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Original Article

The change tendency of PI3K/Akt pathway after spinal cord injury

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Abstract: Spinal cord injury (SCI) refers to the damage of spinal cord's structure and function due to a variety of causes. At present, many scholars have confirmed that apoptosis is the main method of secondary injury in spinal cord injury. In view of understanding the function of PI3K/Akt pathway on spinal cord injury, this study observed the temporal variation of key molecules (PI3K, Akt, p-Akt) in the PI3K/Akt pathway after spinal cord injury by immunohistochemistry and Western-blot. The results showed that the expression of PI3K, Akt and p-Akt display a sharp increase one day after the spinal cord injury, and then it decreased gradually with the time passing by, but the absolute expression was certainly higher than the normal group. These results indicate that the PI3K/Akt signaling pathway is involved in the spinal cord injury and the mechanism may be related to apoptosis.

Keywords: Spinal cord injury, apoptosis, PI3K, Akt

Introduction

Spinal cord injury (SCI) refers to the damage to the spinal cord structure and functions caused by car accidents, high-altitude falling, etc. and it may result in the movement, feeling and autonomic nerve dysfunction below the level of injury. SCI will not only bring physical injury and spiritual burden to the patients, but also impact the life quality of patients. Moreover, it may increase the economic burden of the family and the society [1]. It has been verified that SCI is accompanied by complicated pathological and physiology changes, which mainly consists of two steps: the primary spinal cord injury and secondary spinal cord injury, which determines the final result of SCI [2]. The primary spinal cord injury is caused by the trauma, and it mainly refers to the original, direct and mechanical oppression and local bleeding after the injury. The pathophysiology changes are mainly reflected by the damage to the completeness of myelin sheath, axon, piping system, as well as the tearing of nerve cell membrane and axon membrane. The electrolyte may overflow from the damaged nerve cell, and the nerve cell may degenerate with necrosis, and release several types of inflammatory mediator [3-6]. The secondary spinal cord injury is an initiative process of the interactions and mediating of several factors, and it mainly refers to a series of cellular, molecular and biochemical cascade reaction occurred after the spinal cord injury. Dominated by apoptosis, the main injury mechanism includes the formation of hypoxia free radical, release of protease, excitability poisoning of glutamic acid, lipid peroxidation, Ca^{2+} overload, oxidative stress, angiogenesis, inflammatory responses caused by the activation and aggregation of various kinds of toxin cells (such as neutrophil, polymorphic nucleus leukocyte, astrocyte, etc.). It may further aggravate the injury [7-9]. Owing to the irreversibility of primary injury, the treatment after the spinal cord injury shall target at the secondary spinal cord injury, which plays a critical role in the treatment and recovery of function [10]. At present, many scholars have already conducted studies on the

mechanism of secondary spinal cord injury, proving that apoptosis is the main approach of secondary spinal cord injury, and it mainly refers to the series of cascade activated programmed cell death process stimulated by various death signals. In this process, complicated and thorough signal transduction system were included [11]. During this process, different noxious stimulations have independent apoptosis signal transduction pathway, and some can share one or several accesses with other signal molecules, while a certain access can be activated by several stimulations.

Phosphoinositide 3-kinase/serine-threonine kinase (PI3K/Akt) signal pathway is a significant pathway for the survival of cells mediated by nerve cell. PI3K/Akt signal pathway excitation may restrain the apoptosis induced by several stimulations, and promote the progress of cell cycle, which may be favorable for the survival of cells. Phosphoinositide-3-kinase (PI3K) is a kind of phosphatidylinositol which can phosphorylate the third hydroxyl of inositol ring. Generally, it was consisted by two subunits, P110α and P85β, which is the catalytic subunit and regulatory subunit [12]. In general condition, some factors such as drugs, cell factors, can maintain the survival of nerve cell, and these signals can activate PI3K. Activated PI3K can be transported to the internal surface of the cytomembrane. In this place, it was phosphorylated with three hydroxyl and changing into phosphoinositide 3-phosphoric acid (PI3P), phosphoinositide 3, 4-diphosphonic acid (PI3, 4-P2) and phosphoinositide 3, 4, 5- triphosphoric acid (PI3, 4, 5-P3). The PI3, 4-P2 and PI3, 4, 5-P3 can directly activate serine threonine kinase (AKT) changing to phosphorylation AKT (p-AKT) by stimulating phosphoinositide dependent kinase (PDK). Akt, also known as protein kinase B (PKB), is the main target enzyme of PI3K which can link the pathway. It can mediate several cellular activities and biological effects: such as the cell growth and survival, proliferation and apoptosis, saccharometabolism, genetic transcription, neovascularization and cell migration by phosphorylating a series of apoptosis regulating protein [13-15].

