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·Original Article·

Effect of resuscitation after selective cerebral ultraprofound hypothermia on expressions of nerve growth factor and glial cell line-derived neurotrophic factor in the brain of monkey

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Abstract: Objective To investigate the expression of nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF) in monkeys of resuscitation after selective cerebral ultraprofound hypothermia and blood flow occlusion. **Methods** The monkeys were immediately removed brain after death in operation of group A (identical temperature perfusion group) and group B (ultraprofound hypothermia perfusion group). Immunohistochemical technique was used to determine frontal cellular expression of NGF and GDNF. Statistics were analyzed by ANOVA analyses with significance level at $P < 0.05$. **Results** The expressions of NGF and GDNF in the group B were significantly higher than those in the group A ($P < 0.05$). **Conclusion** NGF and GDNF increased significantly in the monkeys of resuscitation after selective cerebral ultraprofound hypothermia and blood flow occlusion. It may be a protective mechanism for neuron survival and neural function recove**ry.**

Keywords: nerve growth factor; glial cell line-derived neurotrophicfactor; ultraprofound hypothermic circulatory arrest; resuscitation; monkey brain

1 Introduction

The technique of resuscitation after the selective cerebral ultraprofound hypothermia induced by blood flow occlusion can selectively and remarkably lower brain temperature to extend the maximum security time of the brain tissue undergoing ischemia and hypoxia. By this way, the core temperature of body is kept and kinds of systemic complications of profound hypothermia could be avoided. The prospects of its clinical application are broad[1].

Neurotrophic factors (NTFs) are a family of structurally related polypeptides which are the basic material necessary for the body to survive. They play critical roles in promoting neuronal survival, differentiation, growth, maintenance, proliferation, and appear to mediate a protective response in mature animals. Among many neurotrophic factors that act on sensory neurons, nerve growth factor (NGF) and glial cell line-derived neurotrophicfactor (GDNF) have been studied extensively[2]. NGF and GDNF initially interested neurobiologists because of their effects on survival, differentiation and maturation of the developing nervous system. It is now clear that NGF and GDNF function throughout the animal life with a wide repertoire of actions[3].

So far, the impact of resuscitation after selective cerebral ultraprofound hypothermia on the NTFs' expression in monkey's brain was not reported yet. In this study, we investigate the expressions of NGF and GDNF in frontal lobe nerve cells of rhesus monkeys after application of ultraprofound hypothermia.

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2 Materials and methods

2.1 Materials NGF, GDNF polyclonal antibody (rabbit anti-human) were purchased from Wuhan Boster Biotech

Company, China; the second-antibody color system (Envision System) was purchased from DAKO Co., Danmark.

2.2 Preparation of specimens The experimental model was completed in the Second Affiliated Hospital of Kunming Medical College, Kunming, China[4]. Monkeys in the isothermal group (n = 2) received 10-min bilateral internal carotid artery occlusion followed by reperfusion, while monkeys in the ultraprofound hypothermic group (n = 4) received 10-min bilateral internal carotid artery occlusion followed by cold perfusion via bilateral vertebral artery. All brain tissues were taken out immediately after monkey death during perfusion or recovery by craniotomy, then fixed in 4% Multi- paraformaldehyde and paraffin-embedded for immunohistochemistry.

2.3 Immunohistochemical staining Immunohistochemical procedures have been described in detail[5,6]. Immunohistochemistry was performed on formalin-fixed paraffin-embedded brain tissue sections. Tissues were cut into complete series of 10 μm sections. Sections were first immersed for 10 min in 0.30% Triton X-100 at room temperature. We incubated the sections for 30 min in 0.30% hydrogen peroxide and rinsed the sections twice in 10 mmol/L phosphate buffered saline (PBS). After that, the sections were incubated overnight with the primary antibody at a dilution of 1:24 000 rabbit polyclonal NGF and GDNF, respectively (Boster Biotechnology) at 4 ℃ followed by washing. Next, a biotinylated secondary antibody solution Envision reagent (DakoCytomation, Carpinteria, CA; 1:200 dilution) was applied for 30 min.The sections were rinsed again with PBS and reacted for 3 min in substrate medium containing 0.20% 3,3'-diaminobenzidine (DAB, Sigma) and 0.01% hydrogen peroxide until brown reaction products were observed.

