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Original Article

The role of PI3K/Akt/eNOS pathway in spinal cord injury and the recovery of motor function

Longqiang Wang[1*], Haiwei Sun[2*], Song Fu[1], Shize Shao[1], Haitao Hou[1], Xiaolan Liu[2]

[1]The Department of Spine and Spinal Cord, [2]The Department of Limb Traumatology, Shandong Wendeng Orthopaedic Hospital, Wendeng 264400, Shandong, China. [*]Equal contributors and co-first authors.

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Abstract: Objective: To investigate the expression of phosphatidylinositol 3-kinase/protein kinase B/endothelial nitric oxide synthase (PI3K/Akt/eNOS) pathway in patients with spinal cord injury and its correlation with motor function. Methods: A total of 78 patients with spinal cord injury admitted to our hospital during May 2018 - December 2019 were enrolled as the observation group (OG); 61 healthy persons examined in our hospital in the same period were selected as the control group (CG). All patients in the OG were treated with hyperbaric oxygen combined with drugs for 30 days. Motor function was assessed in both groups using the Basso Beattie Bresnahan (BBB) scale. Real-time PCR was used to determine the expression of genes related to the PI3K/Akt/eNOS pathway in both groups. PI3K, Akt, and eNOS proteins in both groups were determined by Western Blot. SPSS Pearson software for correlation analysis was used to measure the correlation of the PI3K/Akt/eNOS pathway with motor function in patients with spinal cord injury. Results: BBB scores for limb movement, gait, coordination function and fine motor skills in the OG were lower than those in the CG ($P < 0.05$). After 30 d of treatment, these scores in OG increased compared with those before treatment ($P < 0.05$). In terms of PI3K, Akt, and/or eNOS, the respective mRNA and proteins in patients in the OG were higher than those in the CG ($P < 0.05$). SPSS Pearson correlation analysis showed negative correlations between the mRNA and proteins of PI3K, Akt, and/or eNOS and the BBB scores in patients with spinal cord injury ($P < 0.05$). Conclusion: The PI3K/Akt/eNOS pathway is abnormally expressed in patients with spinal cord injury, and is related to motor function. Determination of PI3K/Akt/eNOS pathway regulation helps to assess the prognosis and clinical treatment of patients.

Keywords: Phosphatidylinositol 3-kinase/protein kinase B/endothelial nitric oxide synthase, spinal cord injury, motor function, correlation

Introduction

Spinal cord injury is the most serious complication in spinal injury that causes limb dysfunction below the injured section of spinal cord, it impacts the physical and mental health of patients, and also causes social and economic burdens [1]. Surveys showed [2, 3] the incidence of spinal cord injury is 10-40 cases per million in the world, and it is also common in China. Spinal cord injury is often followed by neuropathological changes including primary and secondary injury. Primary injury is irreversible and permanent [4, 5], while secondary injury is reversible because it is caused by the cascade originating from the primary injury and leads to progressive, destruction of tissues [6]. Previous studies indicated that [7, 8] spinal cord injury causes microvascular injuries and hemorrhaging, leading to progressive edema, ischemia and electrolyte imbalance in tissues; bringing about nerve cell death, excessive inflammatory responses, and dysfunction of body movement. PI3K/Akt serves as a regulatory signal upstream in endothelial nitric oxide synthase (eNOS). Akt, a downstream member in PI3K, constitutes the PI3K/Ak signaling pathway to regulate cellular activities. However, its relations with spinal cord injury and motor function are less studied [9, 10]. In this study, the expression of the PI3K/Akt/eNOS pathway and its correlation with motor function in patients with spinal cord injury and healthy subjects were reported below.

