European Review for Medical and Pharmacological Sciences 2015; 19: 3459-3467

Correlation research on the protein expression (p75NTR, bax, bcl-2, and caspase-3) and cortical neuron apoptosis following mechanical injury in rat

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Abstract. – OBJECTIVE: In this study, we aimed to survey the role of p75NTR, bax, bcl-2, and caspase-3 in the progress of traumatic brain injury (TBI).

MATERIALS AND METHODS: A mechanical trauma model of vital neurons was established by putting external pressure, contusion and centrifugal acceleration on neurons. Morphological change, survival rate, assay of LDH activity, and apoptosis rate were evaluated for mild, medium and severe injury models. The expression of bax, bcl-2, caspase-3, p75NTR, p75NTR mRNA was determined by immunohistochemistry, immunofluorescence, Western blotting and RT-PCR.

RESULTS: There was a transient high level Bcl-2 protein within 2 h after injury to increase neuronal tolerance and avoid apoptosis. Subsequently p75NTR, Bax/Bcl-2, and Caspase-3 reached their peaks from 48 to 72 h accompanied with the maximum apoptosis rate.

CONCLUSIONS: Our results suggest that apoptosis ratio in varying degree injury groups are correlated with the expression level of p75NTRmRNA, p75NTR, Caspase-3, Bax/Bcl-2 ratio.

Key Words:
Apoptosis, p75NTR, Bax, Bcl-2, Caspase-3.

Introduction

In the United States, traumatic brain injury (TBI) and spinal cord injury (SCI) together are responsible for an estimated 90,000 disabled person's annually[1]. The high prevalence of adverse outcomes following TBI has been attributed to a large extent to the secondary mechanisms of neuronal cell death[2]. Expression changes of some proteins (*i.e.* Bax, Bcl-2) have been reported in this apoptosis progress as an important component of neuronal response to injury[3,4]. Moreover, the p75 neurotrophin receptor (p75NTR) has been reported to mediate neuronal death and is regulated by Bcl-2 (B-cell lymphoma 2) family members[5]. A study has demonstrated caspase-3 activation after traumatic mechanical injury[6] and a later research has found caspase-3 activated apoptosis mediating by p75NTR[7]. Thus we consider whether the protein expression of p75NTR, Bax (Bcl-2-associated x protein), Bcl-2, Caspase-3 has a bearing on neuron apoptosis after mechanical injury. Furthermore, the role of p75NTR in the pathogenesis and repair of mechanical brain injury is unknown. Thus, a mechanical trauma model of vital neurons has been established by putting external pressure, contusion and centrifugal acceleration on neurons when several *in vivo* and *in vitro* models are investigated[8,9].

Materials and Methods

Dissociation Culture of Cortical Neurons

All animal experiments were approved by the Ethics Committee of Laboratory Animals of Jinan Municipality. The pregnant 17-day Sprague Dawley rats (270 ± 20 g) were provided by Experimental Animal Research Center, Shan Dong University. Six rat embryos were removed from rats under the deep anesthesia by ether, and the cortex tissues were dissected. The cerebral cortex were treated with 0.25% trypsin (Gibco, Grand Island,

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NY, USA) for 4 min and repeated 4 times, then was dissociated. After filtration, dissociated cells were plated on 6-well tissue culture plates (BD Falcon, Lexington, TN, USA) pre-coated with 0.1‰ poly-D-lysine (Sigma, St. Louis, MO, USA) at a density of 15×10^5 cells/well. Cultures were maintained in a 5% CO_2 incubator at 37°C. Cortical neurons were cultured with DMEM-Hi (Dulbecco Modified Ragle Medium-High) glucose medium containing 10% fetal bovine serum (Gibco Carlsbad, CA, USA) with 9.532 mg/ml HEPES, 0.584 mg/ml L-glutamine, penicillin and streptomycin (1 million U/10 mg/ml) 24 h after plating. After 48 h the half medium was changed, and 6×10^{-4} μM cytosine-β-d-arabinofuranoside (Ara-C, Sigma Aldrich, St Louis, MO, USA) was added into each well for 24 h, followed by replacement with fresh DMEM-Hi glucose medium every 2 days. Cultures were exposed to treatment 8 days after seeding. Neuron-specific enolase (NSE) was used as a neuronal marker and combined to antibody.