2.4 Judging results The positive immunohistochemical staining showed as yellow-claybank particles. NGF and GDNF are intracytoplasmic antigens, and mainly distribute in the cytoplasm, nuclear, and neuraxon of nerve cells. Ten regional visions (200×200 μm^2 each) were selected randomly from each section, the positive nerve cells were counted and the positive rate was calculated according to the formula: Positive rate = Number of positive nerve cells/Number of all nerve cells in the regional vision. The mean positive rate of each indicator got from 10 films each slide which represented one monkey.

2.5 Statistical analysis SPSS13.0 statistical package was used to deal with the parameters of experimental data. Data are expressed as mean±SEM, gained from two repeated tests. The significance of difference between mean values was evaluated by the single-factor analysis of variance, and $P < 0.05$ was considered statistically significant.

3 Results

In isothermal experimental group, the NGF and GDNF positive cells in the frontal lobe of monkeys were much less; while in ultraprofound hypothermia group, they increased obviously (Fig. 1).

The positive rate of NGF and GDNF were significantly higher in the ultraprofound hypothermic group that in the isothermal group, indicating that the expressions of NGF and GDNF increased significantly following the resuscitation after selective cerebral ultraprofound hypothermia blood flow occlusion ($P < 0.05$) (Tab. 1).

4 Discussion

4.1 Biological characteristics of NGF and GDNF NGF was discovered by Rita Levi-Montalcini in 1952, and he received the Nobel Prize in Physiology and Medicine in 1986. The developing and developed central nervous systems are always able to produce NGF, particularly in the pyramidal cells of the frontal cortex, basal ganglia cells, hippocampal pyramidal cells and glial cells. Uptaken by cholinergic neurons axon terminals, NGF is transported to neurons of the basal forebrain. In the central nervous system, NGF has a wide range of physiological functions, mainly including maintenance and promotion of developing cholinergic neurons' survival, differentiation, maturation and function execution, and protecting neurons, promoting axonal regeneration and

Tab. 1 Positive rate of NGF and GDNF expressions in different groups (mean±SEM)

Group	Number	NGF (%)	GDNF (%)
Isothermal group	2	5.20±0.26	3.20±0.12
Ultraprofound hypothermia group	4	20.40±0.46*	14.70±0.41*

*$P < 0.05$, *vs* Isothermal group.