Table 1. Primer design

Primer Type	Primer	Length
B-actin	F: 5 'CAGGATGCAGGTGGAAGC 3' R: 5 'TGCTCCAGGCTGTAGTCTGTGG 3'	132
PI3K	F: 5 'AGGTTCCTCTCCTAGCAGATCATTCTC 3' R: 5 'GAGCGGCAACTTCTGAGGTCTTAC 3'	99
Akt	F: 5 'GCTGAGTCCTCTTGCTGTGCTC 3' R: 5 'GTACCTGGAGGCTTGGCATGAC 3'	158
ENOS	F: 5 'TCTGCCCAGAGTCCAGCTGCTG R: 5 'CTGCCCAGGGTCCAGCTGCT	136

Material and methods

Clinical data

A total of 78 patients with spinal cord injury admitted to our hospital during May 2018 - December 2019 were enrolled as the OG. There were 40 males and 38 females. The age was recorded ranged from 22-65 years old, averaged (40.59 ± 5.51) years. The time spent from injury to operation ranged from 1-8 d, and averaged (4.59 ± 0.71) d. Causes of injury: 32 cases of car accident, 31 cases of falling accidents, 8 cases injured by heavy object, and 7 of falling off a ride. Inclusion criteria (1) All patients met the diagnostic criteria for spinal cord injury and were confirmed by imaging examination [11, 12]. (2) All patients had pain and inconvenience of limb movement. (3) All were able to complete the assessment of motor function and PI3K/Akt/eNOS pathway assessment. In addition, 61 healthy subjects, 35 males and 26 females, aged from 21-67 years with an average of (41.22 ± 5.55), were selected as the control group. Exclusion criteria: (1) Patients accompanied by mental disorders, blood system diseases or malignant tumors; or (2) Combined with autoimmune system diseases, other diseases or during pregnancy or lactation were excluded. This study was approved by the Ethics Committee of our hospital. The research subjects and their families were informed and they signed a fully-informed consent form.

Treatment

Patients in the OG were treated with hyperbaric oxygenation combined with drugs. After admission, hormone pellet therapy, dehydration, antioxidants, calcium channel antagonists were routinely given together with pasty drugs to reinforce the acid-base and water-electrolyte balance, etc. The patients were managed with hyperbaric oxygenation therapy, that is, 0.2 MPahyperbaric oxygen inhalation for 60 min, once a day for 30 d.

Outcome measures

(1) Blood collection. Five mL of peripheral venous blood was collected from the OG on the morning of the next day after admission, and also in the CG on the day the patients received physical examination. The collected samples were centrifuged at 3500 rpm for 40 min before the serum was separated and saved in a freezer at -80°C. (2) Detection RNA extraction. Five hundred uL of Trizol was mixed with 1.0 mL of the saved serum in a centrifuge tube and oscillated for 5 min before resting. In both groups of serum samples, Chloroform 200 uL was added followed by vibration for 15 s, rest for 5 min and centrifugation at 1216 (centrifugal force) for 15 min. The supernatant was collected. It was mixed with 1 mL of precooled ethanol (75.0%), and then dried at room temperature for 7 min. Then the Ultraviolet spectrophotometer A260 was used to determine the absorbance in accordance with the kit supplied by Affinity, USA (Lot No: AF3232). The PI3K/Akt/eNOS pathway was measured by real-time fluorescent PCR (model TC-96/T/H (a), Hangzhou Borui Co., Ltd.). PCR parameters: 10 min, 30°C; 30 min, 42°C; 5 min, 99°C; 5 min, 5°C, 35 cycles, 10 min, 72°C. The resulting product was used for 1.5% agarose gel electrophoresis with β-actin as the internal control. See primers in **Table 1** [13, 14]. (3) PI3K, Akt, & eNOS proteins. PI3K, Akt, and eNOS protein levels were determined in both groups by Western Blot. (4) Assessment of motor function. It was done using the Basso Beattie Bresnahan (BBB) scale covering limb movement, gait, coordination function and fine motor skills. The total score was 21 where a higher score indicated better motor function [15, 16]. (5) Correlation analysis. SPSS Pearson was used to find the correlation of PI3K/Akt/eNOS pathway with the motor function in patients with spinal cord injury.

