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Original Article

Spinal ephrinBs/EphBs signaling system mediating diabetic neuropathic pain in rats through the regulation of central sensitization by MAPKs

Diyang Ling[1], Wenjie Yang[2], Youli Zheng[2], Zheyin Wang[1]

Departments of [1]Pain Medicine, [2]Anesthesiology, Shenzhen People's Hospital, Shenzhen City, Guangdong Province, China

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Abstract: Objective: To observe the effect of spinal cord level echrinBs/EphBs signaling system relied on MAPKs to regulate diabetic neuropathic pain in rats. Methods: The SD rats were treated by intraperitoneal injection of streptozocin (75 mg/kg) to establish diabetic models. Models were successful if the blood glucose levels of rats met the test standard after the diabetic models being established. One hundred and twenty rats who had successfully modeled diabetes were randomly divided into three groups: the diabetic group (Db group), the EphB1 inhibitor treatment group (Db+EphB1-FC group), and the p38MAPK inhibitor treatment group (Db+sb203580 group). Another 40 rats were taken as normal (without diabetes) and sham operated control group (Control group). At the 15 th to 21 th day after modeling success, rats in Db+EphB1-FC group were given 10 μg/10 μl of EphB1 receptor antagonist by intrathecal injection, and the EphB1-FC and EphB1-FC groups were treated by intravenous injectionof p38MAPK inhibitor sb203580 1 mg/kg, once a day. Ten rats were randomly selected to detectthe paw withdrawl threshold (PWT) and nerve conduction velocity (NCV) before intrathecal catheterization (day 0, T1), at the 14th day after intrathecal catheterization (day 14, T2) and after the drug treatment (day 22, T3). The levels of spinal cord p38MAPK phosphorylation, p-Akt and p-p100PI3K were detected at the 22th day. Results: Compared with the control group, PWT decreased and NCV slowed down in Db group, Db+EphB1-FC group and Db+sb203580 group on day 0, day 14 (all $P<0.05$). There were no significant differences in PWT and NCV between the Db group, the Db+EphB1-FC group and the Db+sb203580 group at the above three time points. Compared with the Db group, PWT improved (all $P<0.05$) and NCV accelerated (all $P<0.05$) in Db+EphB1-FC group and Db+sb203580 group at the 22th day. There was no significant difference in PWT ($P=0.12$) and NCV ($P=0.23$) between Db+EphB1-FC group and Db+sb203580 group at the 22th day. Compared with the 14th day, PWT ($P=0.032$ and $P=0.021$) improved and NCV ($P=0.025$ and $P=0.023$) speeded up in Db+EphB1-FC group and Db+sb203580 groupat the 22th day. Compared with the control group, the levels of p-p38MAPK in Db, Db+EphB1-FC and Db+sb203580 groups were elevated (all $P<0.01$), but compared with Db group, the levels of p-p38MAPK in Db+EphB1-FC and Db+sb203580 groups were significantly decreased (all $P<0.01$). At the same time, the expression levels of p-Akt and p-p100PI3K decreased in the Db+sb203580 group (all $P<0.01$). Conclusion: Diabetes can activate ephrinBs/EphBs pathway and downstream p38MAPK and AKT signals, and is involved in the formation and maintenance of diabetic neuropathic pain in rats.

Keywords: EphrinBs/EphBs, signaling system, diabetic neuropathic pain, central sensitization, MAPKs

Introduction

Diabetic neuropathic pain is one of the most common, complex and serious complications of diabetes [1]. Chronic hyperglycemia and diabetes are nervous system damages caused by a variety of pathological changes, which can damage any part of the peripheral nervous system, including autonomicnerve, motornerve, and sensory nerve [2]. Clinically, diabetic neuropathic pain extremely serious impact on patients' life quality, which is mainly manifested as limb numbness, limb cold, feeling subsided, sore and pain, spontaneous pain and hyperalgesia, and even lead to diabetic foot ulcer and limb cut off [3]. However, the signal transduction mechanism of diabetic neuropathic pain is unknown.

