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【Key Words】 Ear-acupuncture; Dementia, Vascular; Learning Memory; Hippocampus

GRADUATE FORUM

The Improvement of Memory of Vascular Dementia Rats by Ear-acupuncture and Its Relationship with the Expression of nNOS

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【Abstract】 Objective TO probe into the improvement of learning memory of vascular dementia rats (VD) by ear acupuncture and its relationship with expression of nNOS. **Methods** A rat model of Vascular dementia was established with the method of 4-vessel occlusion, then the rats were treated by acupuncture of ear points kidney and brain, the immunohistochemical method was used to detect the nNOS protein, and the rate of hippocampus neuron loss was calculated by Nissl staining method combined with image analysis. In the meantime, A Y-type maze was also used to test the behavior of the rats. **Results** After acupuncture, the expression of nNOS in CA₁ area of rat's hippocampus was reduced and the lost rate of neurons in CA₁ area of rat's hippocampus is less than that in VD model group, which presents its negative correlation with learning memory ability. **Conclusion**

Ear acupuncture could improve the learning memory of VD rat and its mechanism may be that acupuncture could inhibit the over-increase of nNOS after cerebral ischemia to protect the hippocampus neurons of VD rat.

IN recent years, it has been demonstrated that neuronal nitric oxide synthase (nNOS) participated in the occurrence of vascular dementia (VD)^[1]. Many clinical observations have proved

that acupuncture has obvious therapeutic effect on the injuries of cerebral ischemia and VD, but the mechanism of the treatment of VD by ear acupuncture and its relationship with NO are not clear. In order to exert ear-acupuncture's superiority of safety, no side-effect, and being easy to being popularized and explore the new therapeutic method of non-drug for VD. In present experiment, we observed the change of learning memory ability and nNOS expression in CA₁ area of hippocampus of VD rat, so as to further observe the therapeutic effect of VD treated by ear-acupuncture and investigate its possible mechanism, which provided the experimental evidence for the treatment of VD in clinic.

Materials and Methods

Male Wistar rats, weighing between 200g and 250g, were divided into VD model group, ear-acupuncture treatment group, normal control group which were performed false operation, 10 rats in each groups

1. Preparation of VD model and ear acupuncture treatment

The rat VD model that is similar to learning memory disturbance of human being was established according to the modified Pulsineul's 4-

vessel occlusion method, namely, 24hrs after occlusion of bilateral vertebral arteries, the bilateral common carotid arteries were clamped reversibly for 3 times, each time lasted for 5 minutes, with a 1 hour's interval between two times, then the rat was returned to cage and fed with routine method. According to the method of locating animal acupoint as described by Hua xingbang^[2], the rat's ear-acupoints brain and kidney were selected. After rat VD model was set up successfully, the VD rat was immediately given treated by ear-acupuncture once a day, 15 days in all.

2. Test of rat's learning memory ability

Before and 2 weeks after making model, we adopted Stanes's Y type maze of three-equal-arm to test rat's learning memory ability for 10 times on each morning and afternoon respectively, with 1 min's rest between two times, 8 days in all. The scores of rat's learning memory was expressed by the lightning stroke times that rat needed to have 9 times of correct reaction in 10 successive tests.

3. Immunohistochemical and Nissl staining

After the rat was anesthetized with intraperitoneal injection of 3% pentobarbital sodium (40 mg/kg BW), the rat's brain was taken out, per-

fused and fixed with routine method, then the serial coronal frozen section which is 30 μm thick was made. All the experimental procedures were carried out according to the directions of the immunohistochemical reagent box, the positive neurons appeared brownish red by application of DAB color developing system, and the substitution of PBS for the first specific antibody was performed as the negative control. In the meantime, the adjacent brain sections was given Nissl staining with 10% toluidize blue solution.