Statistical analysis

SPSS 18.0 software was used for statistical analysis. Enumeration data was subject to χ^2 Test and expressed by n (%). Measurement

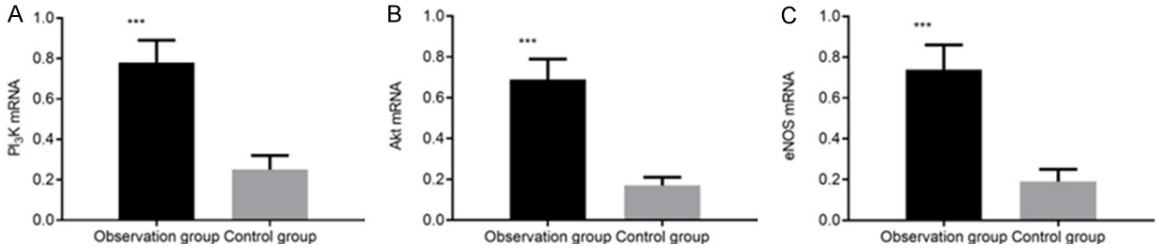

Figure 1. Expression of genes related to PI3K/Akt/eNOS pathway in the two groups. Note: Compared with CG, ***P < 0.001.

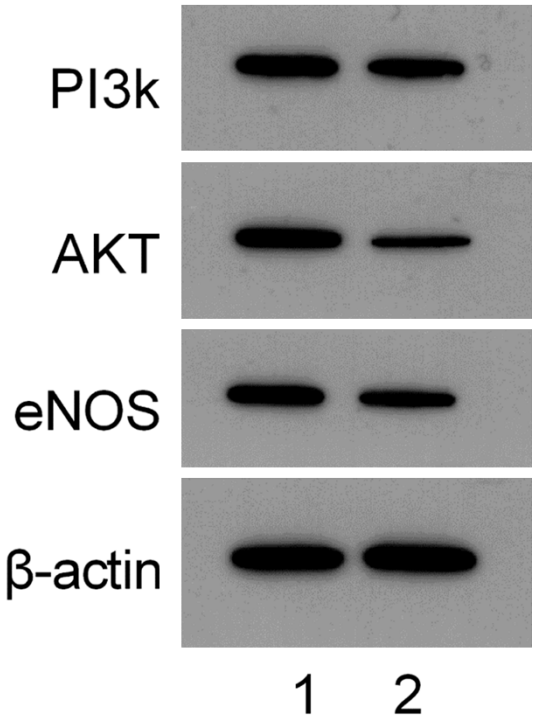

Figure 2. Proteins in the PI3K/Akt/eNOS pathway in the two groups. Note: 1 from the OG; 2 from the CG.

data was inspected by *t* test in ($\overline{X} \pm s$). $P < 0.05$ indicated statistical significance.

Results

Expression of genes related to PI3K/Akt/eNOS pathway in the two groups

The OG reported higher levels of mRNA expressed by PI3K, Akt, and eNOS in the PI3K/Akt/eNOS pathway than the CG (P < 0.05), suggesting elevated expression of PI3K/Akt/eNOS pathway in patients with spinal cord injury. See **Figure 1**.

PI3K, Akt, and eNOS proteins in the two groups

Western Blot showed that the proteins of PI3K, Akt, and eNOS in the OG were higher than those in the CG (P < 0.05), as shown in **Figure 2**.

Assessment of motor function in the two groups

BBB scores of patients in the OG were lower than those in the CG (P < 0.05), as shown in **Figure 3**.

Motor function of patients in the OG before and after treatment

After 30 days of treatment, BBB scores of patients in the OG increased as compared with those before treatment (P < 0.05), as shown in **Figure 4**.