As known to all, PI3K/Akt is directly related to the survival of cells, and it was a significant medium in the process of resisting apoptosis and promoting the survival of cells, as well as a significant signal pathway in the process of cell proliferation and regulation. Recent researches had showed that this pathway also play a vital role in suppressing apoptosis in the ischemia reperfusion injury of several organs, such as the heart, kidney, liver, etc. [16-19]. Some researchers have found that LY294002, the specific inhibitor of PI3k, can restrain the phosphorylation level of Akt, and mitigate the protect function of PI3k in the cerebral ischemia/reperfusion process, which can aggravate the apoptosis and brain damage [20].

At present, how to control the apoptosis and axon degeneration caused by secondary injury, how to maintain the function of residual neural cell, and how to create conditions for the regeneration of axon, is the hot issues in the research of spinal cord injury. Because of its function in anti-apoptosis, regulating and controlling the cell proliferation, PI3K/Akt pathway have already been widely used in the study on cancer therapy and ischemia reperfusion injury. But there have no reports about its function in secondary spinal cord injury. In this study, we aim to evaluate the function and possible mechanism of PI3K/Akt signal pathway in the cell apoptosis after the secondary spinal cord injury. We investigate t he variation trend of PI3K, Akt and p-Akt after the spinal cord injury, which are the key components of PI3K/Akt pathway.

Materials and methods

Ethics statement

All experimental procedures conformed with institutional guidelines for the care and use of laboratory animals at Binzhou Medical University, Yantai, China and the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Experimental materials

48 Female Sprague-Dawley (SD) rats (weighing 200-220 g), were purchased from the experimental animal center of Shandong Yantai Lvye Pharma Co., Ltd.

SABC kit was purchased from Wuhan Boster Bioengineering Co., Ltd. Rabbit-anti rat PI3K polyclonal antibody, rabbit-anti rat AKT polyclonal antibody, rabbit-anti rat p-AKT polyclonal antibody, horse radish peroxidase labelled IgG/