In recent years, the EphB1/ephrinB1 signaling pathway has been shown to play an important role in the modulation of nociceptive informa-

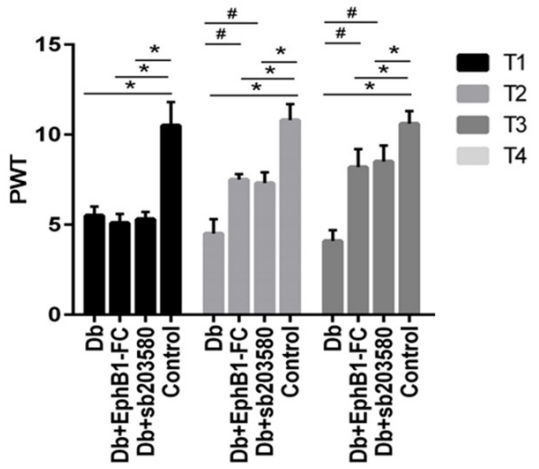

Figure 1. PWT's change of four groups of rats at different time points, vs. Control group, *P<0.05; vs. Db group, #P<0.05. (There were 10 rats respectively at different time points. T1, T2, T3: before sheath built in tube, after 14 days and 22 days of primary administration).

tion in adult rat neural tissue, especially in the spinal ganglia, small diameter neurons and lamellae I-III of spinal dorsal horn [4]. It also plays an important role in many physiological and pathological processes, such as regulating pain threshold and inflammatory reaction [5]. Other studies have confirmed that EphBs can activate MAPK pathway, and MAPK also plays an important role in diabetic neuropathic pain [6, 7]. Downstream PI3K/Akt signaling pathway was involved in various physiological processes, such as cell differentiation, mitosis and cell metabolism [8]. Recent studies have shown that PI3K/Akt signaling pathway plays an important role in capsaicin-induced thermal hyperalgesia, inflammatory pain and mechanical allodynia as well as neuropathic pain caused by nerve ligation and nerve growth factor [9, 10]. However, the mechanism of MAPK/PI3K/Akt signaling pathway involved in ephrinBs/EphBs regulation of diabetic neuropathic pain is unclear.

Materials and methods

Experimental grouping and drug treatment

Three-month-old male SD rats, weighting 180 g (purchased from Guangdong Provincial Animal Experimental Center), were prepared to build diabetes model by intraperitoneal injection of streptozotocin (75 mg/kg). Seven days after streptozotocin injection (day 0), fasting blood glucose was measured, and the blood glucose of 13.5-25 mmol/L rats as successful diabetes models were selected into the formal experiment.

A total of 120 successful diabetes models were randomly divided into three groups: diabetic group (Db group), EphB1 inhibitor treatment group (Db+EphB1-FC group) and p38MAPK inhibitor treatment group (Db+Sb203580 group). Forty rats were chosen as normal (no diabetes) and sham operation controls.

On the 8th after streptozotocin injection (as day 1), all rats were treated with intrathecal catheterization.

From day 15 to day 21, rats in Db+EphB1-FC group were intrathecally injected once a day with 10 ug/10 uL EphB1 receptor antagonist EphB1-FC, and rats in Db+sb203580 group were intravenously injected once a day with 1 mg/kg p38MAPK inhibitor sb203580. Rats in the Db group and the control group both performed intrathecal injection and intravenous injection of the same dose of saline as controls.

Before intrathecal catheterization (day 0), after intrathecal catheterization (day 14) and after drug treatement (day 22), ten rats were randomly chosen to detect the respective paw withdrawal threshold (PWT), nerve conduction velocity (NCV), phosphorylation p38MAPK (P-p38MAPK) and Akt (p-Akt) and P100 PI3K (p-p100PI3k) level.

