·Basic Research·

Neuroprotective effect of Erigeron Breviscapus (vant) Hand -Mazz on NMDA -induced retinal neuron injury in rats

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Abstract

• AIM: To investigate whether Erigeron Breviscapus (vant) Hand-Mazz (EBHM) has neuroprotective effect against N-methyl-D-aspartate (NMDA)-induced neuron death in retinal ganglion cell layer (RGCL).

• METHODS: Sixty healthy SD rats were randomly divided into four groups. 6 animals were normal control group (group A). The others were divided as group B (EBHM group), group C (normal saline+NMDA group) and group D(EBHM + NMDA group), each group had 18 rats. 10nmol NMDA was intravitreally injected to induce partial damage of the neurons in RGCL in the right eyes of Groups C and D. Same volume PBS was intravitreally injected into the left eyes as self-control. Groups B and D were pretreated intraperitoneally with 6g/L EBHM solution at a dose of 150mg/kg body weight/day seven days before and after NMDA treatment. Group C were administrated intraperitoneally with 9g/L normal saline at the same time of EBHM injection. Rats were sacrificed at 4,7,14 day after NMDA treatment. Flat whole retinas were stained with 5g/L cresyl violet and neuron counting in RGCL from both eyes were observed. Each subgroup had 6 rats.

• RESULTS: There was no significant difference of neuron counting in RGCL between the right eye and the left eye in group A. There was no significant difference between normal control group and EBHM group either in the right eyes or in the left eyes at 4, 7 and 14 day respectively after intravitreal injection of 10nmol NMDA in group C and group D. (P= 0.636, P=0.193). Neuron counting of RGCL in group C and D was significantly decreased in the NMDA- treated eyes 4, 7 and 14 days after intravitreal injection (P< 0.001). There was no significant difference between self-control eyes group and normal control group. However, neuron counting was significantly higher in the EBHM+NMDA group than normal saline +NMDA group 14 days after intravitreal injection (P= 0.044), but lower than normal control group (P<0.05).

• CONCLUSION: EBHM has no effect on neuron counting of RGCL when administered alone in normal rats. The results indicate that EBHM plays a partial protective role in NMDA-induced neuron loss in RGCL in rats.

• KEYWORDS: excitatory amino acids; N-methyl-D-aspartate; glaucoma; neuroprotection; erigeron breviscapus (vant) hand-mazz

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INTRODUCTION

Treatments of glaucoma continue to be directed at lowering intraocular pressure (IOP) to decrease the likelihood of disease progression. But now we fully realized it is not enough for protecting the glaucomatous damage of the optic nerve damage. Therefore we should take some other therapeutic measurements to slow down or further prevent death of retinal ganglion cells and their axons to maintain their physiological functions. Neuroprotection is a new conception in glaucoma therapy and would be an adjunctive therapeutic paradigm combined with conventional IOP- lowering treatments[1-4].

Since 1970's Wu *et al* have focused on the study of several Chinese traditional herbs capable of improving neuronal survival of glaucomatous optic nerve damage after medical or surgical treatments for patients whose IOP had been well controlled. It had been found that breviscapus (vant) hand-mazz (EBHM) showed neuron protective

effects. The purpose of this study is to investigate whether EBHM has a neuroprotective effect against NMDA-induced neuron death in the retinal ganglion cell layer (RGCL).

MATERIALS AND METHODS

Materials Sixty healthy SD rats weighed 200~240g (both male and female) were randomly divided into four groups. 6 animals were in normal control group (group A). The other 54 rats were divided into three groups as group B (EBHM group), group C (normal saline+NMDA group), group D (EBHM + NMDA group). Each group had 18 rats.

Methods 10nmol NMDA (Sigma Chemical Co.，St Louis, MO. USA) was chosen for intravitreal injection to induce partial damage of the neurons in RGCL in the right eyes of Groups C and D. Same volume of PBS was intravitreally injected into the left eyes as self-control. Groups B and D were pre-treated intraperitoneally with 60g/L EBHM solution at a dose of 150mg/ (kg•d) seven days before and after NMDA treatment. Group C were administrated intraperitoneally with 9g/L normal saline at the same time of EBHM injection. Rats were sacrificed at 4, 7, 14 day after NMDA treatment. Whole flat mounted retinas were stained with 5g/L cresyl violet (Sigma Chemical Co.，St Louis, MO.，USA) and neuron counting in RGCL from both eyes was conducted. Each subgroup had 6 rats.

All intravitreal injection was guided under an ophthalmic surgical microscope with a self made micro-glass pipette needle. The rats with surgical complications such as cataract or intravitreous hemorrhage were excluded from the study.

