatous visual function protection mechanism and provide experimental evidence for clinical application of bushenhuoxue.

MATERIALS AND METHODS

Materials Thirty, female and male, 8-12 week-old, Sprague Dawley rats (SD rats), weighed about 150-200g, conform the standards of the first class experimental animals, were fed with whole value grain feedstuff. Rats and the feedstuff were provided by Laboratory Animal Center of Chengdu University of Traditional Chinese Medicine. The raising room temperature was 20-25℃, fresh air, circulation day and night, relative humidity was 55%-75%, with 12 consecutive hour light exposure, eat and drink freely. Inclusion criteria: 1) without external ocular diseases; 2) normal binocular direct light reflex and indirect light reflex; 3) without crooked neck.

Reagent Fufangdanshenpian(Batch number: 7122426) and qijudihuangwan (Batch number: 7072153) were purchased from Peking Tong Ren Tang Pharmacy Co., Ltd. BDNF polyclonal antibody(Batch number: BA0565) was purchased from Bausch & Lomb Biological Engineering Co., Ltd.

Methods

Modeling After 3 days adaptive feed, measured the IOP and estimated the normal pressure region which the average was from 9 to 18mmHg. Then 30 SD rats were randomly assigned to control group, model group and treatment group. Model group and treatment group were operated to monocular model, The left eye was not given any treatment. Methods as follows: The rats were anesthetized by 3% pentobabitone sodium 1.5mL/kg through peritoneal injection after weighed and fixed. Then given the rats topical anesthesia of 0.5% proxymetacaine. Under the microscope, the bulbar conjunctiva was cut at 1-2mm on 10 to 2 o'clock from the corneal edge, then exposed the three episcleral veins which nearby the equator 3mm-4mm on 10 o'clock, 12 o'clock and 1 o'clock. Next, the 3 episcleral veins were cauterized by the thermocoagulation device. Finally, reconstructed the bulbar conjunction and coated chiortetracycline eyepaste. When the rats all revived, sent it back to the cage and dropped 0.25% chioromycetin twice a day. The control group had been done the same operation except cauterized the vessels. Four rats died form intragastric administration.

Grouping Each groups were given medicine 8 weeks, methods as follows: Control group, model group were administrated with equivalent 3mL sodium chioride every day; The treatment group were treated by fufangdanshenpian (0.96g/kg daily) and qijudihuangwan suspension(3.0g/kg daily, as 20 times of the adult daily dose). The gastric perfusion was given 8 weeks in the same time once a day. Weighed the rats every two weeks and adjusted the dosage.

IOP detection In this experiment, the IOP was measured at the same time everyday (2:00-5:00 p.m.). Used the handheld tonometer (TONO-PEN) to measure the IOP 3 days before operation. Taken the average as the normal pressure. To measure the IOP during post-operation immediately, one week, two weeks, four weeks, six weeks and eight weeks. This experiment persisted for 8 weeks.

Histopathological observation After operation 8 weeks killed the rats by cervical vertebra dislocation, opened skull, peeled brain tissue and fixed in the complex stationary liquid immediately. Then injected the liquid into the middle of interhemispheric. When the specimen was fixed 72 hours, took the rat cerebral location stereogram [7] as example to open and locate it. Taken out the brain issue (PVC) from 17 distribution in left occipital lobe. The specimens were dehydrated in an ethy alcohol series, xylene transparent and embedded in paraffin. Then sectioned it at 10μm, dryed and stained it by BDNF immunohistochemical method. The BDNF

Table 1 Comparison of IOP before and after modeling in each groups

Group	Eyes	Pre-modeling	Immediately modeling	8 weeks after-modeling
Control	9	10.36±2.4661	11.42±3.1315	12.04±3.8293
Model	9	10.54±3.4946	28.14±7.3919[b,d]	27.58±6.3129[b,d]
Treatment	8	9.52±4.0162	31.74±8.3153[b,d]	25.64±5.5894[b,d]
F		0.253	28.573	22.565
P		0.778	0.000	0.000

[b]$P<0.01$ *vs* pre-models building; [d]$P<0.01$ *vs* control group.

Table 2 Rats' BDNF comparison of each groups in PVC $\bar{x}\pm s$

Group	Total area ($S/\mu m^2$)	Mean optical density	Integrated optical density
Control	132370±7588.47	5365±379.65	35102±2648.5
Model	82438±2597.39[a]	1155.9±123.14[a]	12915±673.28[a]
Treatment	108980±9126.77[a]	3220.4±413.67[a, c]	23821±3431.68[a, c]
F	74.530	24.315	115.116
P	0.000	0.000	0.000

[a]$P<0.05$ *vs* control group; [c]$P<0.05$ *vs* model group.

stained pale brown looks like granular and filamentose. Total area, mean optical density and intergrated optica in every visual field were measured randomly in 6 rat from each group (Each randomly selected from 5 perspectives).