Establishment and Evaluation of Traumatic Neuron injury Model in vitro

Nerve contusion was simulated using stainless steel wire mesh (200 Mesh, Wire diameter: 53 μm, aperture sizes: 74 μm). To simulate injury causing by acceleration, 2000 *rpm* was reached within 30 s to get an angular acceleration of 500 rad/S^2 using low self-balancing centrifuge LDZ5-2. Steel plates (3 g, 6 g, and 9 g) were used for compression injury (mild, moderate, and severe) with vital neuron *in vitro*. The shapes of neurons and axons were observed with inverted phase contrast microscope. Meanwhile trypan blue cell counting (Sigma) and the activity of lactate dehydrogenase (LDH testing kit, Promega, Madison, WI, USA) was measured in culture medium in different groups (control, mild, moderate and severe) at different time-points respectively. Neuron apoptosis of different groups were tested at several time-points by flow cytometry. Detached neuron cells (10^5-10^6 cells/sample) were stained with Annexin V-FITC and propidium iodide (PI) according to the supplier's instructions (Annexin V-FITC Apoptosis Detection kit, BD Pharmingen, Franklin Lakes, NJ, USA). Viable and apoptotic cells were detected by CELL Quest software and counted by Multiset on FACS Calibur Flow Cytometry. Cells with no inducing apoptosis were used as the control. Apoptotic cells include the early apoptotic portion (Annexin V positive) and the late apoptotic portion (Annexin V and PI positive).

Immunofluorescence Analysis for Bcl-2, Bax, and Caspase-3

The expression level of each protein in each injury groups were measured at different time points, rabbit anti-mouse Bcl-2/Bax/Caspase-3 (1:150, Calbiochem, San Diego, CA, USA) and the Alexa Fluor 488 goat anti-rabbit IgG (1:150, Calbiochem) were used as first and secondary antibodies. 10 immunostained images in each experimental group at each time point were captured and quantified to obtain the overall average intensity for each staining. The intensity was compared among control, mild, moderate and severe injury models using Wallac victor3 1420 multilabel counter (Perkin-Elmer, Boston, MA, USA).

Western Blot testing the Expression of p75NTR

To determine the levels of protein expression from control, mild, moderate and severe injury models, soluble proteins were washed with 1‰ phenylmethylsulfonyl fluoride (PMSF) and isolated from each well by adding 125 μl Triple-detergent lysis buffer (300 μl RIPA, 6 μl 100 mM PMSF, 6 μl protease inhibitor). The bicinchoninic acid (BCA) assay method for the determination of protein has been investigated (BCA protein assay kit, Boster Company, Wuhan, China) according to package instructions. Sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis was performed. The electrophoresed proteins were transferred onto polyvinyldene difluoride (PVDF) membranes and subjected to immunoblot analysis with p75 monoclonal antibody (1:500, BD Pharmingen) and a secondary antibody (1:5,000). The reaction was detected with the AlphaimagerTM 2000 (Alpha Innotech Co, San Leandro, CA, USA). β-actin was used as internal control.

Quantitation of p75 mRNA Expression by Real Time Quantitative PCR

Total RNA of cultured cells were isolated by TRIZOL methods. First-strand cDNA was synthesized in 25 μL of RT mixture according to instructions (M-MLV Frist Strand cDNA Synthesis Kit, Fermentas, Leon-Rot, Germany). Upstream primer for the polymerase chain reaction (PCR) reaction was 5'-gCCCCTCTgAACCCTAAg-3', downstream primer was 5'-gAggCATACAgggACAACA-3'. The amplified product of β-actin mRNA (734 bp) was internal reference with 5'-gCCCCTCTgAACCCTAAg-3' as upstream primer and 5'-gAggCATACAgggACAACA-3' as