Intrathecal catheterization methods

Intrathecal catheterization was performed according to the improved Yaksh and Rudy method. Intraperitoneal injection of phenobarbital (40 mg/kg) was performed, and after satisfactory anesthesia, SD rat limbs were fixed in the prone position, then intramuscular injection of 30,000 to 40,000 units of benzylpenicillin sodium was performed to prevent infection. After conventional disinfection of towel, an incision of 1 to 1.5 cm in the posterior occipital was made, then we separated muscle of neck and fascia layer by layer, fully exposed occipito-atlantoaxial membrane, gently made a small cut in the occipito-atlantoaxial membrane, and was able to see extravasation of clear cerebro-

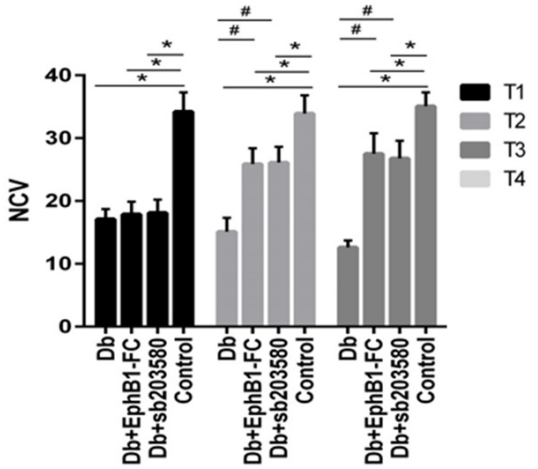

Figure 2. NCV's change of four groups of rats at different time points, vs. Control group, *P<0.05; vs. Db group, #P<0.05. (There were 10 rats respectively at different time points. T1, T2, T3: before sheath built in tube, after 14 days and 22 days of primary administration).

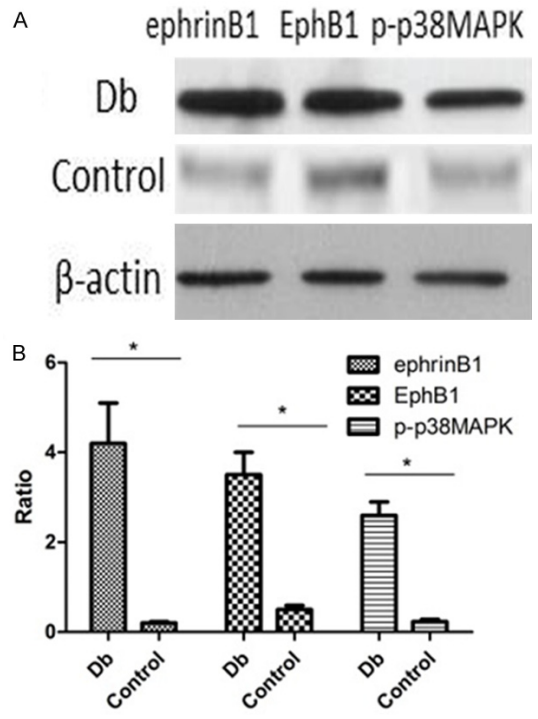

Figure 3. EphrinB1/EphB1/p-p38MAPK protein expression level of the diabetic modeling group vs. control group, *P<0.01.

spinal fluid. With the rat breathing and undulation, we adjusted the rat position to put the spine into a straight line, so that the polyethylene PE-20 catheter could be sent to 7.0-7.5 cm fixed device with the minimum angle. The muscle and skin were sutured layer by layer, and the catheter was fixed by a spin. A total of 10 μL of 2% lidocaine was used to check the catheter position after rats woke up from anesthesia and was able to move about freely. After the injection of lidocaine, if both limbs of rats paralyzed within 30 s, and recovered within 30 min, it signified that the intrathecal catheterization succeeded, and the rats could be used in the experiment. Rats with unsuccessful intrathecal catheterization was removed [11].

Determination of PWT

This experiment used standardized von Frey filaments (Ugo Basile, Italy), and the PWT of rats was measured by the up-down method [12].

Determination of NCV

Rats were injected intraperitoneally with 3% chloral hydrate at a dose of 10 mL/kg. After anesthesia, sciatic nerve in the left leg was exposed. The electrode was placed: the recording electrode was placed in the middle of gastrocnemius, and the stimulation electrode was placed in the middle of the sciatic nerve trunk. After the discharge of the active electrode, the compound action potentials conductive time was recorded, and the nerve length between the recording electrode and the stimulating electrode was measured and recorded. The calculation formula of motor NCV: NCV (m/s) = length/conductive time. NCV determining instrument: cantata electromyography machines were used (Siemens, Germany).