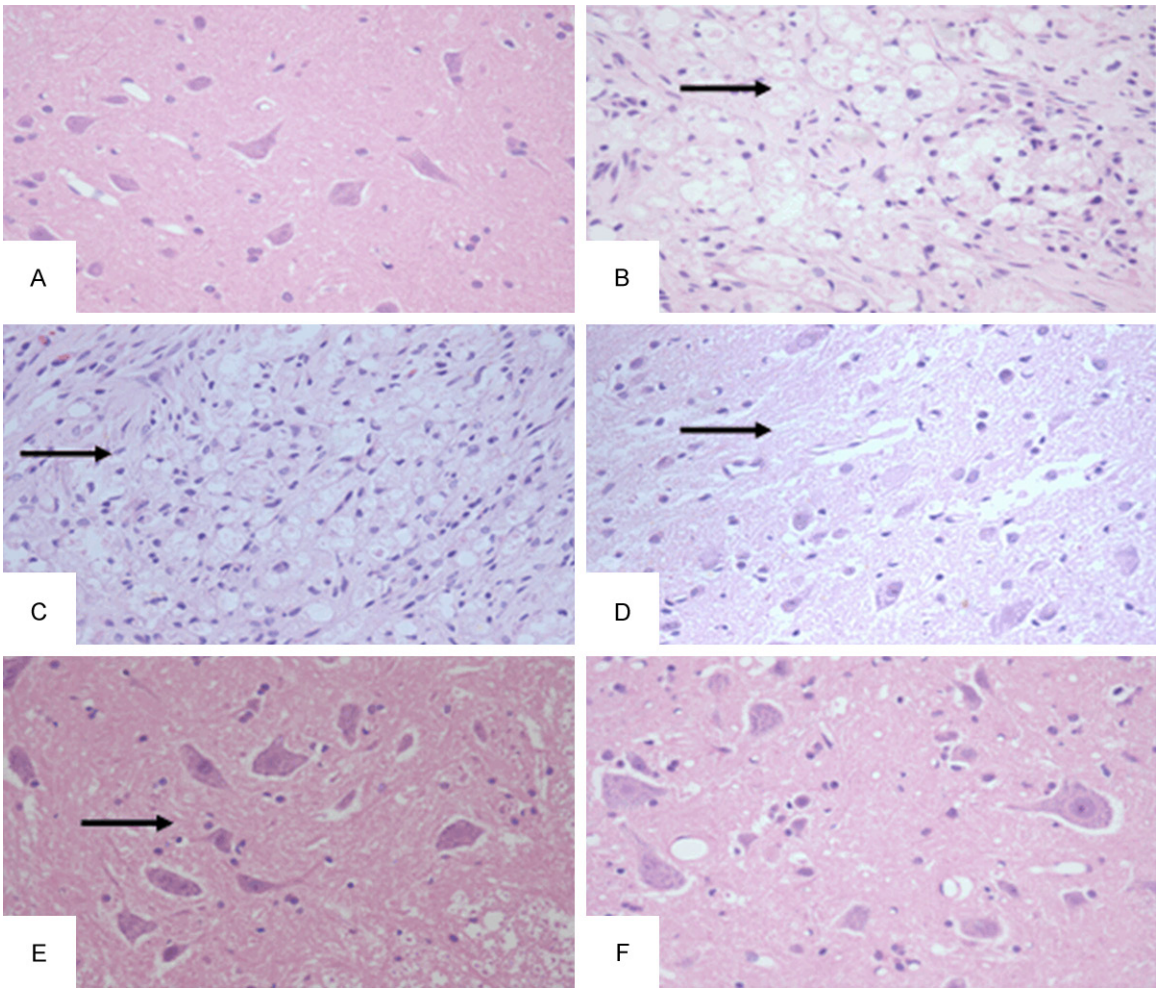

Figure 1. The cellular morphology at different time points after the spinal cord injury in each group (×400). A. Normal group; B. One-day group after spinal cord injury; C. Three-day group after spinal cord injury; D. Seven-day group after spinal cord injury; E. Fourteen-day group after spinal cord injury; F. Twenty-eight-day group after spinal cord injury.

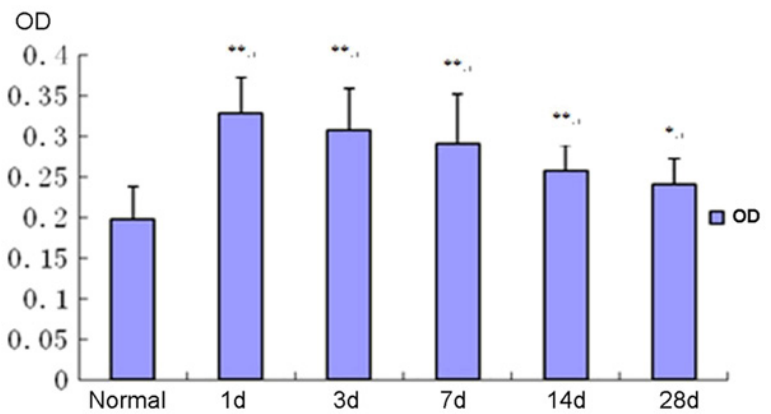

Figure 2. The OD level of PI3K at different time points after the spinal cord injury by immunohistochemistry. *Compared to the normal group, $P<0.05$; **compared to the normal group, $P<0.01$.

TRITC, and RIPA protein lysate were purchased from Shanghai Beyotime Biotechnology Co., Ltd. Rabbit-anti rat β-actin monoclonal antibody was purchased from Cell Signaling Technology, ECL and PVDF membrane were purchased from Millipore Corporation.

Experiment grouping and preparation of spinal cord injury model

48 SD rats were randomly divided into 6 groups: normal control, one day after spinal cord injury, three days after spinal cord injury, seven days after spinal cord injury, fourteen days after spinal cord injury, and twenty-eight days after spinal cord injury. The normal group was not treated, while