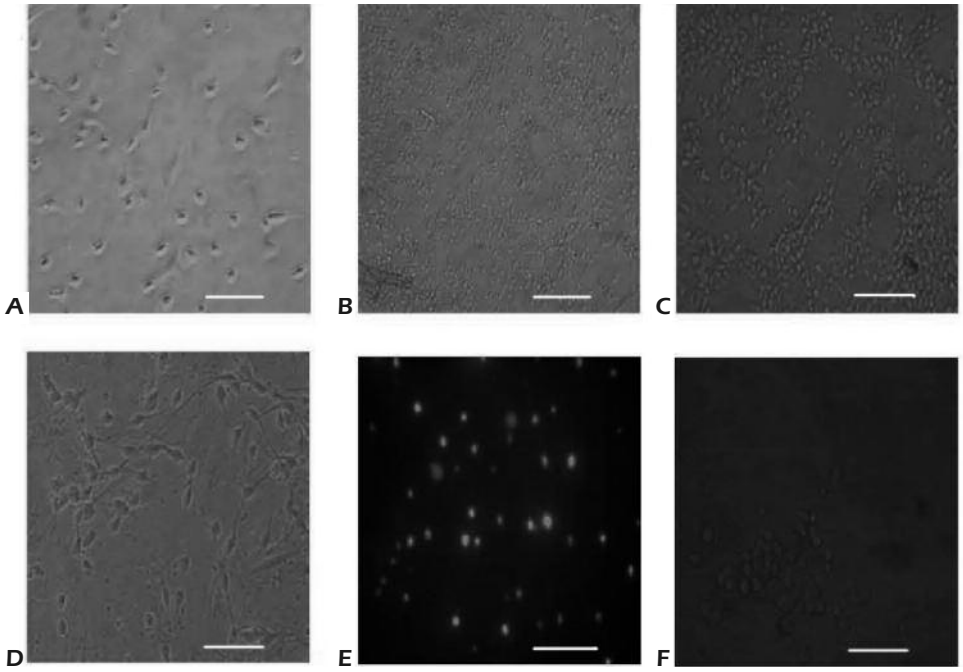

Figure 1. Morphological change of cortical neurons in cultured progress and after injury. ***A,*** normal cells at 24h (20×); ***B,*** normal cells at 72h (20×); ***C,*** normal cells at 72h (40×); ***D,*** normal cells at 7 day (200×); ***E,*** Immunohistochemical staining of NSE in the cytoplasm as positive neurons at 7 day (40×); ***F,*** Injured cells (200×).

downstream primer. The PCR products were resolved on a 2% agarose gel and visualized using ethidium bromide. The size of the bands was confirmed at 500 bp. As a calibrator, we used an obtained cDNA with maximum C_T. Quantification of p75 mRNA was evaluated by using the relative quantity method.

Statistical Analysis

SPSS 11.0 software (SPSS Inc., Chicago, IL, USA) was used in statistical analyses. Data were presented as mean ± SD. The *t* test was performed for pairwise comparisons between experimental groups and control groups. The Pearson correlation was conducted and $p < 0.05$ was considered as statistically significant.

Results

Light microscopic Analysis of Morphological changes to Axons After Centrifugal Injury in vitro

In normal neuron cells, a great number of neuritis became thicker and longer to contact each other and form a complicated network, cells had an increased size, a larger nucleus and more clear cytoplasm with refractivity (Figure 1A-D), at 7th day the percent of cultured neurons was more than 90% (Figure 1E). Apoptosis were discovered after mechanical injury. Axonal swelling, axonal bulb, disruption of axon and neuron were observed by inverted phase contrast microscope in injury group (Figure 1F).

Changes in Survival Rate, Apoptosis ratio and LDH Activity After Mechanical Injury

A significant reduction of survival rate in injury groups started at 30 min compared with control group. A considerable reduction was observed in moderate and severe injury groups compared to mild groups with a significant different from 1 h to 72 h (Figure 2A, Table I).

A significantly higher LDH activity in injury groups began at 30 min and lasted to 72 h compared with control group; LDH activity in moderate and severe injury groups was higher compared with mild groups; the bimodal curve of LDH activity reach the first peak at 30 min and the second peak at 72 h (Figure 2B, Table I).