Test the level of p-p38MAPK, p-Akt and p-p100PI3k by means of western blot

The process is as follows: (1) sample preparation: after rat died by excessive anesthesia euthanasia, took quickly its spinal cord tissue and cut it into small pieces. Then added 150-250 μl lysate (protease and phosphatase inhibitors are included) to every 20 mg spinal cord tissue, and stirred until it had thoroughly melted. Centrifuged melted sample at the speed of 12,000 r/min and for 15 min at 4°C, took supernatant and processed quantitatively protein, then kept it in the refrigerator of -80°C; (2) protein quantification; (3) the preparation of PAGE glue; (4) sample addition and electrophoresis; (5) transfer membrane; (6) the detection

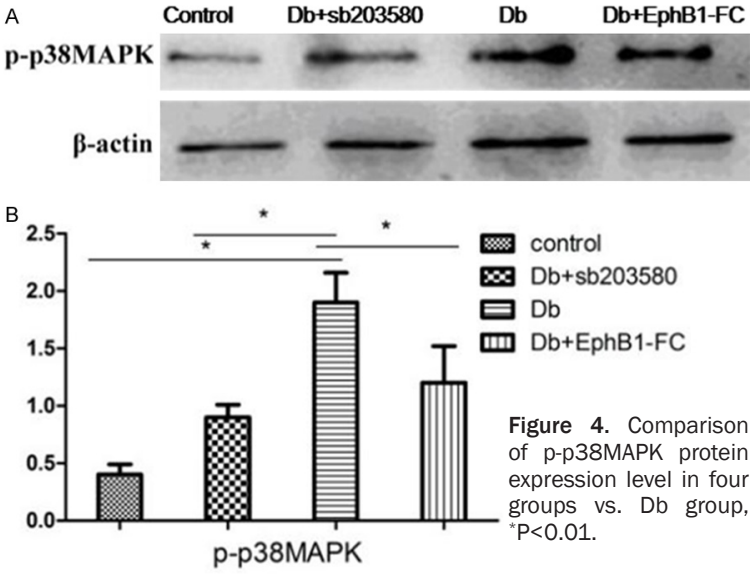

Figure 4. Comparison of p-p38MAPK protein expression level in four groups vs. Db group, *P<0.01.

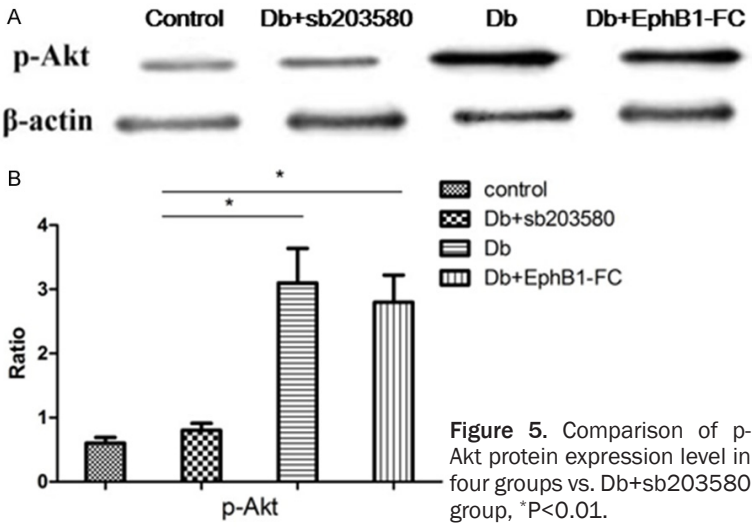

Figure 5. Comparison of p-Akt protein expression level in four groups vs. Db+sb203580 group, *P<0.01.

of membrane protein; (7) the close of membrane and antibody incubation: preserve 5% SMP (Skimmed milk powder) at room temperature for one hour. Primary antibodies (Abcam company, USA): according to directions, diluted antibodies with P-p38MAPK, p-p100PI3K 1:150, p-Akt 1:200, β-actin 1:1,000, put antibodies into confining liquid and diluted it to the required concentration. Then hatched it and membrane at room temperature for 2 hours. Secondary antibodies (Abcam Company, USA): washed membrane which hatched primary antibodies with TBST for 5 minutes by 3 times. Then according to dosage and the proportion of 1:1,000, diluted secondary antibodies marked by HRP, hatched it with membrane at the temperature of 37°C for one hour, and washed it for 5 minutes with TBST by 3 times; (8) developing; (9) ECL chemiluminescence detection. Take ??? as internal reference, express relative expression value of protein with relative gray ration.