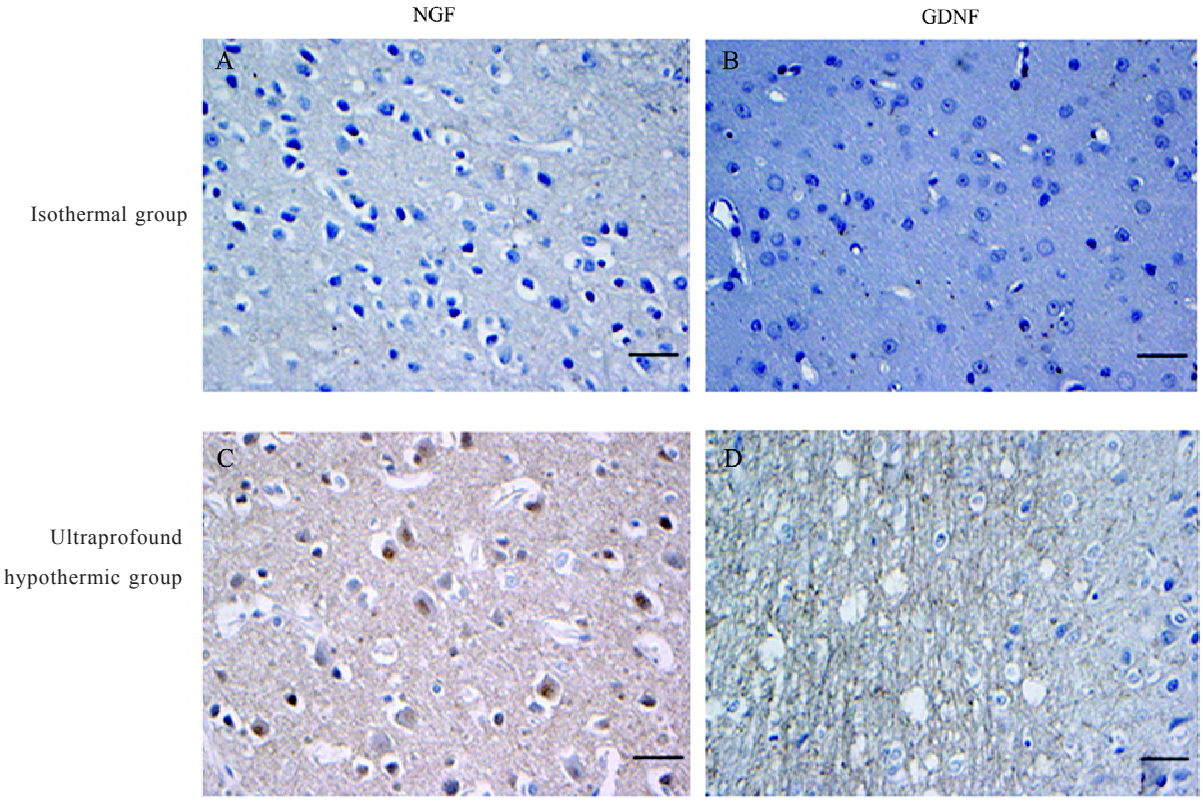

Fig. 1 Immunohistochemistry staining of NGF (A, C) and GDNF (B, D) proteins in different groups. The positive cells showed as yellow-claybank particles. The numbers of NGF-positive cells and GDNF-positive cells in ultraprofound hypothermia group were much more than those in isothermal group, indicating that the expressions of NGF and GDNF proteins in ultraprofound hypothermia group were up-regulated. Scale bar, 50 μm.

reconstructing neural connections after brain injury. Different mechanisms have been reported[7,8]. The nutrition role of NGF gene was also confirmed by gene knockout[9]: in the peripheral nervous system, dorsal root ganglion neurons and sympathetic neurons were reduced after NGF gene knockout; in the central nervous system, the cholinergic neurons in the basal forebrain decreased and atrophied, and the cholinergic neurons in the hippocampus were reduced after *NGF* gene knockout.

GDNF was a new NTFs discovered by Lin *et al*. in 1993[10]. As could significantly promote the survival of dopaminergic neurons in substantia nigra, it was called glial cell-derived neurotrophic factor. GDNF mRNA is expressed in the substantia nigra, striatum basal ganglia, cerebral cortex, cerebellum, thalamus, locus coeruleus, and so on. Its biological activity including the following four areas: to participate programmed cell death, to promote the survival of dopaminergic neurons, to participate in the nerve repair after axonal injury, and to contribute to the survival and differentiation of Purkinje cell. GDNF functions through its functional receptor tyrosine kinase (c-Ret)-mediated phosphorylation, and the ligand GDNF family receptorα 1 (GFR-α-1) is necessary to be involved. The protective mechanism of GDNF against cerebral ischemia-reperfusion injury is relevant to inhibiting apoptosis in the ischemic penumbra[11]. GDNF is the most effective NTFs so far to support growth of motor neurons *in vitro*, not only to prevent the development of motor neuronal programmed death, but also to promote the survival of cortex neurons after spinal cord injury and to prevent the delayed neuronal death after cerebral ischemia[12,13].

4.2 Deep hypothermic circulatory arrest and NTFs A substantial amount of researches have confirmed that among both cerebral ischemia or focal cerebral ischemia cases, expressions of a variety of NTFs and their receptor could be seen in the ischemic area, which suggested that NTF is related to neuronal survival and nerve repair. An in vitro study[14] indicated that NTF could protected the nerve cells in the course of ischemic cascade reaction: intravenous injection

and local brain injection of certain exogenous NTFs can improve neurological function, promote regeneration and rehabilitation of neurons after brain injury, and reduce infarction volume and cerebral edema after brain ischemia. Damaged mature axons could grow with application NGF, while would not grow in the lack of NGF, suggesting that NGF can overcome the surrounding preventive factors for axon growth and promote axon regeneration, neuron survival and transmitter release. The high expression of NGF can promote neurons' survival, and local nerve tissues' repair[15]. In the peripheral nervous system injury, the increased NTFs play an active role in the regeneration of myelin and axon. In the central nervous system injury, NTFs are expressed quickly by specific neurons, astrocytes, local microglia cells, and invasive inflammatory cells. Cerebellum Purkinje cells expressed little NGF in mature; the hippocampus pyramidal neurons re-express NGF and its receptors after injury; NTFs activate the cell signal transduction systems and promote the growth of axons[16].