Cellular relative apoptosis ratio in each group was shown after FCM analysis. Apoptosis began

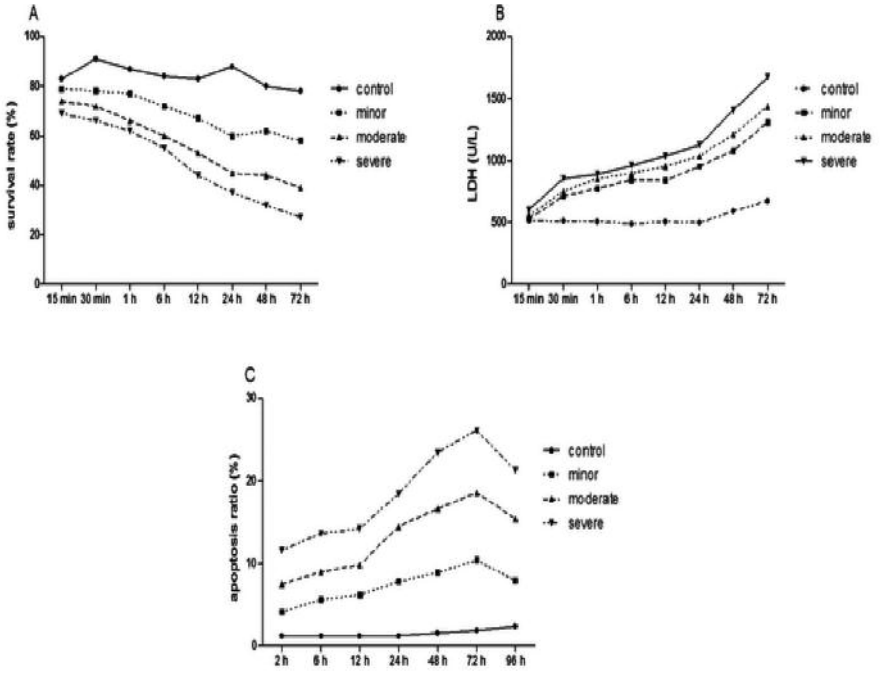

Figure 2. Evaluation of mechanical injury models between mild, medium, and severe groups. ***A,*** Survival rate in different groups; ***B,*** LDH activity in different groups; ***C,*** Apoptosis Ratio in different groups, $*p < 0.05$ compared with minor injury group.

Table I. Survival rate (%) (mean ± SD) and LDH (μ/l) (mean ± SD) activity for cortical neurons after injury at different time points.

		Group			
Time	**Control**		**Minor injury**	**Moderate injury**	**Severe injury**
15 min	SR	83 ± 6.2	79 ± 7.5	74 ± 7.0	69 ± 7.3
	LDH	516 ± 110	530 ± 106	556±103	603 ± 108
30 min	SR	91 ± 7.2	78±6.5a	72 ± 6.4a	66 ± 5.6a
	LDH	510 ± 84	710 ± 132a	750 ± 146a	854 ±1 38abc
1h	SR	87 ± 6.5	77 ± 7.6a	66 ± 5.7ab	62 ± 5.8abc
	LDH	509 ± 103	773 ± 135a	852 ± 96ab	893 ± 130abc
6h	SR	84 ± 5.1	72 ± 5.1a	60 ± 5.6ab	55 ± 6.3abc
	LDH	486 ± 105	842 ± 95a	895 ± 130a	962 ± 133abc
12h	SR	83 ± 3.6	67 ± 4.8a	53 ± 6.2ab	44 ± 5.7abc
	LDH	506 ± 125	839 ± 109a	956 ± 155a	1036 ± 108abc
24h	SR	88 ± 4.1	60 ± 4.3a	45 ± 4.8ab	37 ± 4.9abc
	LDH	496 ± 122	954 ± 150a	1034 ± 165ab	1125 ± 164abc
48h	SR	80 ± 5.2	62 ± 4.5a	44 ± 5.6ab	32 ± 6.1abc
	LDH	591 ± 114	1078 ± 107a	1211 ± 151ab	1407 ± 140abc
72h	SR	78 ± 4.7	58 ± 5.1a	39 ± 6.6ab	27 ± 4.3abc
	LDH	676 ± 102	1307 ± 178a	1436 ± 195ab	1670 ± 174abc

Results are mean ± SD (n = 5); SR: survival rate; LDH: LDH activity; ap < 0.05 vs. control; bp < 0.05 vs. minor injury group; cp < 0.05 vs. moderate injury group.

at 2 h, increased at 12 h and reached peak at 72 h, then decreased. The apoptosis ratio in severe groups was significantly higher than other groups ($p < 0.05$) (Figure 2C, Table II). These results confirmed our injury model was successful.

