



The role of the PI3K/AKT signalling pathway in bFGF/PDGF composite hydrogel promoting the repair of spinal cord injuries

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OBJECTIVE: *This study aimed to explore the role of the PI3K/AKT signaling pathway in bFGF/PDGF composite hydrogel promoting the repair of spinal cord injuries.*

METHODS: *In this study, the spinal cord injury rat model was established using Allen's punch method. Healthy male Sprague Dawley rats of the clean grade were randomly divided into four groups (n=18, each): sham operation group (group S), bFGF/PDGF composite hydrogel group (group A), bFGF/PDGF composite hydrogel + LY294002 (PI3K/AKT signaling pathway inhibitor) group (group B) and bFGF/PDGF composite hydrogel + IGF-1 (PI3K/AKT signaling pathway agonist) group (group C). After the operation, the motor function of the posterior limbs, the apoptosis of the spinal cord cells and the expression of PI3K, Akt and phosphorylated Akt (p-Akt) in the spinal cord tissues of the rats in each group were detected.*

RESULTS: *BBB joint score were significantly higher (P<0.05).*

CONCLUSION: *bFGF/PDGF composite hydrogel can significantly promote the repair of spinal cord injuries and the mechanism is closely correlated to the activation of the PI3K/AKT signaling pathway.*

KEY WORDS: BFGF, Cell apoptosis, PDGF, PI3K/Akt signaling pathway, Spinal cord injury

Introduction

Spinal cord injuries (SCI) are some of the most serious injuries in the nervous system. They can not only cause disabilities such as paraplegia and quadriplegia, serious-

ly affecting the quality of life of the patient, but can also impose heavy burdens on families and society^{1,2}. Therefore, promoting the repair of SCI has important clinical significance. The results of previous studies have revealed that vascular endothelial growth factor (VEGF)³, epidermal growth factor (EGF)⁴, nerve growth factor (NGF)⁵, brain-derived neurotrophic factor (BDNF)⁶, neurotrophic factors 3 (NT-3)⁷ and other growth factors can repair SCI by promoting cell proliferation, differentiation, anti-apoptosis and functionalization.

However, the role and mechanism of basic fibroblast growth factor (bFGF)/platelet derived growth factor (PDGF) composite hydrogel in promoting the repair of SCI remains unclear. Therefore, the aim of this study was to explore the role of the PI3K/AKT signaling pathway in bFGF/PDGF composite hydrogel promoting SCI repair through animal experimentation.

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Materials and methods

EXPERIMENTAL ANIMALS AND GROUPING

Seventy-two healthy male Sprague Dawley (SD) rats of 8-10 weeks old and weighing 250-300g were selected for this study. All experimental animals were provided by the Experimental Animal Center of Nanchang University. In this study, these rats were randomly divided into four groups (n=18, each) using the random number table method: sham operation group (group S), bFGF/PDGF composite hydrogel group (group A), bFGF/PDGF composite hydrogel + LY294002 (PI3K/AKT signaling pathway inhibitor) group (group B) and bFGF/PDGF composite hydrogel + IGF-1 (PI3K/AKT signaling pathway agonist) group (group C). The Ethics Committee of our hospital has approved this study.

