

Effect of estrogen on recovering the injured nervous system

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Abstract: Estrogen plays an important role in the recovery of injured nervous system. This study aims to discuss the effect of estrogen on glial cells in spinal cord and apoptosis of neuron at different time points with a hope to lay theoretical basis on treating acute spinal cord injury (SCI) in clinic. Totally 72 adult rats were divided into a simple injury group and an estrogen group. Then several animal models with SCI were prepared. The estrogen group was treated with intramuscular injection of 100µg/kg estrogen every day till the death of animal models, while the simple injury group was treated with intramuscular injection of 0.5mL saline every day. Then these animals were put to death in the 1st d, 3rd d, 5th d, 8th d, 14th d and 21st d after SCI respectively and tissue sections were prepared, followed by B-cell lymphoma-2 (Bcl-2) detection, Terminal Deoxynucleotidyl Transferase-mediated dUTP nick-end labeling (TUNEL) and detection of the cell apoptosis in animal models after SCI. In the 14th d after the injury of spinal cord nervous system, Gale grading and inclined plate maintenance tests were carried out. In the 1st d after SCI, there was a higher expression of Bcl-2 protein in the SCI tissues. Bcl-2 protein reached the peak in the 3rd d after SCI in the simple injured group, while the estrogen group reached the peak in the 8th d. At that time, Bcl-2 protein was both expressed in nerve cells, and in glial cells in a higher level. The expression began to decline in the 14th d after SCI, and with only a little expression in the 21st d after SCI ($p < 0.05$). TUNEL detection results showed that, positive cells dominated by glial cells emerged in simple injured group only 24th h later; they reached the peak after 3~8 days, and then began to reduce. In the 21st d, positive cells still existed, and there was less cell apoptosis after treated with estrogen ($p < 0.05$). Two weeks after SCI, Gale score and inclined plate maintenance rate were higher in the estrogen group than in the simple injured group ($p < 0.01$). Adverse effects that occurred in injury group included blood dryness, necrosis, cyst cavity and cavity, while in estrogen group, adverse effects included focal bleeding, hydropic degeneration of neuron, disappearance of partial nissl bodies and neuraxial edema. All these findings suggest that, estrogen used for treating SCI can effectively inhibit the apoptosis of early nerve cells and glial cells in injured spinal cord nervous system by improving the micro-circulation, enhancing the expression of Bcl-2 protein, removing the free radicals and inhibiting the antioxidation. Thus, it can reduce the secondary SCI and promote the recovery of injured spinal cord nervous functions.

Keywords: Estrogen; nervous system; spinal cord injury.

INTRODUCTION

Estrogen plays a key role in many physiological activities, such as sustaining the normal operation of reproduction, bone homeostasis, immunity and cerebra vascular physiological activities and protecting the nervous system as well. In particular, estrogen leaves great significance on regenerative diseases in central nervous system, like stroke, Alzheimer's disease (AD), and Parkinson's disease (PD), as well as diseases like spinal cord injury (SCI), sciatic nerve injury, acute intracerebral hemorrhage, cerebral ischemia and neurotrauma.

In nervous system, the estrogen is of great significance as the key signal molecule. It plays a great role in promoting the growth and development of nerves, combination of plasticity with neurotransmitter and promoting the survival, regeneration of Myelin sheath and Axon. Hu Jiali *et al.* (2011) once pointed out that estrogen played neuroprotection function through multiple ways. It can resist apoptosis; promote expression, antioxidation and

anti inflammatory of nerve growth factor and its receptor. Meanwhile, it provides new therapy for treating diseases in nervous system, such as AD, PD, meningioma, multiple sclerosis, and autoimmune encephalomyelitis (Jiali and Xiaofei, 2011).

Ma Min *et al.* (2014) mentioned that apoptosis was one of important causes of fewer neurons in AD brain tissues in the neuroprotection mechanism of AD, while estrogen could obviously protect the neurons (Min and Bin, 2014). Zhu Jiaying *et al.* acquired the nervous cell injury model induced by glutamic acid from the reorganization of lentivirus intervention by estrogen and its receptor in their paper. This model could reduce the excitatory neurotoxicity, while this mechanism was just complemented by inhibiting the N-methyl-D-aspartate receptor-1of glutamic acid receptor and vesicular glutamate transporter protein1 (VGLUT1) (Jiaying *et al.*, 2013).