The Expression of Bax, Bcl-2 and Caspase 3, p75NTR and p75NTRmRNA After Mechanical Damage of Neurons

Immunofluorescence photos of moderate injury groups showed Bcl-2 localized in the outer mito-

Table II. The apoptosis ratio (%) (mean ± SD) for cortical neurons after injury at different time points.

		Group		
Time	**Control**	**Minor injury**	**Moderate injury**	**Severe injury**
2h	1.25 ± 0.12	4.14 ± 0.31[a]	7.50 ± 0.60[ab]	11.57 ± 1.39[abc]
6h	1.23 ± 0.20	5.62 ± 0.58[a]	8.96 ± 0.82[ab]	13.64 ± 1.32[abc]
12h	1.27 ± 0.11	6.22 ± 0.61[a]	9.86 ± 0.94[ab]	14.23 ± 1.36[abc]
24h	1.24 ± 0.22	7.81 ± 0.67[a]	14.51 ± 1.11[ab]	18.48 ± 1.32[abc]
48h	1.60 ± 0.24	8.90 ± 0.81[a]	16.62 ± 1.31[ab]	23.47 ± 1.19[abc]
72h	1.91 ± 0.14	10.41 ± 1.31[a]	18.61 ± 1.12[ab]	26.14 ± 1.18[abc]
96h	2.40 ± 0.16	7.91 ± 1.03[a]	15.41 ± 1.32[ab]	21.31 ± 1.11[abc]

Results are mean ± SD (n = 5); [a]$p < 0.05$ vs. control group; [b]$p < 0.05$ vs. minor injury group; [c]$p < 0.05$ vs. moderate injury group.

chondrial membrane, endoplasmic reticulum, and nuclear envelope (Figure 3A), Bax and Caspase-3 located in cytoplasm (Figure 3B, D), Protein expression of Bax, Bcl-2, and Caspase-3 increased significantly at 2 h, 1 h, and 1 h respectively, their expression reached peak at 24 h, 2 h, and 48 h respectively (Figure 4). Bax in severe injury group was higher compared to other groups in early injury period ($p < 0.05$) (Figure 4A). Bcl-2 in injury groups was significantly lower than control group after 24h ($p < 0.05$) (Figure 4B). Moreover, the ratio of Bax/Bcl-2 increased and reached peak from 48h to 72h in different injury group and the ratio increased with the degree of injury severity (Figure 4C). Meanwhile the severe injury group showed the highest expression (Figure 4D).