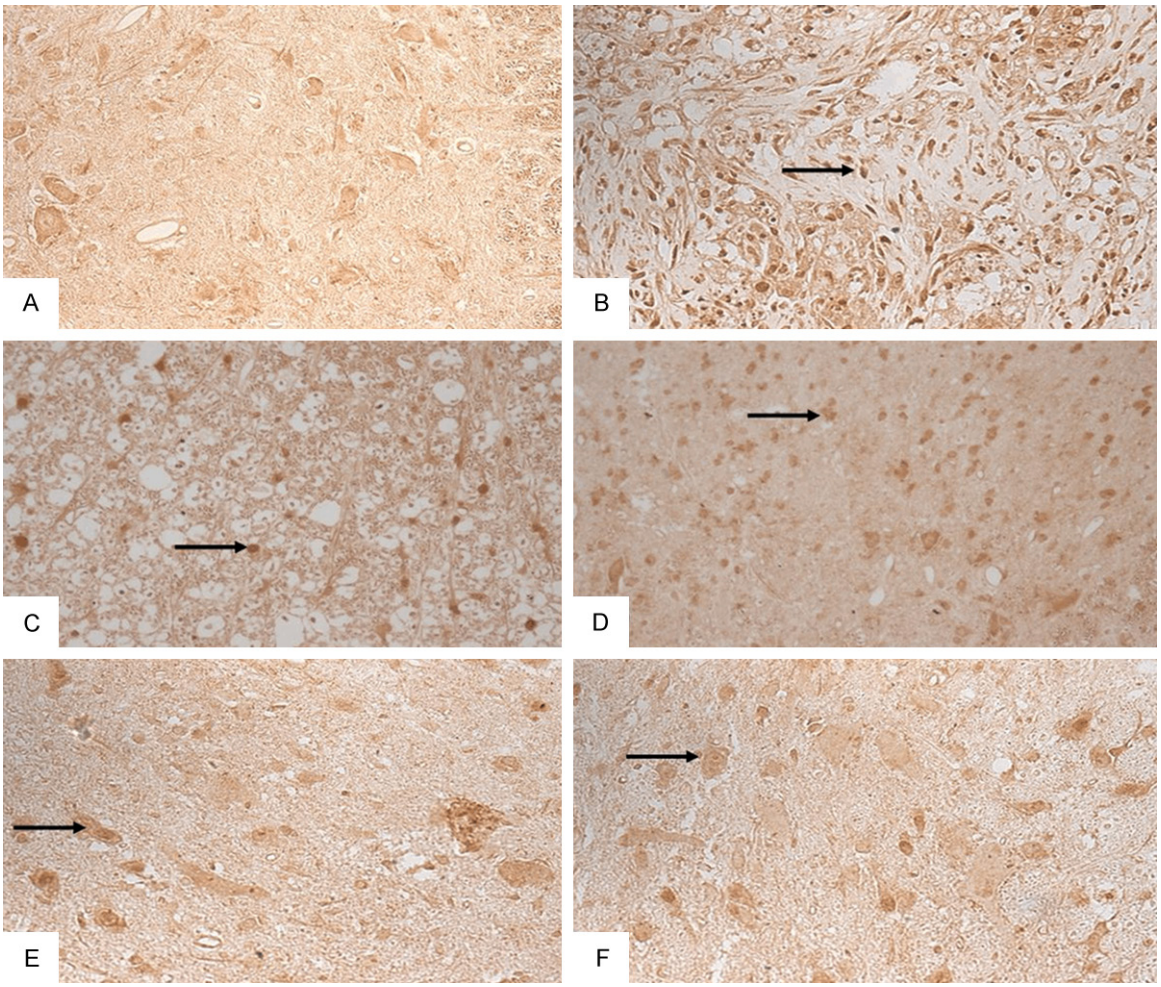

Figure 3. The expression of PI3K at different time points after the spinal cord injury by immunohistochemistry (×400). A. Normal group; B. One-day group after spinal cord injury; C. Three-day group after spinal cord injury; D. Seven-day group after spinal cord injury; E. Fourteen-day group after spinal cord injury; F. Twenty-eight-day group after spinal cord injury.

the acute spinal cord injury model was manufactured in all spinal cord injury groups: The rats were kept under standard conditions in a 12/12 h light/dark cycle and no food were provided 8 hours before the operation. All rats were deep anesthesia by 3.5% chloral hydrate (1 ml/100 g) and median incision about 2.5 cm in the thoracic segment of the back were cut, then the skin and subcutaneous tissue were cut gradually for exposing the T7-T8 vertebral plate. The spinal cord between T7 and T8 were completely sheared with iridectomy scissors in order to making complete transection ventrally and dorsally spinal cord injury model.

The rats were fed in single cages after the surgery, and padding were changed every day for keeping clean and dry. After fasting for 12 hours, food and water were given for improving the nutrition. Penicillin were injected through the muscle (50,000 U/kg/d), and the rats were kept warm. Bladders were manually emptied four to five times daily until autonomous urination recovery, so as to promote the formation of reflexive bladder and help recover the micturition reflex.