Ultrastructure of primary visual cortex Randomly selected one SD rats each group, fixed, anetheticed, decapitated, and cut PVC into $2mm^3$ pieces. Next, put it into the liquid with glutaraldehyde (3%), refrigerated it 2 hours at 4℃ and fixed it 30 minutes with osmium acid, the ultrastructure of primary visual cortex was observed. This observation process finished in the Electron microscopy room of west China medical center of Sichuan university.

Statistical Analysis All analysis were performed with SPSS statistical software version 13.0. Paired *t* test was used for before and after comparison, while one-way analysis of variance was conducted for comparison between groups. Data were presented as mean ± standard deviation. Statistically significant difference was set at $P<0.05$.

RESULTS

IOP compared between each group As shown in Table 1, the IOP had no significant difference between groups before operation ($P>0.05$). Immediately modeling, the IOP of model and treatment group were highly significant different compared with control group ($P<0.01$), it indicated modeling success. While model group and treatment group had no significant difference ($P>0.05$), there were equilibrium between groups. After 8 weeks modeling, we found the IOP of the model group and treatment group had significant difference compared with control group and pre-modeling ($P<0.01$), it showed that the elevated intraocular pressure maintained good, while the model group and the treatment group had no significant difference ($P>0.05$).

Rats' BDNF comparison of each groups in PVC As shown in Table 2 and Figure 1A-C, the influence of bushenhuoxue on the expression of PVC's BDNF as follows: 1) The total area, mean optical density and intergrated optical density of model group were significantly less than the control group ($P<0.05$); 2) Compared with the model group, the total area, mean optical density and intergrated optical density of the treatment group were higher ($P<0.05$). The results demonstrated that TCM of bushenhuoxue can improve the expression of BDNF in PVC.

Ultrastructure of each groups in PVC's Neuronal cells (Figure 2A-C).

DISCUSSION

Glaucoma is a multiple factors disease. Recently years, many scholars had been made a lot of experiment and application studies on the visual function damage. Although some progress had been made, the pathogenesis of glaucoma was still unclear. The artificial damage of PVC have shown that can induce the RGCs happened retrograde trans-neuronal degeneration. And the mechanism of it was considered to be due to the decrease of the neurotrophic factor what the RGCs get from PVC[8,9]. On the other hand, when the RGCs further reduced, it must further decreased the visual afferent impulses and aggravated the disuse atrophy degeneration of PVC. The vicious circle existed in RGCs and the damage of PVC maybe played an important role in the progress of visual function damage[5]. BNDF is a member of the neurotrophic factor family. Fournier *et al*[10] confirmed that BNDF can increase the expression of the related gene of reborn axon such as GAP-43, L1 and TAG-1mRNA in order to promote the rebirth of the broken axon. Unoki and Lavail [11] observed that BNDF was injected into vitreous at 2 days before modeling rat of EIOP (160mmHg, lasted for 90 minutes) can effectively reduced the damage of inner retina. They also observed that the BNDF can protect the retinal tissue levels and RGCs quantity by histological method. Liu *et al*[12] observed that: Used BNDF before modeling can significantly promote the recover of ERG b wave with acute EIOP. This suggested that BNDF have remarkable promotion effect on the recover of retinal electrophysiological function.

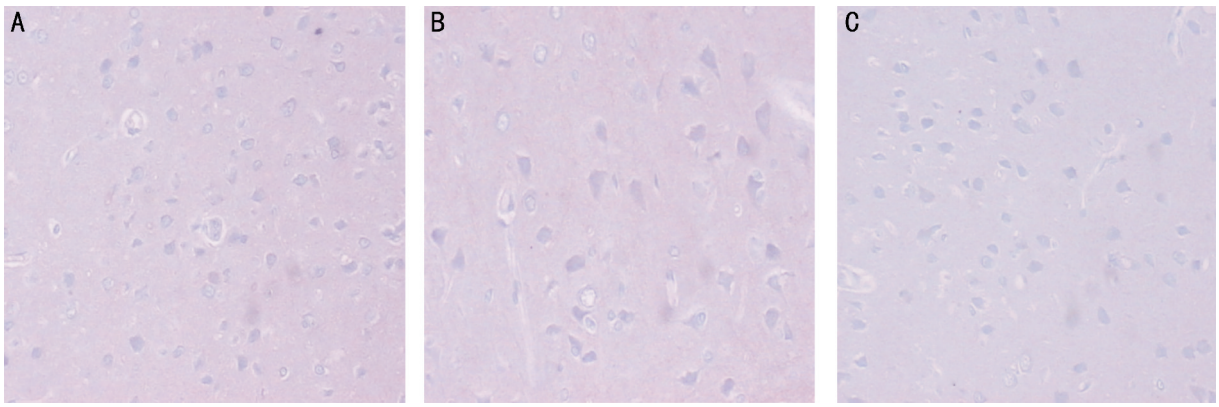

Figure 1 BDNF stained in PVC (200×) A: Control group; B: Model group; C: Treatment group.