Based on SCI rat models, this study observed the effect of estrogen on glial cells in spinal cord and apoptosis of neurons and then it further proved the protective and

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recovery effect of estrogen on the spinal cord nervous system.

MATERIALS AND METHODS

Experimental animals and grouping

Totally 72 healthy adult Sprague-Dawley rats provided by Qingdao Drug Administration with weight ranged from 250 to 310g were selected. They were divided into a simple injury group and an estrogen group with 36 rats in each group. All animals used for test have undergone molecular and pathological analysis before this study and the test has approved by the ethics committee of Maternal and Child Health Hospital of Zhengzhou, China.

Preparation of animal models with SCI

Improved Allen's impact method was used to make SCI model. Intraperitoneal anesthesia was performed with 0.3% pentobarbital sodium (30mg/kg), and the standard of anesthesia effect was disappearance of corneal reflex and hindlimb contraction after stimulated by pain. Fur on the back of rats was cut. Then rats were fixed on the operating table, sterilized by 10% povidone iodine and covered by hole towel later. An incision with a length of 4 cm was cut in the middle of back centering on T8 spinous process to expose T7~T11 spinous process and lamina. All animal models were established with Allen impact equipment with 25g/cm wounding power. The standard of successful impact was as follows: partial edema and hyperemia in spinal cord, a spasmodic twitch in the tail of animal models together with a contraction-like flutter in their two hind limbs, and then a gradually paralyzed hind limbs. After inspection about hemorrhage, surgery field was sutured; when rats woke up, they were fed with water and food, and then bladder massage or puncture helpful to urination was performed every 12 hours. The estrogen group was treated with intramuscular injection of estrogen (100µg/kg) every day until all rats in this group were put to death, while the simple injury group was treated with intramuscular injection of 0.5mL saline every day.

Preparation of tissue slices

After successful impact of animal models, put those rats to death in batches in the 1st d, 3rd d, 5th d, 8th d, 14th d, and 21st d. Resect the injured spinal cord tissues from each animal model at 4mm of both up and down the center of injured spinal cord, and then carry out the myocardial perfusion fixation with 4% paraformaldehyde for these resected tissues. Twenty-four hours later, soak them in 20% sucrose for 12h and then embed them in paraffin. Finally take the tissue slices in depth of 4µm or so.

Detection of bcl-2

Carry out the inspection of Bcl-2 monoclonal antibody according to the instructions of SP reagent. Record the number of positive cells under microscopy.

TUNEL

Carry out the TUNEL by employing the detection kits of apoptosis according to the instructions of SP reagent. Record the number of positive cells under microscopy.

Image analysis and data statistics

Apoptotic cells showing brown yellow after in situ end labeling were considered as positive cells. Each tissue section was observed at ten high power fields respectively. Proportion of positive cells accounting for all cells was calculated.

STATISTICAL ANALYSIS

Apoptosis index (AI) = (number of died cells/ total number of cells under microscopy)*100%. The data from each group was expressed by mean ± SD and t test was used for comparison between the two groups. When p<0.05, difference was considered as statistically significant.

RESULTS

Detection of bcl-2 protein

In the first day after SCI, there was a higher expression of Bcl-2 in tissues, and it reached the peak in tissues from simple injury group in the 3rd d while it reached the peak in tissues from estrogen group in the 8th d. Bcl-2 was expressed in both nerve cells and glial cells meanwhile. However, there was more inhibition in the glial cells and there was less expression until the 14th d. There was only a little expression in the 21st d after injury (p<0.05). Please refer to table 1.

Inspection of TUNEL

There were positive cells 24h later in the simple injury group, which were mainly the glial cells. It reached the peak from the 5th d to 8th d, and then came down. There were still some positive cells in the 21st d, but there were much fewer died cells above all (p<0.05). Please refer to table 2.

Gale grading and rate of inclined plate maintenance two weeks after SCI

Two weeks after SCI, the Gale grading and calculation for the rate of inclined plate maintenance were carried out among those acquired experimental data, as shown in table3. It showed that the estrogen group was much better than the simple injury group in Gale grading, so was the estrogen group as to the rate of inclined plate maintenance.