The protective mechanisms of NTFs in cerebral ischemia including: inhibit neuronal apoptosis by antagonizing caspase activity; repair damaged neurons, promote neurons' regeneration, and regulate neuronal plasticity; promote endogenous neural stem cell proliferation and differentiation; reduce ischemia-reperfusion injury, and so on.

In our experiments, two monkeys in the isotherm group died after reperfusion directly due to hemodilution, increased brain metabolism, no lowering of brain temperature, stress reaction induced by surgical trauma, and thus the experimental monkeys died of serious brain ischemia and hypoxia. The positive rates of NGF and GDNF were in a low-level expression: (5.20±0.26)% and (3.20±0.12)%, respectively. We considered that sharp devastating hypoxia-ischemia led to the loss of self-protection mechanism for brain nerve cells, a large number of brain nerve cells necrosed and were unable to express NTFs.

Four monkeys in the ultraprofound hypothermia group were not recovered successfully, and died after reperfusion in the experiments. Though the brain temperature dropped to 16 ºC and the brain metabolism decreased to about 8% of the normal state[17], the vasospasm made the blood supply of vertebral-basilar artery system ineffective, and the ischemic brain stem dysfunction led the monkeys to death. The expressions of NGF and GDNF increased significantly, and the positive rates were (20.40±0.46)% and (14.70±0.41)%, respectively. The difference was statistically significant compared with the isothermal group ($P < 0.05$), suggesting that deep hypothermic circulatory arrest can increase expressions of NGF and GDNF. The reason may be that low temperature increase the tolerance of brain against ischemia and hypoxia, the self-protection of the brain could be exerted, and the survival time of nerve cells extended relatively. Moreover, hypothermia, in addition to cerebral ischemia, is also a predisposing factor for NTF expression. Previous animal experiments confirmed that after focal cerebral ischemia, the number of NTF-immunoreactive cells in the ischemic cerebral cortex noticeably increased compared with that in the non-ischemic cortex. The increase of NGF and GDNF may promote damaged neurons to self-recover to a certain level, and reduce infarct size[18-20]. This study could reach the same conclusion. However, because of the limited protective effect of NTFs on the survival of nerve cells, as well as other reasons, four monkeys ultimately died of the cerebral ischemia that was out of the compensatory ability of the monkeys' brain. The result also shows that cerebral ischemia for 10 min at 37 ºC by simultaneous occlusion of bilateral internal carotid arterys and bilateral vertebral arterys followed by selective ultraprofound hypothermic perfusion for 60 min to the brain is not feasible in the current circumstances.

To sum up, the technology of resuscitation after selective cerebral ultraprofound hypothermic blood flow occlusion can reduce cerebral metabolism, increase expression of NGF and GDNF, improve neurons tolerance to ischemia and hypoxia, inhibite apoptosis, and repair damaged neurons in a certain range. It is an important protection mechanism of the brain against cerebral ischemia.

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选择性超深低温断血流复苏促进猴脑中神经生长因子和胶质细胞源性神经营养因子的表达

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摘要：目的 观察常温缺血10 min后选择性超深低温断血流复苏后猴脑中神经生长因子(nerve growth factor，NGF)和胶质细胞源性神经营养因子(glial cell line-derived neurotrophicfactor，GDNF)表达的变化。**方法** 等温组及超深低温组实验猴于灌注或复苏死亡后立即开颅取脑，用NGF和GDNF抗体进行免疫组化染色；对额叶恒定视野内NGF和GDNF的阳性细胞记数求阳性率，并统计学分析。**结果** 等温组2只实验猴额叶NGF和GDNF有微量表达，超深低温组4只实验猴额叶NGF和GDNF表达明显上调，与等温组比较差异均极显著($P < 0.01$)。**结论** 猴脑选择性超深低温断血流复苏实验可引起NGF和GDNF表达上调，这可能是防止脑缺血的重要保护机制之一。

关键词：神经生长因子；胶质细胞源性神经营养因子；超深低温断血流；复苏；猴脑