Histology analysis

Rats in one-day group, three-day group, seven-day group, fourteen-day group and twenty-eight-day group were anaesthetized with 3.5% chloral hydrate (1 ml/100 g) respectively, then they were fixed onto the operating table for

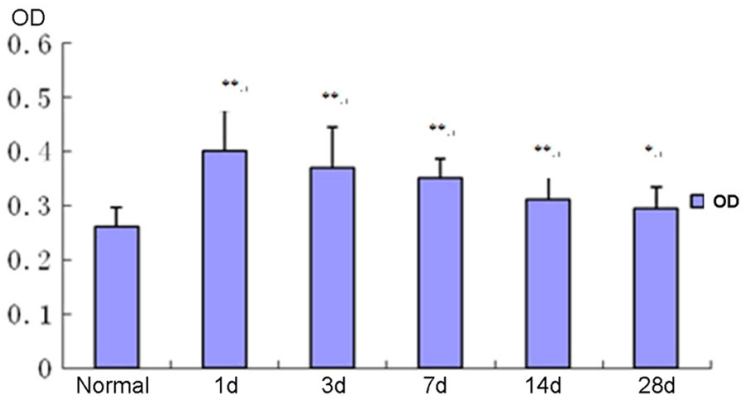

Figure 4. The OD level of AKT at different time points after the spinal cord injury by immunohistochemistry. *Compared to the normal group, *P*<0.05; **compared to the normal group, *P*<0.01.

opening the chest and exposing the heart. Later, blunt needle were used running through from the apex cordis to the left ventricle and then to the aorta. The rats were perfused normal saline (NS) followed by 4% paraformaldehyde into the heart. 2 cm spinal cord tissue at the center of the injury was taken. Part of tissue was embedded in paraffin for histology analysis. For histopathological analysis, paraffin sections (5 um) of each group were stained with hematoxylin and eosin (HE), then the cellular morphology were observed by Microscope.

Immunohistochemistry analysis

For immunohistochemical analysis, the paraffin section of different groups were incubated with antibodies against PI3K and Akt at 4°C overnight, after washing the sections, secondary antibodies were applied and incubated according to the SABC Kit. At the end, the expression of PI3K and Akt at different time points after the spinal cord injury was analyzed.

Western blot analysis

The other part of the spinal cord tissues at the center of the injury were lysed in RIPA lysis buffer containing protease and phosphatase inhibitors. Tissues and cell debris were cleared by centrifugation at 12,000 g for 10 min at 4°C. After concentration, total protein was detected using the BCA method, and protein extracts were boiled with SDS sample buffer. Western blotting analysis was carried out as below: 25 μg of protein was subjected to SDS-PAGE and electro-transferred to PVDF membranes. After blocking with nonfat dry milk, the membranes were incubated with primary antibodies against PI3K, Akt and p-Akt overnight at 4°C. The membranes were then incubated with secondary antibody after three rinses. The blot signal was detected using an ECL detection kit and analyzed with Image J software.

Statistical analysis

Two-tailed Student's test was applied to analyze the expression of PI3K and Akt in different group compared to the normal group. All analysis was performed by SPSSI3.0 statistical analysis software. Significant differences were considered at a threshold of $P < 0.05$.

Results

The survival rate of rats after the surgery

Within a week after the surgery, the rats with spinal cord injury were in bad state: taking less food, having retention of urine or urinary incontinence, some even had blood urine. In order to prevent infection, penicillin was injected in muscle. There was no death in the control group, while two rats died in the spinal cord injury model groups because of retention of urine and wound infection. One week later, all rats turned into good state, but some rats still had retention of urine or urinary incontinence.