DISCUSSION

Estrogen E is mainly produced from ovary. It sustains the reproductive cycle and physiological feature of female together with progesterone (Pengwei and Qingcheng,

Table 1: The number of positive cells in Bcl-2 of rats after SCI (Number of positive cells/under microscopy, mean± SD)

Time of SCI	Simple injury group	Estrogen group
1d	18.93±3.61	21.85±6.03
3d	20.84±6.39	36.78±5.23*
5d	16.37±3.87	38.61±4.07*
8d	10.61±3.03	41.35±7.68*
14d	8.78±2.47	23.27±6.32**
21d	7.86±2.87	13.91±2.48

Table 2: AI of rats after acute SCI in the simple injury group and estrogen group at different time points (mean ± SD)

Time of SCI	Simple injury group	Estrogen group
1d	20.08±4.01	11.27±3.31*
3d	36.92±4.82	17.93±4.63**
5d	39.98±5.63	18.23±5.37**
8d	41.03±4.98	19.61±5.06*
14d	19.83±2.03	11.29±1.96*
21d	5.03±1.29	3.07±1.09

Note: compared with the simple injury group, * refers to $p < 0.05$, ** refers to $p < 0.01$.

Table 3: Gale grading and rate of inclined plate maintenance two weeks after SCI (n=12)

Number of rats	Gale grading*		Rate of inclined plate maintenance **	
	Simple injury group	Estrogen group	Simple injury group	Estrogen group
No. 1	2.00	4.00	0.351	0.425
No. 2	2.00	5.00	0.342	0.517
No. 3	2.00	3.00	0.321	0.406
No. 4	2.00	5.00	0.307	0.417
No. 5	2.00	3.00	0.361	0.483
No. 6	2.00	3.00	0.309	0.397
No. 7	2.00	3.00	0.379	0.436
No. 8	2.00	4.00	0.392	0.459
No. 9	3.00	4.00	0.381	0.438
No. 10	2.00	5.00	0.312	0.491
No. 11	2.00	3.00	0.306	0.473
No. 12	3.00	5.00	0.311	0.345

Note: *refers to $p < 0.01$ when compared with the simple injury group, **refers to $p < 0.01$ when compared with the simple injury group.

2013). At present, it is mainly applied to treat some obstetrics and gynecology diseases in clinic. Estrogen, as the key signal molecule in the nervous system, is no longer confined within the reproduction. It also plays a great role in the synthesis and secretion in nervous system, as well as its structure and functions. There are so many studies nowadays showing that 17- β estradiol (17 β E2) can protect the nerve cells and prevent from the neurodegeneration (Xun *et al.*, 2006).

Distribution of estrogen receptor in periphery and central nervous system

When estrogen would like carry out the biological behavior evaluation in the nervous system, it should be mediated by estrogen receptor (ER) at first. Target tissues affected by estradiol can be divided into a classical and a

non-classical type, while ER also includes two subtypes, namely α and β . The classical target tissues include uterus, breast, placenta, liver, central nervous system, cardiovascular system and skeleton, which all contain a large amount of ER α . The non-classical target tissues include prostate, testicle, ovary, pineal body, thyroid, parathyroid, adrenal gland, pancreas, gallbladder, skin, urinary tract, lymphocytes and red blood cells (Chunxia and Jiqiang, 2010). There are quite a number of studies showing that ER is widely distributed in the basal forebrain, diencephalon, mesencephalon, hippocampus, amygdaloid body, cerebral cortex, cerebellar cortex. Besides, there is a difference in sex (Lili and Shengchun, 2004). Except for neurons, there are also some receptors of estrogen in neuroglial cells (Ru *et al.*, 2009).

Effect of estrogen on nerve cells

Estrogen and its receptor produce effect mainly in three methods: firstly, the classical nuclear receptor of estrogen (genomic mechanism); secondly, the receptor mechanism of film surface (non-genomic mechanism); thirdly, antioxidation mechanism. In recent years, there were lots of studies showing that estrogen carried out the evaluation for different biological behaviors of nervous system based on glial cells. By contrast, it confirmed the method of mediating the estrogen by glial cells (Tao *et al.*, 2012).