Results

1. Results of behavior test

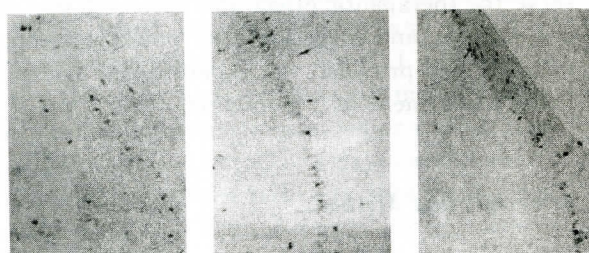
Before 4-vessel occlusion, the rat's learning memory ability in each group was basically same. In false operation control group, the rat's learning memory ability had no obvious change all the time, indicating that rats in control group showed no learning memory disturbance. While the rat's lightning stroke times in VD model group is obviously increased after operation as compared with that in control group and treatment group and before operation ($P < 0.01$), indicating that rats in VD model group suffered from severe disturbance of learning memory abil-

Attached table Rat's learning -memory scores tested by Y-maze

($\bar{x} \pm s$)

Groups	Control group	Model group	Treatment group
Pre-operation	5.68 ± 1.29	6.07 ± 1.67	5.86 ± 1.71
Post-operation	5.81 ± 1.51	18.06 ± 2.68 ⁽¹⁾	8.31 ± 1.85 ⁽²⁾

Note: 10 rats in each group, In comparison with control group 1) $P < 0.01$, In comparison with model group 2) $P < 0.01$



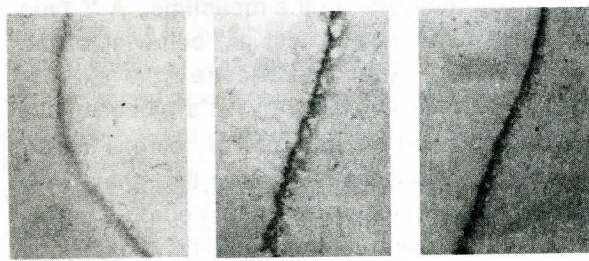
(A1) (B1) (C1)

Fig. 1 microphotograph showing nNOS immunohistochemical positive cells in CA₁ area of hippocampus in rats (×100)

Note: A₁ represents control group, microphotograph showing a few of scattered weak positive cells in CA₁ area of hippocampus

B₁ represents VD model group, showing that nNOS positive cells in various layers of CA₁ area of hippocampus were stained heavily, and the number of nNOS positive cells is increased with obvious process

C₁ represents treatment group, showing that the number of nNOS positive cells is obviously reduced



(A2) (B2) (C2)

Fig. 2 microphotograph showing Nissl staining of brain section in CA₁ area of hippocampus in rat (×100)

Note: A₂ represents control group

B₂ represents model group, showing that neurons in pyramidal cell layer of CA₁ area of rat's hippocampus were severely lost, and many cavities were also seen

C₂ represents ear-acupuncture treatment group, were no obvious difference with control group

ity of spatial resolution. The lightning stroke times in ear-acupuncture treatment group is less than that in VD model group, but compared with control group, no significant difference is found, indicating that ear-acupuncture could strikingly improve the learning memory ability of spatial resolution of VD rats (see attached table)

2. Results of immunohistochemical staining

In control group, a few of nNOS positive neurons with weak and moderate staining were seen in pyramidal cell layer, molecular layer and multiform cell layer of CA₁ area of rat's hippocampus, and the neuron process with weak staining were also found; In VD model group, the number of nNOS positive neurons in pyramidal cell layer, molecular layer and multiform cell layer was obviously increased, the positive neurons were heavily stained, with many long process, and many scattered Aspiking neurons (prickle cell) and fibrous network were also found. In ear-acupuncture treatment group, the number of nNOS positive cells was obviously reduced, only a few of positive cells with weak or moderate staining scattered in the pyramidal cell layer, molecular layer and multiform cell layer of CA₁ area, and a few of Aspiking neurons were occasionally seen (see A1, B1, C1 in Fig1); Nissl staining showed that neurons in pyramidal cell layer of CA₁ area of hippocampus were severely lost, and many cavities were also found in VD model group; As treatment group was compared with control group, no significant difference was found in the number of neuron in pyramidal cell layer of CA₁ area of hippocampus (see A2, B2, C2 in Fig2).