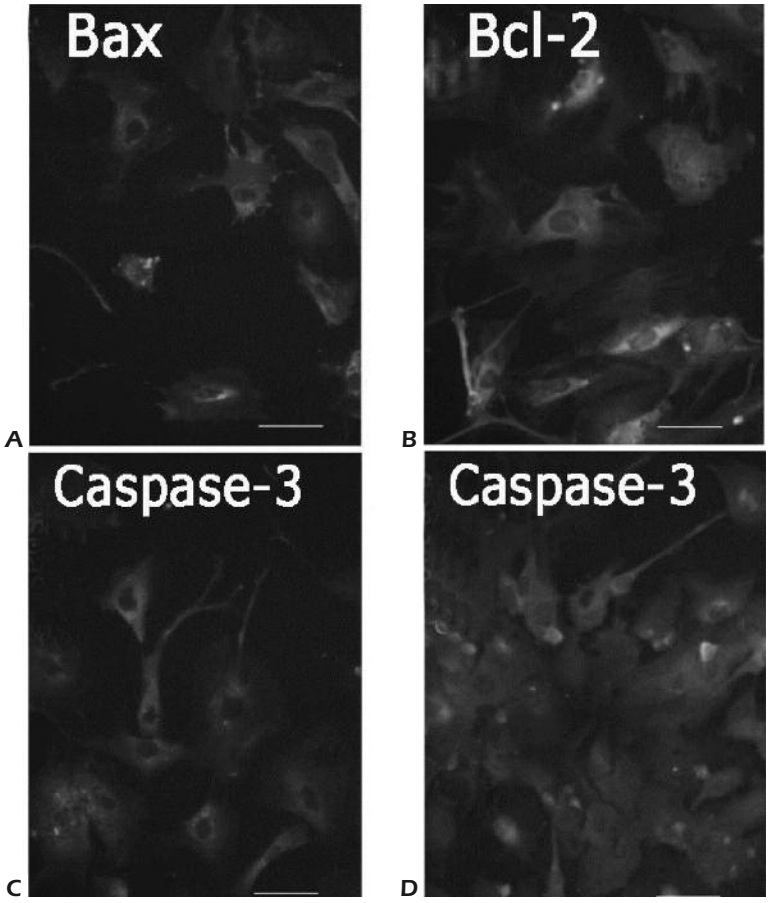

Figure 3. Immunofluorescence photos for Bax, Bcl-2 and Caspase 3: ***A,*** Bax in moderate injury group at 72h (200X); ***B,*** Bcl-2 in moderate injury group at 72h (200X); ***C,*** Caspase-3 in control group at 72h (200X); ***D,*** Caspase-3 in moderate injury group at 72h (200X).

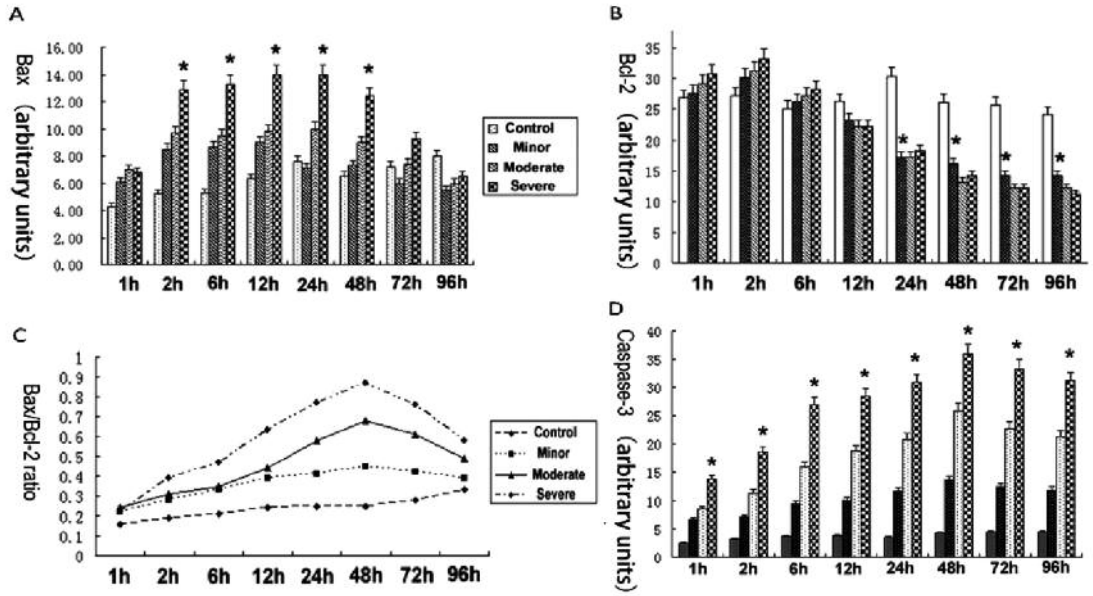

Figure 4. The protein expression changes within 96 hours after mechanical damage of neurons (*$p < 0.05$ compared with control group). ***A,*** Bax at different injury groups; ***B,*** BCL-2 at different injury groups; ***C,*** the bax/bcl-2 ratio at different injury groups; ***D,*** Caspase-3 at different injury groups