Neuroprotection of estrogen

Neurotrophic factor is a kind of protein, and its specificity can produce some certain effect on the nerve. Nerve growth factor in brain and brain-derived neurotrophic factor (BDNF) can effectively promote and sustain the differentiation, growth and survival of nerve cells, and it can greatly improve the cognitive functions (Li *et al.*, 2011). Insulin-like growth factor-1(IGF-1) can promote the differentiation and survival of special nerves in development stage. As a neuromodulator, it has an effect on the synaptic plasticity of mature nerves, takes part in the reflection of injury from nerve tissues and protect the nerves against the stimulation of neural degeneration (Zhongqing and Aili, 2013). Heat shock protein (HSP) acts as a molecular chaperone, which can take part in the process of growth, development and differentiation of cell at room temperature. When organism suffers different harmful stimulation, such as ischemia and high temperature, there is going to be more HSPs (Yuping and Jiandong, 2010). Both protein kinase β and estrogen can protect the neurons of hippocampus. Bcl-2 is the proto-oncogene sustaining the survival of cells in many tissues, and estrogen can promote the survival of cells by Bcl-2 in many non-nerve tissues (Xiangjun *et al.*, 2011).

Effect of estrogen on the rebirth of periphery nerves

There are studies showing that estrogen can promote the recovery of sciatic nerve functions in rats, and protect the neurons by inhibiting the lipid peroxidation reaction of spinal cord induced by sciatic nerve injury (Xiao *et al.*, 2007). Besides, Progesterone can protect the nerves by promoting the generation of Myelin sheath in periphery nerves (Xianming and Yuntao, 2014). This study proved that estrogen was able to promote the micro-circulation in SCI, promote the expression of Bcl-2 protein and remove the oxyradical. The antioxidation effect can inhibit the death of early nerve cells and glial cells in the injured spinal cord nervous system. Therefore, it can reduce the secondary SCI, and promote the recovery of nerve functions of injured spinal cord.

The relation of estrogen with PD

PD is caused by dopamine (DA) neuron injury, and the degeneration DA neuron in compacta of substantia nigra (SNc) plays a key role in the incidence of PD. A proper application of estrogen can remove dyskinesia in the early

stage of PD (Xueping, 2011). The effect of estrogen on DA is mainly expressed in its relation with release and behavior. Because the ER α and ER β are found in DA neurons in mesencephalon, the effect of estrogen on the nigrostriatal system in adults can be understood. As to the action mechanism of estrogen on DA, estrogen can also regulate synthesis and release of DA through presynaptic membrane D2 receptor besides promoting synthesis and release of DA by genomic mechanism and changing neurilemma effect by non-genomic regulation mechanism, improve sensitivity of receptor by improving density of subsynaptic membrane D1 and D2, reduce absorption of neurotoxic substance by affecting material transport by DA.

Effect of estrogen on acute neurotrauma

This study can prove that estrogen can promote the recovery of neural functions for rats with SCI in experiment by reducing the apoptosis. Therefore, treatment with some estrogen can protect and promote the recovery after suffering acute neurotrauma.

Effect of estrogen on acute cerebral ischemia

Endothelial nitric oxide synthase (eNOs) can effectively increase the blood circulation in ischemic penumbra, and protect the residual neurons in ischemic region (Oge *et al.*, 2003). It was reported in the experiment of acute cerebral ischemia (Dena B *et al.*, 2001), estrogen was able to reduce the volume of infarction, protect neurons in the injured contra lateral cortex and corpus striatum (hippocampal excluded) based on the ER α (Yongting *et al.*, 2010).

Effect of estrogen on cerebral hemorrhage

In studying the effect of estrogen in the model of acute intracranial hematoma in basal ganglia of rats, this paper raised that estrogen produced effect by its receptor in cerebral cells. The animal models with injection of estrogen spent less time on assimilating hematoma and edema in cerebral tissues as well as recovery of neural functions than other animal models. Besides, it enjoyed the cerebral protective effect on the male animals (Yaqiong and Ping, 2010).

Although there are various discussions over the study of the neuroprotective effect of estrogen and its recovery effect on injured nerves, there is no doubt that a proper application of estrogen can eliminate the neural injury caused by cerebral ischemia and leave a great effect on the memory and cognition functions for both human beings and other lower animals. However, there are quite a number of questions without solution at present, such as confirming the optimal estrogen formula, timing for replacing estrogen, administration of estrogen and duration. In addition, the targets of estrogen in cell and subcell have also joined in the neuroprotective effect of estrogen, such as regulating the energy balance of cerebral

tissues and the metastasis of neurovascular unit (NVU). In fact, their effect in this process is far from clear and definite. Thus, more studies are required to evaluate the effect of estrogen on precaution, treatment and recovery of injured central nervous system, and more studies to illustrate the mechanism of estrogen, so that it can provide more benefits to the clinical practice with the greatest effect. .

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