ANIMAL MODEL PREPARATION

In this study, the SCI model of rats was established using the modified Allen punch method⁸. Before they were operated on, all the experimental animals underwent fasting for 12 hours and water deprivation for four hours, then were intraperitoneally injected with 3 ml/kg of 10% chloral hydrate for anesthesia. The rats were fixed in the Trendelenburg position, the heads fixed with a stereotaximeter, and the backs shaved. The operation area was routinely sterilized three times with iodine complex and covered with aseptic drape. With T8 spinous process as the center, a dorsal median incision with a length of approximately 3 cm was made. The skin and subcutaneous tissue were cut off in turn and the paravertebral muscles attaching to the spinous process and lamina were separated to expose the T7-T9 spinous processes and laminae. T8 and partial T7 and T9 spinous processes and laminae were removed with a rongeur to expose this segment of dural sac, forming a punch area with a size of approximately 4 mm × 3 mm. The wound was washed with adrenaline gentamicin saline. The self-designed Allen punching frame was placed and the punching tube was aligned with the punching center so that it was vertical and close to the spinal dura mater. A punching rod weighing 5g with a head 2.5 mm in diameter fell freely from the height of 5.0 cm. After punching, it was observed that the rat tail showed spastic swing and the lower limbs and the body retracted and fluttered, then completely relaxed. Local spinal cord swelling and hematoma could be seen. This was an indication that the punching was successful. Rats in groups A, B and C were intraspinally injected with 1 μ l of bFGF/PDGF composite hydrogel, bFGF/PDGF composite hydrogel + LY294002, bFGF/PDGF composite hydrogel + IGF-1 into the injured spinal cord segment (T8 plane) respectively. Then the wound was sutured in

the order of muscle-fascia-skin. After the operation, rats were injected intraperitoneally with penicillin and assisted to urinate in the morning and evening until they resumed reflex urination. For rats in group S, only the corresponding laminae were removed, but the segments of spinal cord were not punched and the other operations were the same.

ASSESSMENT OF THE MOTOR FUNCTION OF RATS

Six rats were randomly selected from each group on post-operative days 1 (T1), 7 (T2) and 14 (T3). BBB joint score and the inclined plate test score were used to evaluate the motor function of the posterior limbs of the rats based on the method in literature^{9,10}. BBB joint scoring method: its contents include the range of motion of each joint of the posterior limb, gait and coordination function of the posterior limb, fine movement of the claw during movement, with a full score of 21 points. Inclined plate test method: The rat was placed on a special inclined plate with shallow grooves on the test surface, then the ability of the rat to maintain posture, the ability to grasp and the maximum angle at which the rat could stay on the inclined plate for at least five seconds were recorded and defined as the functional values. The inclined plate test score of normal rats is 75. Each rat was measured three times, and its mean value was finally adopted.

DETECTION OF EXPRESSION LEVELS OF PI3K, AKT AND P-AKT IN SPINAL CORD CELLS BY WESTERN BLOT

At the end of the assessment of the motor function of rats, T8 spinal cord was taken under anesthesia, and the spinal cord specimen was placed at -80°C for reserve. Fresh frozen T8 spinal cord were added to the lysate, homogenated, then underwent full lysis for 30 minutes, centrifuged at 12,500 rpm in a tube with a radius of 12.28 cm, centrifuged for 15 minutes, then supernatant was obtained, added with lysis buffer, and boiled at 100°C for five minutes. Spinal cord tissue protein was extracted. Forty μ g of protein was taken, subjected to 12% polyacrylamide gel electrophoresis, then transferred on PVDF membrane, blocked for nonspecific antigen with 5% dried skimmed mil sealing solution for two hours, mixed with 1:1000 diluted anti-PI3K antibody, rabbit anti-mouse Akt antibody and rabbit anti-mouse p-Akt antibody and 1:10000 diluted GAPDH antibody, respectively, and incubated at 4°C overnight. The membrane was washed with tris buffered saline (TBS) containing 0.1% Tween 20, then mixed with horseradish peroxidase-labeled secondary antibody and incubated at 37°C for one hour. The membrane was washed then colored with diaminobenzidine (DAB). IPP6.0 software was used to analyze the band results. The ratio of the

gray value of the target protein band to the gray value of the GAPDH band was used to represent the expression level of the target protein. Each experiment was repeated three times, and an average value was obtained.

DETECTION OF APOPTOSIS OF SPINAL CORD CELLS BY TUNEL ASSAY

Paraffin sections were made from fresh frozen T8 spinal cord. Paraffin sections were dewaxed and rehydrated, then the apoptosis of spinal cord cells was detected by an in-situ cell apoptosis detection kit (Promega, USA). Specific operation steps were performed according to the instructions of the kit. Under a light microscope, the apoptotic nuclei were tan, and the non-apoptotic nuclei were blue purple. At least 500 cells were observed in each section, the number of apoptotic cells per 100 cells was calculated and expressed as a percentage (%), which is the apoptosis index (AI).