The cellular morphology at different time points after the spinal cord injury

We observed the cellular morphology with light microscope, in one-day group, three-day group and seven-day group, the morphological structure of the spinal cord injury region was incomplete, and the nervous tissue was fragmentary. There were obvious cavities, foam cell and inflammatory cell infiltration in sight, the neuron in grey matter region was irregular, the nissl bodies of the nerve cell decreased, the fiber in white matter decreased and distributed unevenly, the myelin sheath was chaotic in arrangement, the myelin sheath interval enlarged, and the structure was incomplete (**Figure 1B-D**). In fourteen-day group and twenty-eight-day group, the nerve cell was regular in form, the cell nucleus and nucleolus can be seen, but the

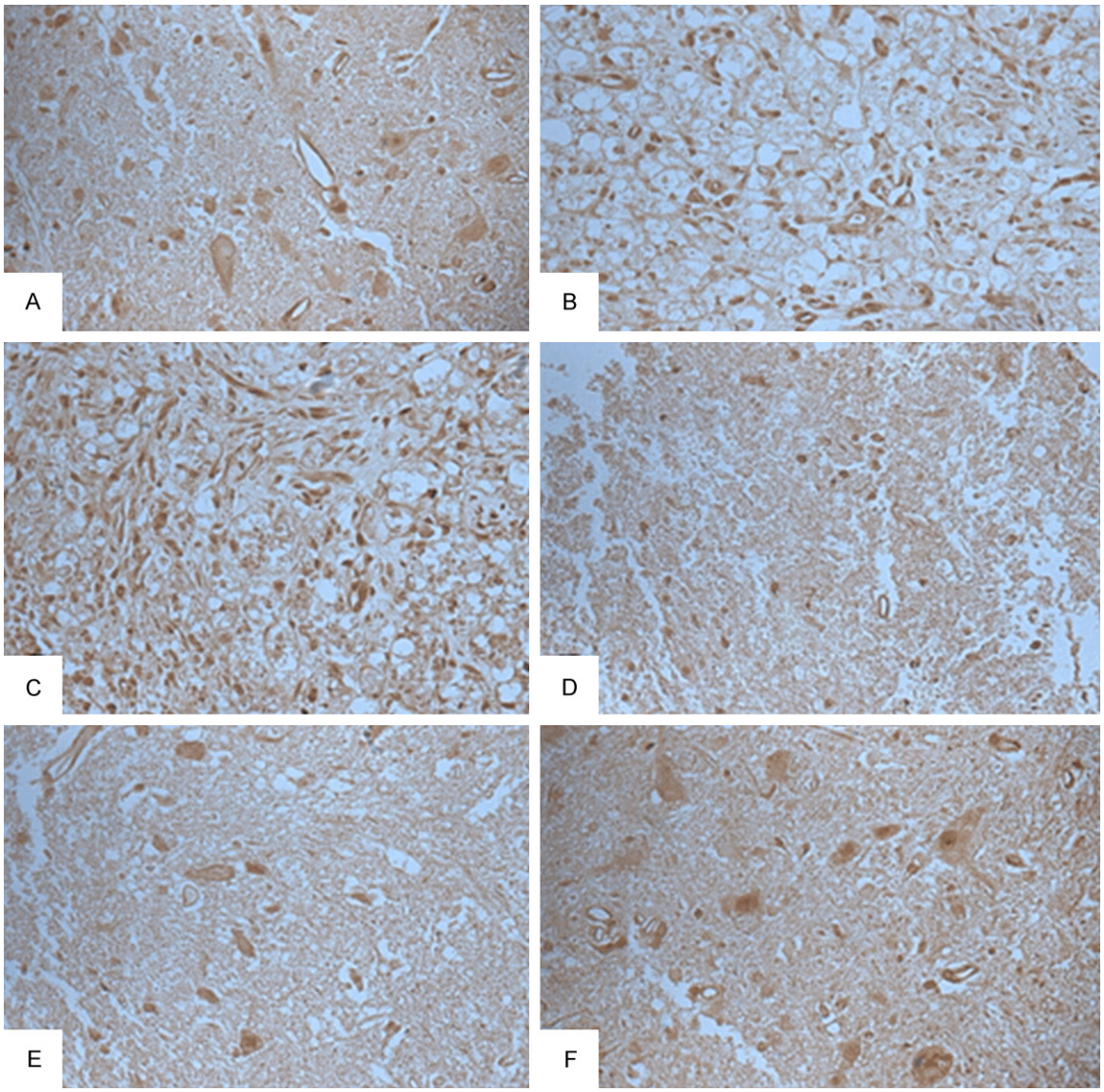

Figure 5. The expression of AKT at different time points after the spinal cord injury by immunohistochemistry (×400). A. Normal group; B. One-day group after spinal cord injury; C. Three-day group after spinal cord injury; D. Seven-day group after spinal cord injury; E. Fourteen-day group after spinal cord injury; F. Twenty-eight-day group after spinal cord injury.

nissl bodies in nerve cell was relatively small, and the distribution was relatively uneven (**Figure 1E**, **1F**).