Correlation of PI3K/Akt/eNOS pathway with motor function in patients with spinal cord injury

SPSS Pearson showed a negative correlation between mRNA of PI3K, Akt and eNOS and BBB scores of patients with spinal cord injury (P < 0.05, **Table 2**).

Discussion

Spinal cord injury is considered to be a serious central nerve injury, and most develop impairment of central nervous function caused by primary or secondary injury of the spinal cord due to external mechanical forces [17]. In recent years, the development of the construction and transportation industry in China played a role in the increasing occurrence of spinal cord injury that affects the health and life of patients [1]. Foreign scholars have found that [18] spinal

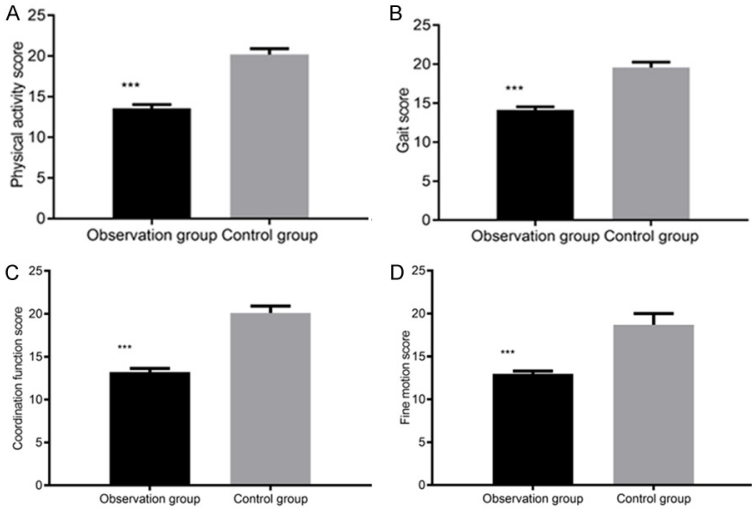

Figure 3. Assessment of motor function in the two groups. Note: Compared with CG, *P < 0.001.

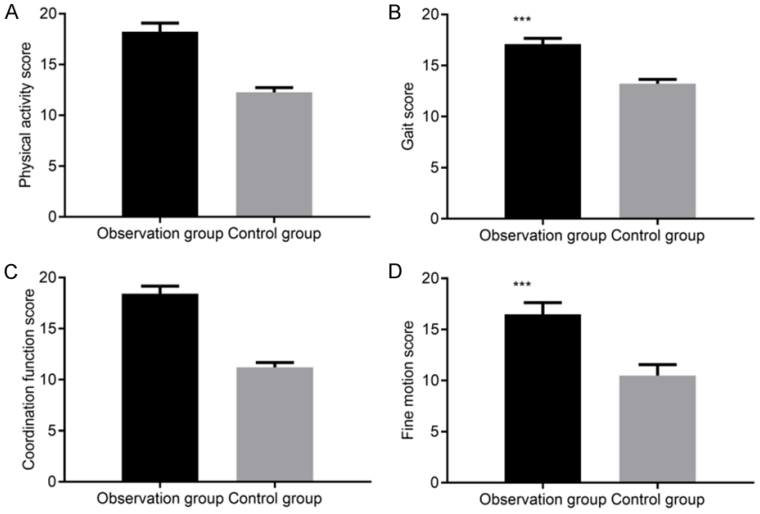

Figure 4. Motor functions of patients in OG before and after treatment. Note: Compared with CG, *P < 0.001.

Table 2. Correlation analysis of PI3K/Akt/eNOS pathway with motor function in patients with spinal cord injury (r, P)

Correlation	Limb movement	Gait	Coordination function	Fine motor skills
PI3K	-0.769 (0.000)	-0.633 (0.000)	-0.678 (0.000)	-0.651 (0.000)
Akt	-0.712 (0.000)	-0.711 (0.000)	-0.753 (0.000)	-0.713 (0.000)
ENOS	-0.678 (0.000)	-0.782 (0.000)	-0.631 (0.000)	-0.672 (0.000)

cord injury is the most common and serious complication in spinal injury that causes loss of motor and sensory function below the injured section of spinal cord, with the clinical manifestations of qua-driplegia and paraplegia and even more disability.