After nucleation, 12 o'clock of the conjunctiva of the eyeball was marked by a suture, then the eye ball was fixed in 100g/L formalin solution for 1 hour. The anterior segment was removed and retina was carefully take out. Let retina dry and make 4 cuts (top, bottom, left and right) by a micro-surgical scissor and then spread on the glass slide. The retina was stained with 5g/L Cresyl violet solution for 2 to 20 minutes and rinsed quickly in 950mL/L ethyl alcohol, then placed in n-butyl alcohol, two changes of two minutes each, cleared in xylene and mounted.

Mean densities of neuron in the RGCL were estimated using a computerized image analysis system. Retina was divided as superior-temporal, inferior-temporal, superior-nasal and inferior-nasal quadrant. Then every quadrant was divided averagely into three regions as central, mid-central and peripheral area. Take three views of photographs randomly in each region and every viewing was 18483.47μm^2. The number of neurons in the 36 photos were counted and converted into neurons per mm^2 for each retina.

Statistical Analysis All data were analyzed by SPSS 10.0 statistical analysis software. Analysis methods were One-Way ANOVA, Two-Way ANOVA and independent-sample t test. $P< 0.05$ was considered significant.

RESULTS

Neuron Counting from RGCL of the Normal Control Group The mean of RGCL neurons was 3531.5±84.3/mm^2 in the right eye, and 3427.8 ±164.7/mm^2 in the left eye. There was no significant difference between the right eye and the left eye（$F = 1.884$，$P = 0.200$）.

Neuron Counting from RGCL of the Normal Eyes Treated with EBHM Group The mean of RGCL neurons was 3473.8 ±145.3/mm^2, 3438.0 ±172.2/mm^2, 3443.0 ± 137.1/mm^2 in the right eye and 3541.7±149.3/mm^2, 3362.7± 84.5/mm^2, 3414.8±149.5/mm^2 in the left eye at 4, 7 and 14 day respectively. There was no significant difference between EBHM group and normal control group (Right eye: F=0.578, P= 0.636;Left eye: F=1.729，P= 0.193).

Neuron Counting form RGCL with Normal Saline Intraperitoneal Injection and NMDA Intravitreal Injection Group The mean numbers of RGCL neurons were 2599.3 ±137.3/mm^2, 2337.7 ±143.5/mm^2, 2141.7 ± 91.4/mm^2 in the NMDA-treated eyes （right eyes）and 3385.0±115.2/mm^2, 3542.8±112.8/mm^2,3252.3±66.5/mm^2 in the PBS-injected eyes （left eyes）at 4, 7 and 14 day after intravitreal injection and the difference between two eyes was significant (F = 123.849,266.290,423.076 respectively, $P< 0.001$). The percentage of neuron loss was 26.4%, 33.8%, 39.4% at above survival time in the NMDA-treated eyes respectively. The difference was significant between NMDA treated eye and control group （F=132.063，$P<$ 0.001). The difference was significant between any two groups by q test （$P< 0.05$）. There was no significant difference between self-control eyes and normal control group（F=1.365，P = 0.282）.

Neuron Counting from RGCL of EBHM Intraperitoneal Injection and NMDA (10nmol) Intravitreal Injection Group The mean numbers of RGCL neurons were 2557.2± 106.7/mm^2, 2460.3 ±94.5/mm^2, 2288.8 ±87.6/mm^2 in the NMDA-treated eyes and 3392.5 ±90.5/mm^2, 3459.7 ± 128.5/mm^2, 3312.5±116.9/mm^2 in the PBS-injected eyes at 4,7 and 14 day after intravitreal injection and the difference was significant (F=213.741，235.441，294.450 respectively，

Table 1 Neuron counting of RGCL from each group (/mm^2,mean ± SD)

	EBHM group		nmNMDA+normal salin group		NMDA+EBHM group	
	right eye	left eye	right eye	left eye	right eye	left eye
4d	3473.8 ± 145.3	3541.7 ± 149.3	2599.3 ± 137.3*	3398.7 ± 110.0	2557.2 ± 106.7*	3392.5 ± 90.5
7d	3438.0 ± 172.2	3362.7 ± 84.5	2337.7 ± 143.4*	3493.2 ± 97.5	2460.3 ± 94.5*	3459.7 ± 128.5
14d	3443.0 ± 137.1	3414.8 ± 149.5	2141.7 ± 91.4*#	3252.3 ± 111.5	2288.8 ± 87.6*#	3312.5 ± 116.9

*The difference is significant compared with self-control eye or control group(P<0.001);#The difference is significant between two groups(P=0.044)

P<0.001). The percentage of neuron loss was 27.6%, 30.3%, and 35.1% above survival time in the NMDA-treated eyes respectively. There was no significant difference between self-control eyes and control group (F=1.469, P=0.253).