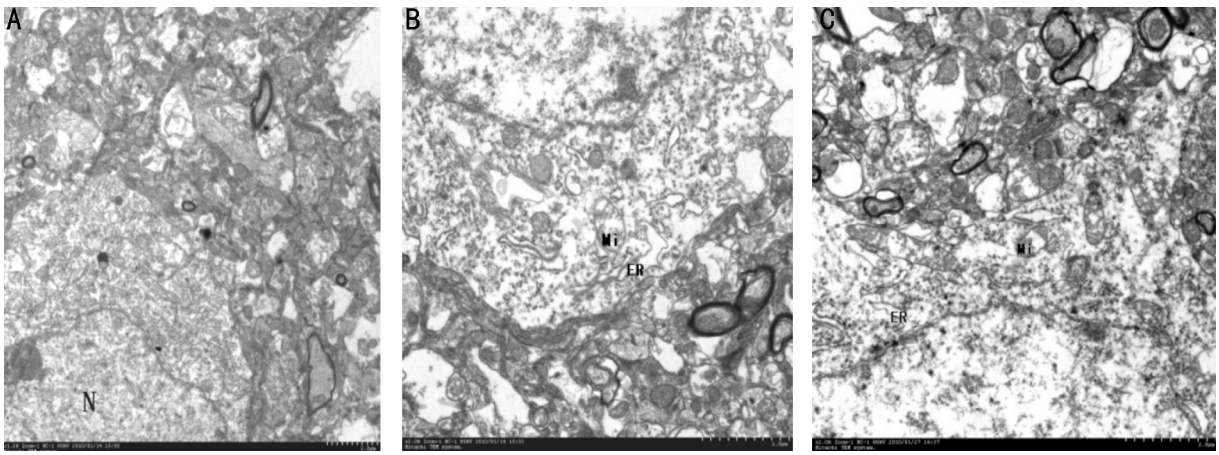

Figure 2 Ultrastructure of each groups in PVC(12000×) A: In control group, the axon lined uniformity and close. The nucleus looks like round or ovoid, the organelles were rich in cytoplasm. Mitochondria, spine and the endoplasmic reticulum can be seen clearly; B: In model group, the dilatation of endoplasmic reticulum looks like vacuole, swollen mitochondria with cristae loss and vacuolation; C: In treatment group, the axon lined uniformity with loose structure and mild edema. The organelle in cytoplasm were light decreased. The mitochondria swell to varying degrees with hypertrophic and dilated rough surfaced endoplasmic reticulum.

Glaucoma belongs to "wufengneizhang" of traditional Chinese medicine. It is syndrome was similar to "qingmang" such as Specificoptic nerve atrophy and visual field defect. This experiment founded that chronic EIOP can reduce the BDNF expression of PVC in SD rats, reduce survivability and increase death. On the contrary, TCM of bushenhuoxue can increase BDNF of PVC, raise survivability, decrease the death and improve the ultrastructure of neuron cell in rat model of chronic EIOP. Previous study[13-17] also showed that TCM of bushenhuoxue contribute to recover the rats' total wave of mfERG (multifocal electroretinogram), the response density of total wave and wave P1 on 1, 2, 3, 4 loop, wave N1 on 2, 3 loop, the peak latency of total wave P1 and wave N1 on 3, 4 loop. It also had positive effects on the expression of Bcl-2 and Bax, restrain the apoptosis of gangliocyte, restraint the RNFL (retinal nerve fiber layer) and RGCL (retinal ganglion cells layer) getting thinner, improve the ultrastructure of RCGs. TCM of bushenhuoxue also can repaired injury LGN in the rat model of chronic EIOP. The visual function of glaucoma patients who controlled the IOP with TCM of bushenhuoxue were improved in different degree. Qijudihuangwan is a famous prescription of nourishment for kidney, liver and eyesight in TCM. Fufangdanshenpian can resolve blood-stasis and unblock collaterals. Both the two were loaded into the book of Chinese Medicine, used as OTC (over-the-counter) medicine, high quality and inexpensive. According to the previous-study and our experiment results, both the two prescription can be widely used as clinical glaucoma optic nerve protection medicine.

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