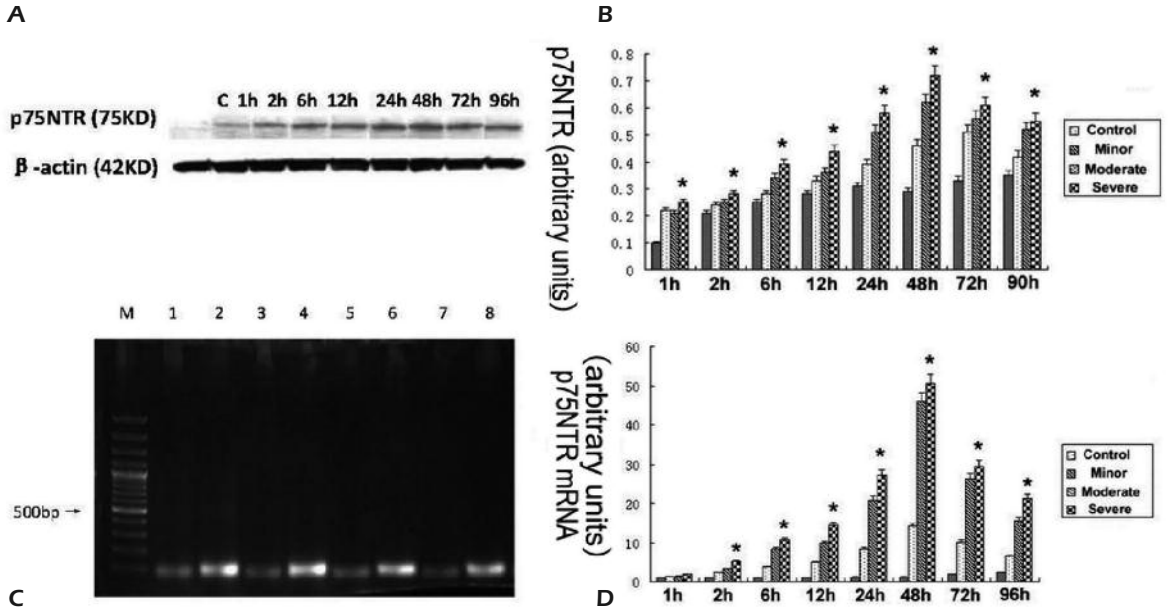

Figure 5. The expression of p75NTR and p75NTRmRNA after mechanical damage of neurons. ***A,*** p75NTR expression in medium injury group at different time points; ***B,*** p75NTR expression in different groups at different time points; ***C,*** p75NTR mRNA expression, M- standard DNA marker, gene represented by 1,3,5,7- p75NTR, gene represented by 2,4,6,8- actin; ***D,*** p75NTR mRNA expression in different groups at different time points.

Table III. Correlation coefficient matrix of apoptosis, p75NTR mRNA expression, p75NTR protein expression, Bax/Bcl-2, and Caspase-3.

Group			Apoptosis	RQP75	WP75	Caspase3	Bax/Bcl2
control	Pearson Correlation						
Sig. (1-tailed)	Apoptosis		0.968	0.690	0.790	0.890	
		RQP75	0		0.650	0.698	0.867
		WP75	0.029	0.041		0.927	0.921
		Caspase3	0.01	0.027	0		0.902
		Bax/Bcl2					
		Apoptosis	0.002	0.003	0.001		
				0.000	0.029	0.010	0.002
Light injury	Pearson Correlation	Apoptosis		0.886	0.991	0.947	0.917
		RQP75	0.002		0.896	0.933	0.888
		WP75	0	0.001		0.944	0.899
	Sig. (1-tailed)	Caspase3	0	0	0		0.964
		Bax/Bcl2	0.001	0.002	0.001	0	
		Apoptosis		0.002	0.000	0.000	0.001
Medium injury	Pearson Correlation	Apoptosis	0.831	0.971	0.923	0.94	
		RQP75	0.005		0.906	0.883	0.928
	Sig. (1-tailed)	WP75	0	0.001		0.971	0.972
		Caspase3	0.001	0.002	0		0.964
		Bax/Bcl2					
		Apoptosis	0	0	0	0	
				0.005	0.000	0.001	0.000
Serious injury	Pearson Correlation						
Sig. (1-tailed)	Apoptosis		0.851	0.929	0.908	0.866	
		RQP75	0.004		0.961	0.868	0.915
		WP75	0	0		0.953	0.953
		Caspase3	0.001	0.003	0		0.946
		Bax/Bcl2					
		Apoptosis	0.003	0.001	0	0	
				0.004	0.000	0.001	0.003