STATISTICAL ANALYSIS

In this study, data were analyzed using statistical software SPSS20.0. Measurement data were expressed as mean ± standard deviation ($\bar{x} \pm SD$). Count data were expressed as percentage (%). The test of normality was conducted using a W-test. The homogeneity of variance was tested using an F-test. Multi-group comparison was conducted using univariate analysis of variance. The back testing was conducted using the least significant difference (LSD). Non-normally distributed means of multiple samples or normally distributed means of multiple samples with heterogeneity of variance were compared using a nonparametric test. Counting data were compared using a Chi-square test. $P < 0.05$ was considered statistically significant.

TABLE I - Comparison of inclined plate test score and BBB joint score of rats of each group at different time points after operation (n=18, points, ±s)

Group	T1	T2	T3
BBB joint score			
S	17.3±1.2	18.2±0.8	18.7±1.1
A	4.5±0.6*	7.8±1.0*	10.6±1.3*
B	2.7±0.3*#	4.6±0.8*#	7.2±1.4*#
C	7.3±1.2*#	11.5±1.8*#	15.7±1.5*#
Inclined plate test score			
S	71.6±2.8	70.5±3.1	72.5±3.0
A	37.4±2.1*	45.5±2.4*	50.3±2.8*
B	29.6±1.8*#	34.9±2.1*#	42.4±2.5*#
C	48.3±2.5*#	55.1±3.0*#	61.8±3.4*#

Note: Compared with group S, * $P < 0.05$; compared with group A, # $P < 0.05$

Results

POSTOPERATIVE MOTOR FUNCTION OF POSTERIOR LIMBS OF RATS IN EACH GROUP

Compared with group S, the inclined plate test score and BBB joint score at T1, T2 and T3 in groups A, B and C were significantly lower ($P < 0.05$). Compared with group A, the inclined plate test score and BBB joint score at T1, T2 and T3 in group B were significantly higher, and the inclined plate test score and BBB joint score at T1, T2 and T3 in group C were significantly higher ($P < 0.05$, Table I).

EXPRESSION OF PI3K AND P-AKT IN RATS IN EACH GROUP

The difference in Akt expression among the four groups at each time point was not statistically significant. Compared with group S, the expressions of PI3K and p-Akt in spinal cord cells of rats at T1, T2 and T3 in groups A, B and C were significantly lower ($P < 0.05$). Compared with group A, the expressions of PI3K and p-Akt in spinal cord cells of rats at T1, T2 and T3 in group B were significantly lower, and the expressions of PI3K and p-Akt in spinal cord cells of rats at T1, T2 and T3 in group C were significantly higher ($P < 0.05$, Table II).

AI IN RATS OF EACH GROUP AT T1, T2 AND T3

Compared with group S, AI of rats in groups A, B and C at T1, T2 and T3 was significantly higher ($P < 0.05$);

Table II - Expression levels of PI3K and p-Akt in rats of each group (n=6, ±s)

Group	T1	T2	T3
PI3K			
S	2.18±0.13	2.22±0.15	2.27±0.12
A	1.53±0.09*	1.62±0.11*	1.58±0.10*
B	1.03±0.05*#	1.10±0.07*#	1.04±0.05*#
C	1.87±0.13*#	1.95±0.16*#	1.91±0.12*#
Akt			
S	1.12±0.08	1.06±0.05*	1.15±0.06
A	1.07±0.06	1.11±0.04	1.17±0.08
B	1.19±0.05	1.13±0.07	1.09±0.06
C	1.20±0.04	1.16±0.05	1.14±0.05
p-Akt			
S	2.54±0.17	2.68±0.21	2.57±0.18
A	1.64±0.11*	1.72±0.14*	1.67±0.12*
B	1.11±0.09*#	1.08±0.11*#	1.13±0.10*#
C	1.94±0.15*#	2.01±0.13*#	1.97±0.15*#