Clinical studies indicated [19] NO plays an important physiological role as a gaseous signaling molecule in the human body. Previous studies have shown that [20] NO regulates the activity of iron protein-related bio-enzymes and serves as the NF-kB signal in activated peripheral blood mononuclear cells. Hence, NO is an effective vasodilator that regulates vascular tension. Continuous stress responses in patients with spinal cord injury promote the release of NO which is directly involved in the occurrence and development of the disease [21]. NO is mainly catalyzed by NOS and is widely distributed in the human body to form eNOS, iNOS, and nNOS. Phosphorylation sites at Ser177 regulate eNOS activity, and its expression is regulated by a variety of kinases, including: Akt, PKA, etc. though [22]. PI3K/Akt is a regulatory pathway in eNOS where activated PI3K phosphorylates lipids on cell membranes to release the second messenger Phosp-hatidylinositol trisphosphate (PIP3), and activates Akt by recruiting it to the cell membrane through interacting with the anchored point of phosphatidylinositol [23]. PI3K, on the other hand, activates lipositol on phosphorylated inositol rings to release lipids and binds to intracellular proteins to regulate signal transduction and subcellular fraction localization. The primary structures, mode of regulation or substrate specificity of lipid kinases plays a role in signal transduction. Clinical studies indicated [24]: that activated Akt mediates downstream signaling and constitutes the PI3K/Akt pathway. Akt is the only enzyme regulating eNOS by activating its locus to promote NO synthesis, also regulates downstream signaling to constitute the PI3K/Akt/eNOS pathway. Spinal

cord injury is complex and often develops into abnormal motor functions affecting the health and life of patients. In this study, the BBB score of patients with spinal cord injury in the OG were inferior to those in the CG ($P < 0.05$); and they increased after 30 days of treatment ($P < 0.05$), indicating that patients with spinal cord injury often suffer from abnormal motor function, and therapeutic intervention is helpful for the recovery of limb function. In order to find the expression of PI3K/Akt/eNOS pathway in spinal cord injury and its relationship with motor function recovery, we performed SPSS Pearson correlation analysis in this study, which showed that the expression of PI3K, Akt, and eNOS mRNA in patients with spinal cord injury were negatively correlated with their BBB scores for limb movement, gait, coordination function and fine motor skills ($P < 0.05$). There is a close relationship between PI3K/Akt/eNOS pathway and motor function in patients with spinal cord injury. Therefore, for patients with spinal cord injury, more attention needs to be paid to the PI3K/Akt/eNOS pathway so as to predict the recovery of motor function as well as according to it adjust the treatment plan for earlier and better recovery, which is quite important.

In summary, the PI3K/Akt/eNOS pathway is abnormally expressed in patients with spinal cord injury and related to motor function. Determination of the PI3K/Akt/eNOS pathway activation helps predict the prognosis and therefore the necessary clinical treatment.

However, there are also some limitations in this study. For example, with a small number of cases and short observation time, we were not able to observe the changes of the PI3K/Akt/eNOS pathway after the treatment of spinal cord injury in people of different ages and genders. At the same time, the specific mechanism of the pathway was not discussed. In the next study, the number of cases and observation time will be expanded to further explore the specific mechanism of the PI3K/Akt/eNOS pathway in spinal cord injury.

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Disclosure of conflict of interest

None.

Address correspondence to: Xiaolan Liu, Three Department of Limb Traumatology, Shandong Wendeng Orthopaedic Hospital, No. 1, Fengshan Road, Wendeng 264400, Shandong, China. Tel: +86-0631-8480996; E-mail: jahrir@163.com

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