Statistical analysis

Arranged and analyzed collected data by means of SPSS 17.0, which is a common tool in statistics. Expressed measurement data by means of mean ± sd. Tested measurement data of two independent samples by means of t-test. Compared three groups through the analysis to F by means of one-way ANOVA.

Results

The neuropathic pain situation of diabetic rats

Compared with the control group, tested PWT (paw withdrawal threshold) by means of vonfrey fiber after 4 weeks of intraperitoneal injection with STZ (streptozotocin). Results showed: the PWT of 32 molding rats decreased significantly (**Figure 1**), NCV slowed obviously (**Figure 2**), which testified that there were obvious neuropathic pain in 80% diabetic rats (both P<0.01).

The protein expression of ephrinB1/EphB1/p-p38MAPK in Db group

Compared with the control group, the protein expression level of ephrinB1/EphB1/p-p38-MAPK in Db group increased obviously (**Figure 3**).

Comparison among PWT, NCV and other relative protein

Meanwhile, compared with the control group, the PWT of Db+EphB1-FC group and Db+sb203580 group decreased too during T1-T3

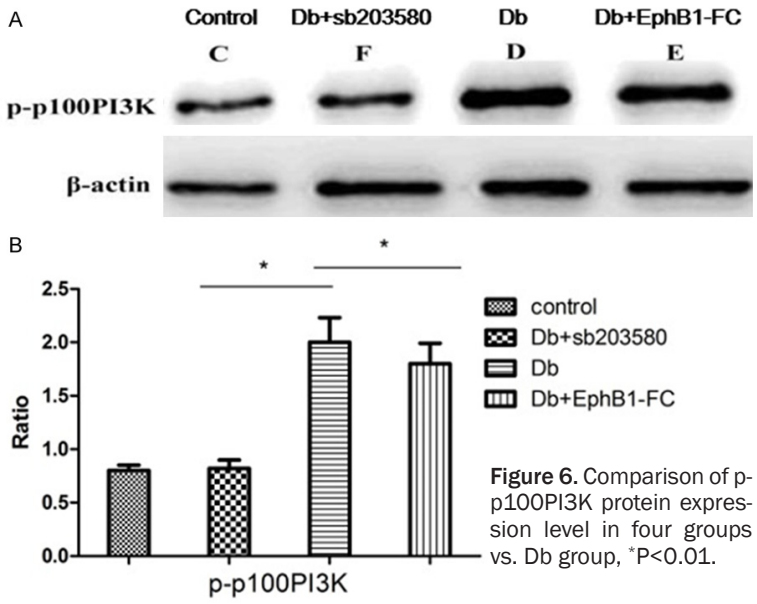

Figure 6. Comparison of p-p100PI3K protein expression level in four groups vs. Db group, *P<0.01.

(P<0.05) (**Figure 1**), so did NCV (P<0.05) (**Figure 2**), and the p-p38MAPK level in Db group, Db+EphB1-FC group, and Db+sb203508 group also increased (**Figure 4**); compared with Db group, the PWT of Db+EphB1-FC group and Db+sb203508 group increased at T2 and T3 (P<0.05) (**Figure 1**), and NCV increased (P< 0.05) (**Figure 2**). The p-p38MAPK level in Db+ EphB1-FC group and Db+sb203508 group decreased obviously (P<0.01) (**Figure 4**). Meanwhile, the expression level of p-Akt (P<0.01) (**Figure 5**) and p-p100PI3K in Db+sb203508 group decreased (P<0.01) (**Figure 6**).

Discussion

Clinically, the main symptoms of diabetic neuropathic pain are paresthesia, persistent spontaneous pain [13], and significantly decline in pain threshold [14]. Diagnosis should meet the typical symptoms of pain and be confirmed with the nerve pathology and physiological function examinations [15]. In this study, it was demonstrated that the PWT of diabetic modeling rats was apparently reduced, caused by DNP, suggesting that diabetic rats had obvious DNP phenomenon. It was similar with Sun's study [16] that diabetic rats with successful model would suffer from significant DNP.