Discussion

The learning memory disturbance induced by cerebrovascular disease is the main manifestation of vascular dementia, this disease belongs to the category of "amnesia" in TCM, and TCM holds that the deficiency of the kidney and the brain marrow is the fundamental cause of this disease. It is said in *INTERNAL CLASSIC* that ear is the meeting of all meridians, and twelve meridians all arrive at ear region. Acupuncture of ear-acupoint may regulate functions of general Zang-fu organs. Many clinical studies have proved that acupuncture obvious improve the learning memory ability of VD patients^[3]. Ear-acupoint

brain functions to regulate the excitation or inhibition of cerebral cortex, and the ear-acupoint kidney may tonify the kidney and replenish the essence and strengthen the brain marrow, promote flow of qi and blood circulation. In present experiment, we selected ear-acupoints kidney and brain to treat VD, so as to observe the therapeutic effect of VD treated by ear-acupuncture and further probe into its treatment mechanism.

It has been proved by experiment that NOS inhibitor or hemoglobin might block the formation of LPT in CA₁ area of hippocampus and NO might increase the spontaneous release of cultured hippocampus pyramidal cell transmitter, so we may guess that NO might serves as "retrograde" transmitter between postsynaptic membrane and presynaptic membrane in the course of LPT formation, so as to participate in the synapse plasticity regulation of learning memory^[4]. In another study, it also has been proved that nNOS might mediate the excitatory amino acid in brain to involve in the pathologic process of ischemic brain injury^[5]. The excitatory amino acid in brain may act on the NMDA receptor to increase the level of intracellular Ca²⁺, so as to promote nNOS in brain to produce NO, subsequently, the increased level of cGMP leads to the excitation of postsynaptic neuron, so as to bring about neurons death or necrosis, resulting in learning memory disturbance, the behavior test in present experiment also proved the effect of NO on the learning memory ability.

The results obtained in present experiment showed that the number of nNOS positive neurons in CA₁ area of hippocampus in VD model group is increased, and the neurons in CA₁ area of hippocampus were severely lost, which presents negative correlation with the VD rat's learning memory, indicating that the learning memory disturbance of VD was related to the severely lost neurons induced by the increased level of nNOS in brain. Our previous experiment also proved the effect of NO on the learning memory of VD. The results in present experiment still showed that after ear-acupuncture, the learning memory ability of VD rats is remarkably improved, the number of nNOS positive cells in CA₁ area of hippocampus is decreased, and the number of lost neurons of CA₁ area of hippocampus is also reduced, suggesting that ear-acupuncture might inhibit the over-synthesis of nNOS to prevent toxic action of NO on

the hippocampus neuron, so as to improve the learning memory disturbance of VD. Some researchers have observed that acupuncture had definite therapeutic effect on cerebral ischemia, it might increase the blood flow volume in ischemic area, the proliferation of capillary and gliocyte in ischemic and necrotic focus. It was found that under electron microscope that acupuncture had protective action on the neuron injuries after cerebral ischemia^[6]. Another experimental results showed that acupuncture of scalp acupoint might reduce the level of serum lipid peroxide(LPO)and raise the level of superoxide dismutase (SOD) in whole blood, thus acupuncture had the functions of eliminating oxygen free radical, blocking lipid peroxidation reaction^[7]. According to the above experiment, we may guess that the ways by which the ear acupuncture improve the learning memory disturbance of VD are as follows: First, ear-acupuncture could control proper synthesis of NO in brain of VD rat to regulate brain blood flow, improve blood and oxygen supply of the brain, alleviate cerebral edema, increase usage rate of glucose, reduce pile-up of lactic acid of cerebral tissue, inhibit the free radical reaction induced by cerebral ischemia, recover the balance between oxidation and antioxidation, alleviate membrane lipid peroxidation, so as to prevent NO from destroying cell membrane, improve membrane permeability, reduce intracellular Ca^{2+} overload after cerebral ischemia to protect neurons. Second, ear-acupuncture could keep proper level of NO in brain of VD rat to prevent the increase of nNOS-mediated excitatory amino acid induced by repeated cerebral ischemia in hippocampus, so as to protect hippocampus neurons to improve learning memory. Third, ear-acupuncture might change the release of Ach in brain, regulate the ratio of cAMP/cGMP to improve energy metabolism of brain tissue, promote the repair and regeneration of injured brain tissue to enhance learning memory

ability. In addition, ear acupuncture might regulate genetic expression of mitochondrion, increase the energy synthesis of hippocampus neuron, reduce injury of nerve cell to protect hippocampus neuron, improve VD intelligence. Ear acupuncture also might reduce the production of nNOS and induce the production of hippocampus LPT to improve the learning memory disturbance of VD.

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