Note: Compared with group S, * $P < 0.05$; compared with group A, # $P < 0.05$

TABLE III - Comparison of AI in four groups of rats at different time points (n=18, %, ±s)

Group	T1	T2	T3
S	3.5±0.6	3.2±0.5	3.3±0.5
A	24.6±3.1*	32.0±3.5*	18.6±2.6*
B	33.2±5.3*#	41.2±4.7*#	29.7±3.8*#
C	15.7±2.4*#	23.5±2.9*#	11.4±1.7*#

Note: Compared with group S, *P<0.05; compared with group A, #P<0.05

compared with group A, AI of rats in group B at T1, T2 and T3 was significantly higher. AI of rats in group C at T1, T2 and T3 was significantly lower (P<0.05, Table III).

Discussion

The results of this study revealed that, compared with group S, the expression levels of PI3K and p-Akt in groups A, B and C were significantly lower, AI was significantly higher, and the inclined plate test score and BBB joint score were significantly lower; compared with group A, the expression levels of PI3K and p-Akt in group B were significantly lower, AI was significantly higher, and the inclined plate test score and BBB joint score were significantly lower, while the expression levels of PI3K and p-Akt in group C were significantly higher, AI was significantly lower, and the inclined plate test score and BBB joint score were significantly higher.

The pathophysiological processes of SCI include primary and secondary injuries. Primary injuries are usually irreversible mechanical injuries to the spinal cord tissues, and secondary injuries are complex patterns of pathophysiological dynamic damage, which can further aggravate the primary injuries. Studies have confirmed that apoptosis induces the expansion of spinal cord and the loss of motor and sensory functions by inducing the death of some functional cells^{11,12}. Therefore, in this study, it is of great clinical significance to explore specific molecular pathways that mediate apoptosis and to design effective therapeutic approaches to promote SCI recovery.

The PI3K/AKT signaling pathway is considered to be the most important survival pathway in cells which can regulate the process of apoptosis and inhibit apoptosis. Insulin-like growth factor 1 (IGF-1) is a multifunctional cytokine. Its role in promoting cell proliferation and inhibiting cell apoptosis is realized mainly through activating the PI3K/AKT signaling pathway^{13,14}. LY294002 can specifically block the phosphorylation of PI3K/AKT, accordingly inhibit the activation of this pathway^{15,16}. In this study, the SCI rat model was established by the classical modified Allen punch method⁸. After punch-

ing, it could be observed that the rat tail showed spastic swing, the lower limbs and the body retracted and fluttered, then completely relaxed, and local spinal cord swelling and hematoma could be seen. The model is simple in its operation, does not cause complete paraplegia of rats and at present it is the main method to simulate SCI. The BBB joint score and the inclined plate test score are classical methods for evaluating the motor function of the posterior limbs of rats, which can reflect the recovery of SCI^{17,18}.

The results of this study showed that, compared with group S, the expression levels of PI3K and p-Akt in groups A, B and C were significantly lower, AI was significantly higher, and the inclined plate test score and BBB joint score were significantly lower. The result suggests that after SCI, the expressions of PI3K and p-Akt in spinal cord cells are significantly lower, apoptosis is higher, and motor ability is lower.

The results of this study also revealed that, compared with group A, the expression levels of PI3K and p-Akt in group B were significantly lower, AI was significantly higher, and the inclined plate test score and BBB joint score were significantly lower, while the expression levels of PI3K and p-Akt in group C were significantly higher, AI was significantly lower, and the inclined plate test score and BBB joint score were significantly higher. The result suggests that the inhibition of the PI3K/AKT signaling pathway can significantly reduce PI3K expression in spinal cord cells, decrease Akt phosphorylation and increase apoptosis, weakening the motor ability of rats with SCI. Contrarily, activation of the PI3K/AKT signaling pathway can significantly increase PI3K expression in spinal cord cells, increase Akt phosphorylation and decrease apoptosis, enhancing the motor ability of rats with SCI. This reveals that bFGF/PDGF composite hydrogel promoting the repair of SCI is associated with activating the PI3K/AKT signaling pathway and promoting the expression of Akt phosphorylation.