The expression of PI3K and Akt at different time points after the spinal cord injury by immunohistochemistry

According to the result of immunohistochemistry staining, PI3K positive reaction appeared in graininess state, and it was mainly expressed in the cytoplasm of the cell. There was little expression of PI3K in normal group. The expression of PI3K in one-day group was the highest, and then it was in a decreasing trend, but the absolute expression was higher than the normal group ($p<0.05$) (**Figures 2** and **3**).

According to the result of immunohistochemistry staining, Akt positive reaction appeared in graininess state, and it was mainly expressed in the cytoplasm of the cell. There was little expression of Akt in normal group. The expression of Akt in one-day group was the highest,

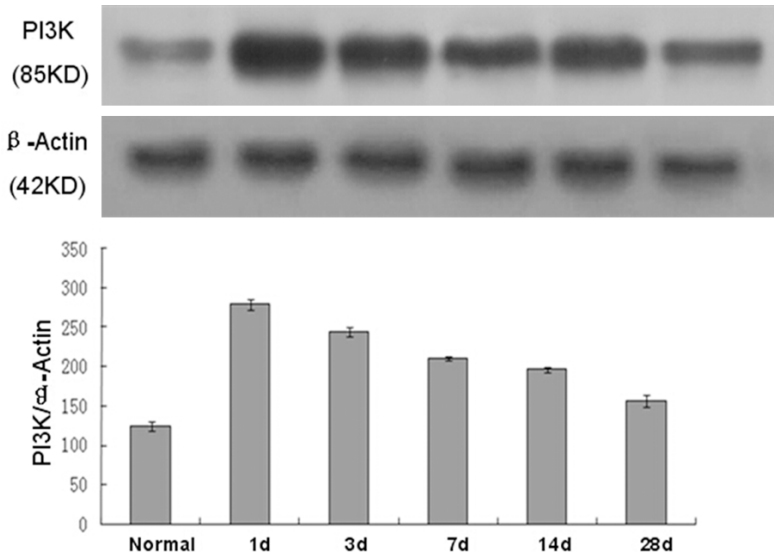

Figure 6. The expression of PI3K at different time points after the spinal cord injury by Western Blot.

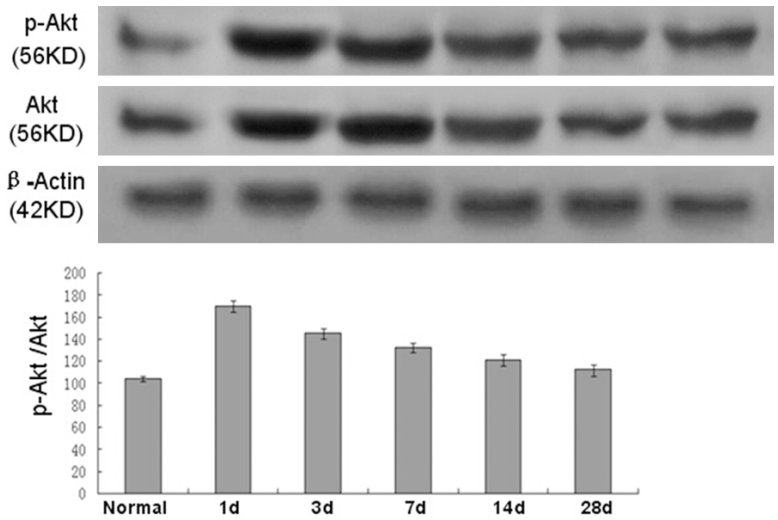

Figure 7. The expression of Akt and p-Akt at different time points after the spinal cord injury by Western Blot.

and then it was in a decreasing trend, but the absolute expression was higher than the normal group ($p<0.05$) (**Figures 4** and **5**).

The expression of PI3K, Akt and p-Akt at different time points after the spinal cord injury by Western Blot

According to the result of Western Blot, the expression of PI3K and p-Akt/Akt in one-day spinal cord tissue was the highest, and then it was in a decreasing trend. But the absolute expression was higher than the normal group ($p<0.05$) (**Figures 6** and **7**).