EphrinBs/EphBs signaling system mediates peripheral pain sensitization and maintenance, and involves in modulation of nociceptive information in the spinal cord. In this study, through intrathecal injection of EphB1-Fc in rats, the receptor antagonist of EphB1, it was found that the pain threshold of these rats were higher than that of the untreated DNP group. This phenomenon indicated that ephrinBs/EphBs signaling system mediated the rats' DNP, which was consistent with Kitamura's study [17].

EphrinBs/EphBs comes into play mainly by activating p38-MAPK. The preceding experiment has shown that the formation of p38MAPK information channel is involved with a variety of neuropathic pain formation, including diabetes and neuropathic pain [18]. The present study uncovered similar results with preceding studies about the DNP caused by p38MAPK [19].

Akt/PI3K is located in the lower segment of p38MAPK pathway, which is responsible for biological information transmission of p38MAPK [20]. As a specific MAPK inhibitor, SB203580 is able to block the transmission of information from lower segment and reduce the phosphorylation of Akt/p100 PI3K [21]. Therefore, it can cut the information pathway of lower Akt/PI3K and exert its biological effects. In this study, it was discovered that after the MAPK was inhibited, expression of Akt/PI3K was significantly reduced. This fact illustrated that Akt/PI3K information pathway may play a role in the occurrence and development of neuropathic pain mediated by ephrinBs/EphB/MAPK, which was analogous to the research outcome of Song [22].

At the meantime, because of the relatively small sample size and comparatively inadequate observation time of this research, the results may be slightly different. In the future, prospective study will be carried on and confirmed with a more sufficient sample size and longer observation time.

In summary, the spinal ephrinBs/EphBs may activate the MAPK pathway, and thus participate in the formation and maintenance of neuropathic pain in diabetes rats. It will provide a

theoretical basis for diabetic patients with neuropathic pain in clinical.

Disclosure of conflict of interest

None.

Address correspondence to: Zheyin Wang, Department of Pain Medicine, Shenzhen People's Hospital, No.1017 Dongmen North Road, Luohu District, Shenzhen City, 518020, Guangdong Province, China. Tel: +86-775-22943488; Fax: +86-775-22943488; E-mail: zywang1998@yeah.net