The Difference of Neuron Counting among Normal Control Group, EBHM Group, EBHM+NMDA Group and Normal Saline Solution +EMDA Group There was no significant difference between normal control group and EBHM group either in the right eyes or in the left eyes (P=0.636, P=0.193). Neuron counting was significantly higher in the EBHM+NMDA group than normal saline +NMDA group (P=0.044) at 14 day after intravitreal injection. However, it was obvious that the difference was still significant between normal control group and EBHM+ NMDA group (P<0.05). The data indicated that EBHM had no effect on neuron counting of RGCL when administered alone in normal rats, but it had partial protective effect on NMDA-induced neuron loss in RGCL in the rat (Table 1).

DISCUSSION

EBHM, a plant grows in 2 500 meters above the sea level at the plateau region in southwest of China. It posses the efficacy to inhibit blood coagulation, enhance the microcirculation and dilate brain blood vessels, as evidenced by its clinical application in treatment of brain thrombosis and associated paralysis, myocardial ischemia and coronary arteriosclerosis. Some clinical studies showed that EBHM could restore and/or improve visual defects in patients with IOP controlled glaucoma [5-7]. Some lab studies also demonstrated it had neuroprotective effects in some animal models of glaucoma [8-11]. EBHM was approved by Chinese Medicine and Drug Administration Bureau and has been used to treat patients with IOP controlled glaucoma. It has been marketing in China since 2001.

Quigley *et al* confirmed glaucomatous damage large RGC in the early stage. Recent research indicated that greater sensitivity of larger RGC to NMDA-mediated cell death and elevated glutamate levels in the vitreous body of human and monkeys with glaucoma. Excitotoxins is a possible new mechanism for RGC loss from glaucoma. Maybe protect RGC from EAA damage is a potential neuroprotective therapy for glaucomatous optic neuropathy. So some people try to save RGC through the pathway of EAA and practice a neuroprotection in glaucoma patient.

10nmol NMDA was chosen for intravitreal injection to cause partial damage of the neurons in RGCL. There was no difference between normal control group and EBHM group of neuron counting of RGCL. It shows that EBHM has no effect on neuron counting of RGCL when administered alone in normal rats. The mean of neuron counting of RGCL is statistical significantly higher in EBHM+NMDA group than in normal saline+NMDA group at 14 day after intravitreal injection. It is suggested that EBHM has partial protective effect on NMDA-induced neuron loss in RGCL in the rat.

Mitochondrion is not only the power manufactory of cellular oxidation and energy supply, but also plays an important role in the process of cell death. Ankarcrona *et al* [12] demonstrated that the maintenance of mitochondrial function may be a decisive factor in determining the degree and progression of neuronal injury caused by excitotoxins. Due to absence of corresponding hydrolytic enzyme at extracellular matrix, the only way to clear the extracellular EAA is to remove EAA transporters (EAAT) located at presynaptic membrane and glia cytoplasmic membrane, which is energy depended. EAAT is significant in recycle of EAA, termination of exciting signal and protection of neurons from excitotoxicity. Additionally, decreases in production of energy resulted from suppression in electron transport in mitochondria, may make neurons hypersensitive to glutamate[13].

Jia *et al* [8] reported EBHM possess the protective and/or improving effects on the Cytochrome oxidase (CO) active retinal ganglionic cells of rat under artificial acute high IOP.

In the study, the data of density and the grey value of CO active ganglionic cell in the experimental models treated with EBHM are significantly different from those of the control group. CO is mainly located at the spine and intramembrane of mitochondria. It transports electrons during the process of biologic oxidation and ATP production. ATP provides the energy which guarantees the normal function of Na^+-K^+ pump and then sustains resting potential of plasma membrane, axoplasmic transport and so on. The level of CO suggests the functional state of neurons. We infer the neuroprotective effects of EBHM against EAA may enhance the microcirculation, EBHM shows neuroprotective effect only four weeks after its injection in our study. The mechanism of EBHM is quite different from the inhibitor of NMDA receptor directly. We infer that the partial protective effects of EBHM against NMDA induced neuron damage maybe due to the enhance of the microcirculation, survival stimuli (eg increase cAMP), maintaining energy of retina and increase of the remove of extracellular EAA according to the results of our study.

However, our study did not show its protective effect in the early stage of NMDA insult. There maybe exists some other mechanisms which should be further studied or it may related with the difference between EAA animal model and glaucomatous damage, relatively short observing period, fewer animal numbers in each group and did not analysis large RGC which is sensitive to EAA. Neuroprotection was confirmed in animal model or experiments *in vitro* in some ophthalmic or nerve degeneration diseases. We just observed a phenomenon without any clinical trial to confirm it in this study. As glaucomatous optic neuropathy is a slowly progressive disease, clinical trials of neuroprotection will necessarily be of long duration and need a large number of patients enrolled. It's very hard to practice. Although EBHM shows neuroprotective effects in some extent in some lab study and clinical trial, it is not clear about its mechanism and effective elements of its main integrant. We need further investigation to confirm its clinical effect. In a word, we need more sophisticated new techniques to confirm EBHM as a neuroprotective drug.

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