Discussion

With the gradual increase of trauma incidence, patients with spinal cord injury increase day by day. The cause of injury is mostly related to the sport injury and car accidents [21], therefore, most patients with SCI are young adults. After the spinal cord injury, there may be local functional disorder or paraplegia, which may bring a huge burden to the society and family [22]. Currently, the functional recovery and treatment after the spinal cord injury is a great problem, the pathophysiology changes and mechanism after the spinal cord injury become the hot issue in medical research. It has been verified by research that the neurology damage after the spinal cord injury is caused by two mechanisms, namely primary injury and secondary injury [23]. The secondary injury may occur within several minutes or several days after the injury [24, 25].

Lipson AC discovered that there may be short time interval and small-scale apoptosis after the spinal cord injury, but afterwards, numerous astrocytes and oligodendrocytes deceased [26]. After being paralyzed for eight hours, the amount of apoptotic cell reached the highest. There are also researchers showed that within 24 hours after the spinal cord injury, there may be acute inflammatory response, and some may have toxic effect (for instance IL-6, TNF, etc.) [27]. The content of inflammatory factor, lysosome, toxicant, and some unknown molecule increases drastically, which may cause autophagy. The autophagy of spinal cord is mainly reflected by

degeneration, necrosis and the formation of cavitation. The process may last for seven to nine days [28, 29].

In this study, we found that the form of the spinal cord injury and substantial nervous tissues were incomplete in the one-day group, three-day group and seven-day group. And there were substantial foam cells, inflammatory cell infiltration and obvious cavities in tissues, the neuron was irregular in arrangement, the nissl bodies of the nerve cell and the nerve fiber decreased, and distributed unevenly. The myelin sheath was chaotic in arrangement, and the myelin sheath interval enlarged. In fourteen-day and twenty-eight-day group, the cells were arranged regularly. Cell nucleus and nucleolus can only been seen in few neurons, and few nissl bodies can be seen and distributed unevenly. These results were in accordance with other report in literatures, which can prove that there may be secondary injuries within several minutes or several days. With the increase of injury days, there would be certain compensation repair with the mediation of the body, which can alleviate the expression of secondary injury.

Apoptosis plays a vital role in the secondary injury of spinal cord injury, while there are many factors resulting in the apoptosis. The main mechanism mainly includes the combined effect of imbalance in gene control, the substantial generation of apoptotic related enzyme, nitric oxide, excitatory amino acid, inflammatory factor, etc. In recent years, there were a lot of studies on the apoptosis mechanism after spinal cord injury, their results proved that apoptosis was the main approach of secondary spinal cord injury. The recent studies had shown that PI3K/Akt signal pathway was the main survival-promoting signal, and its activation was crucial in protecting the nerve cell against the ischemia and anoxia neuron damage [30-34]. PI3K/Akt signal pathway also is the main approach for transduction the membrane receptor signal into the cell, which can play a vital role in maintaining the cell survival and restraining the apoptosis. It is crucial in restraining apoptosis and promoting proliferation of cell by influencing the activated process of effector molecules, such as apoptosis-related protein, cell period-control protein [35-38]. PI3K may be activated by extracellular signals, such as the tyrosine kinase receptors, non-tyrosine kinase receptors, insulin receptor, and the activated PI3K is located in the cell membrane, which may further activate the series of downstream protein kinase, such as PKA, AKT and PKC, and regulate several pathophysiology process, including the proliferation, apoptosis, migration and vicious transformation. Akt is one of the significant downstream target kinase in the PI3K signal pathway. When cells are stimulated by the extracellular signal, activated PI3K may produce PIP3, which can interact with the PH structure of Akt, transform Akt to the cell membrane and activate it into p-Akt, so as to increase the cascade reaction of signal pathway for regulating the apoptosis.

In this study, the expression of PI3K, Akt and p-Akt at different points after the spinal cord injury was detected by immunohistochemistry and Western-blot. From our results, we found that the expression of PI3K, Akt and p-Akt reached to the peak one day after the spinal cord injury, and then it decreased gradually with the time passing by, but the absolute expression was certainly higher than the normal group. These results were in conformity with the concept 'window stage' proposed by Dobkin [39], namely, there was a 'window stage' after the spinal cord injury. Within seven to fourteen days after the spinal cord injury, it is a critical period for repairing the defense mechanism, and it may receive better effect if ectogenic intervention treatment is given within this period, for instance, stem cell transplantation, drug therapy, etc. These treatments can not only avoid the unfavorable environment of acute injury period, but also can create good micro-environment for the tissue repair, regeneration of stem cells and drug effect, then prevent the formation of glial scar, and promote the recovery of function.

Acknowledgements

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Disclosure of conflict of interest

None.

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