References

[1] Said G. Diabetic neuropathy-a review. Nat Clin Pract Neurol 2007; 3: 331-40.
[2] Forbes JM and Cooper ME. Mechanisms of diabetic complications. Physiol Rev 2013; 93: 137-188.
[3] Feldman EL, Nave KA, Jensen TS and Bennett DL. New horizons in diabetic neuropathy: mechanisms, bioenergetics, and pain. Neuron 2017; 93: 1296-1313.
[4] Kozulin P and Richards LJ. Axonal guidance: making connections. Springer New York 2016.
[5] Zhou XL, Zhang CJ, Wang Y, Wang M, Sun LH, Yu LN, Cao JL and Yan M. EphrinB-EphB signaling regulates spinal pain processing via PKC-gamma. Neuroscience 2015; 307: 64-72.
[6] Steinle JJ, Meininger CJ, Chowdhury U, Wu G and Granger HJ. Role of ephrin B2 in human retinal endothelial cell proliferation and migration. Cell Signal 2003; 15: 1011-1017.
[7] Tatsumi E, Yamanaka H, Kobayashi K, Yagi H, Sakagami M and Noguchi K. RhoA/ROCK pathway mediates p38MAPK activation and morphological changes downstream of P2Y12/13 receptors in spinal microglia in neuropathic pain. Glia 2015; 63: 216-228.
[8] Sever R and Brugge JS. Signal transduction in cancer. Cold Spring Harb Perspect Med 2015; 5: a006098.
[9] Lin JH, Chiang YH and Chen CC. Research strategies for pain in lumbar radiculopathy focusing on acid-sensing ion channels and their toxins. Curr Top Med Chem 2015; 15: 617-630.
[10] Lee TM, Chang NC and Lin SZ. Inhibition of infarction-induced sympathetic innervation with endothelin receptor antagonism via a PI3K/GSK-3beta-dependent pathway. Lab Invest 2017; 97: 243-255.
[11] Zhou XL, Zhang CJ, Wang Y, Wang M, Sun LH, Yu LN, Cao JL and Yan M. EphrinB-EphB signaling regulates spinal pain processing via PKC-gamma. Neuroscience 2015; 307: 64-72.
[12] Lim DW, Kim JG and Kim YT. Analgesic effect of Indian gooseberry (emblica officinalis fruit) extracts on postoperative and neuropathic pain in rats. Nutrients 2016; 8: E760.
[13] Bouhassira D, Wilhelm S, Schacht A, Perrot S, Kosek E, Cruccu G, Freynhagen R, Tesfaye S, Lledo A, Choy E, Marchettini P, Mico JA, Spaeth M, Skljarevski V and Tolle T. Neuropathic pain phenotyping as a predictor of treatment response in painful diabetic neuropathy: data from the randomized, double-blind, COMBO-DN study. Pain 2014; 155: 2171-2179.
[14] Baron R, Maier C, Attal N, Binder A, Bouhassira D, Cruccu G, Finnerup NB, Haanpaa M, Hansson P, Hullemann P, Jensen TS, Freynhagen R, Kennedy JD, Magerl W, Mainka T, Reimer M, Rice AS, Segerdahl M, Serra J, Sindrup S, Sommer C, Tolle T, Vollert J and Treede RD. Peripheral neuropathic pain: a mechanism-related organizing principle based on sensory profiles. Pain 2017; 158: 261-272.
[15] Grace PM, Hutchinson MR, Maier SF and Watkins LR. Pathological pain and the neuroimmune interface. Nat Rev Immunol 2014; 14: 217-231.
[16] Sun W, Miao B, Wang XC, Duan JH, Wang WT, Kuang F, Xie RG, Xing JL, Xu H, Song XJ, Luo C and Hu SJ. Reduced conduction failure of the main axon of polymodal nociceptive C-fibres contributes to painful diabetic neuropathy in rats. Brain 2012; 135: 359-375.
[17] Kitamura T, Kabuyama Y, Kamataki A, Homma MK, Kobayashi H, Aota S, Kikuchi S and Homma Y. Enhancement of lymphocyte migration and cytokine production by ephrinB1 system in rheumatoid arthritis. Am J Physiol Cell Physiol 2008; 294: C189-196.
[18] Ruan JP, Zhang HX, Lu XF, Liu YP and Cao JL. EphrinBs/EphBs signaling is involved in modulation of spinal nociceptive processing through a mitogen-activated protein kinases-dependent mechanism. Anesthesiology 2010; 112: 1234-1249.
[19] Xu M, Bruchas MR, Ippolito DL, Gendron L and Chavkin C. Sciatic nerve ligation-induced proliferation of spinal cord astrocytes is mediated by kappa opioid activation of p38 mitogen-activated protein kinase. J Neurosci 2007; 27: 2570-2581.
[20] Liu Y, Peng TS, Hu XD, Li SJ, He YH, Hu F and Jiang J. Oxypeucedanin hydrate monoacetate isolated from angelica dahurica induces apoptosis in Caco-2 colon carcinoma cells through the mediation of PI3K-signalling pathway and inhibition of cancer cell migration. Bangladesh Journal of Pharmacology 2016; 11: 402-407.

[21] Gil-Araujo B, Toledo Lobo MV, Gutierrez-Salmeron M, Gutierrez-Pitalua J, Ropero S, Angulo JC, Chiloeches A and Lasa M. Dual specificity phosphatase 1 expression inversely correlates with NF-kappaB activity and expression in prostate cancer and promotes apoptosis through a p38MAPK dependent mechanism. Mol Oncol 2014; 8: 27-38.

[22] Song XJ, Zheng JH, Cao JL, Liu WT, Song XS and Huang ZJ. EphrinB-EphB receptor signaling contributes to neuropathic pain by regulating neural excitability and spinal synaptic plasticity in rats. Pain 2008; 139: 168-180.