A previous study revealed that bFGF is a member of the fibroblast growth factor family and highly expressed in the nervous system, can regulate a variety of biological functions through complex signal transduction systems including proliferation, morphogenesis and the inhibition of cell apoptosis¹⁹. PDGF is a member of the transforming factor D superfamily in the neurotrophic factor family. It is a growth regulator with many functions. It can inhibit the expression of c-fos after SCI, and it can accordingly reduce apoptosis and avoid secondary injury⁸. Moreover, the bFGF/PDGF combination has a strong synergistic effect in promoting nerve cell repair and angiogenesis²⁰. These are consistent with the results of this study.

This study still has the following limitations. Firstly, in this study, the expression levels of PI3K and Akt were examined only by Western blot; immunohistochemical and reverse transcription polymerase chain reaction (RT-PCR) methods were not used. Secondly, in this study,

the apoptosis of spinal cord cells was detected by TUNEL assay, the expression levels of apoptosis-related proteins were not detected, it still needs to be further improved in future experiments. Finally, this study lacks the relevant data such as pathological images of spinal cord tissue. Further in-depth research is needed.

Conclusion

bFGF/PDGF composite hydrogel can inhibit apoptosis, improve the motor ability of rats with SCI and promote the repair of SCI. The mechanism may be closely correlated to the activation of the PI3K/AKT signaling pathway and promoting the expression of p-Akt. This conclusion still needs further research for verification.

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Riassunto

Questo studio è finalizzato all'esplorazione del ruolo della via di segnalazione PI3K / AKT nell'idrogel composito bFGF / PDGF che promuove la riparazione delle lesioni del midollo spinale.

Per questo studio è stato utilizzato il modello di lesione del midollo spinale sul ratto utilizzando il metodo del punzone di Allen.

Sono stati utilizzati ratti maschi sani Sprague Dawley, "clean grade" suddivisi casualmente in quattro gruppi di 18 esemplari ciascuno: gruppo di intervento simulato (gruppo S); gruppo trattato con idrogel composito bFGF / PDGF (gruppo A); gruppo trattato con idrogel composito bFGF / PDGF + LY294002 (PI3K / AKT signaling pathway inhibitor) (gruppo B); e gruppo trattato con bFGF / PDGF idrogel composito + IGF-1 (PI3K / AKT signaling pathway agonist) (group C).

Dopo l'operazione sono state rilevate la funzione motoria degli arti posteriori, l'apoptosi delle cellule del midollo spinale e l'espressione di PI3K, Akt e Akt fosforilato (p-Akt) nei tessuti del midollo spinale dei ratti di ciascun gruppo.

RISULTATI: rispetto al gruppo S, i livelli di espressione di PI3K e p-Akt nei gruppi A, B e C erano significativamente più bassi, l'indice di apoptosi (AI) era significativamente più alto e il punteggio del test della piastra inclinata e il punteggio dell'articolazione BBB erano significativamente più bassi ($P < 0,05$). Rispetto al gruppo A, i livelli di espressione di PI3K e p-Akt nel gruppo B erano significativamente inferiori, AI era significativamente più alto e il punteggio del test della piastra incli-

nata e il punteggio del giunto BBB erano significativamente inferiori. I livelli di espressione di PI3K e p-Akt gruppo C erano significativamente più alti, AI era significativamente più basso e il punteggio del test della piastra inclinata e il punteggio del giunto BBB erano significativamente più alti ($P < 0,05$).

CONCLUSIONE: l'idrogel composito bFGF / PDGF può promuovere in modo significativo la riparazione delle lesioni del midollo spinale e il meccanismo è strettamente correlato all'attivazione della via di segnalazione PI3K / AKT.

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