RQP75: p75NTR mRNA expression; WP75: p75NTR protein expression. Upper triangular matrix: Pearson Correlatio.

p75NTR mRNA expressed after varying degree injury at different period of time (Figure 5A, B, D) and reached peak at 48 h. The expression were higher in moderate, severe injury groups than minor injury and control groups ($p < 0.05$) (Figure 5D). The size of the bands was confirmed by at 500 bp (Figure 5C).

The Correlative analysis result

The correlation analysis result showed that apoptosis ratio in varying degree injury groups are correlated with the expression level of p75NTRmRNA, p75NTR, Caspase-3, Bax/Bcl-2 ratio ($p < 0.05$) (Table III) while had no correlation with control group.

Discussion

Traumatic Brain Injury (TBI) is neuronal cellular and axonal injury caused by shearing stress[10]. One of the adverse outcomes following TBI is the secondary mechanisms of neuronal cell death. It was reported previously that apoptotic and necrotic death occur after experimental traumatic brain injury[11,12]. Many *in vivo* and *in vitro* models have been developed for simulating the secondary mechanisms. Most models simulated one or more pathological mechanisms of TBI. In our study, Morphological changes and self-repairing membranes of injured neurons here were consistent with previous study[13,14]. The model is induced by single impairment and has

key advantages of intuitive and affordable because of no confounding effects, and it provides a power tool to study cell morphology, molecular pathology and drug therapy in traumatic and secondary brain injury.

In this study transient high level Bcl-2 protein within 2 h after injury to increase neuronal tolerance and avoid apoptosis, which was consistent with previous study[15]. Subsequently the peak in cell apoptosis from 48 to 72 h accompanied with maximum ratio of Bax/Bcl-2, thus the ratio of protective Bcl-2 to apoptotic Bax determined susceptibility of cells to apoptosis. Caspase-3 had a long duration time and reached peak at 48-72 hour when the cell apoptosis reached maximum as well, which demonstrated that increasing caspase-3 expression after injury played an important role in neuronal apoptosis execution as previous description[16,17]. p75NTR had a closely relationship with neuronal growth inhibitory factor (myelin-associated protein, myelin-associated glycoprotein, oligodendrocyte myelin glycoprotein)[14,18-20].

Our study confirmed that the balance between the pro-apoptotic factor Bax protein and anti-apoptotic factor Bcl-2 protein was broken after mechanical injury of neurons, Bax/Bcl-2 ratio increased. Bcl-2 could not continue to increase to be heterodimers with Bax and thus lost the ability of anti-apoptosis[21,22]. At the same time, Bax protein transfered from the cytoplasm to the mitochondria and caused the mitochondrial permeability transition, and thereby activated a cascade of apoptosis. Meanwhile the increasing expression of p75NTR activated the Jun N-terminal Kinase (JNK) pathway and increased expression of phosphorylated JNK, which was an early signal of apoptosis. The activation of JNK also made Bcl-2 lost its anti-apoptotic effects by promoting its phosphorylation[20,23].

Conclusions

In summary, to the best of our knowledge, this is the first study to investigate the chronological expression changes of p75NTR, Bax, Bcl-2, Caspase-3, and p75NTR mRNA in cortex neurons after mechanical injury, and it demonstrates that apoptosis ratio in varying degree injury groups and expression level of p75NTRmRNA, p75NTR, Caspase-3, Bax/Bcl-2 are two-related. But it has some disadvantages, for an example, cell changes during acceleration could not be determined; Model validity was empirically assessed by trypan blue dye and assay of LDH activity to determine the extent of nerve damage[24], and we hope exploring them in the future research work.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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