Nimodipine alleviates apoptosis-mediated impairments through the mitochondrial pathway after spinal cord injury

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Abstract Spinal cord injury (SCI) remains an unsolved human health challenge. To alleviate the impairments of SCI, we studied the therapeutic effect of nimodipine (an L-type Ca2+ channel antagonist) on functional recovery from SCI using Nystrom’s method in a mouse model. Eighty-four mice were divided into three groups: control group in which only vertebral plates were cut off without causing any spinal injuries; SCI; and SCI with nimodipine treatment. We assessed the histopathology, apoptosis detection, cell cycle, mitochondrial transmembrane potential, bcl-2/bax and caspase-3 levels of tissue 8 h, 1 d, 3 d and 4 d after trauma to evaluate rehabilitation. Behavioral performances were also assessed before and after nimodipine treatment. Results from inclined plane tests, motor score assessment and histological observations indicated that mice in the nimodipine-treated group rehabilitated better than those in the SCI group. The ratio of apoptosis, caspase-3 and bax expression in the nimodipine-treated group were significantly lower than those in the SCI group. The mitochondrial membrane potential and bcl-2 expression were up-regulated in the nimodipine-treated group. Taken together, our results indicate that the inhibition of calcium flux by nimodipine could reduce apoptosis processes and tissue damage through a mitochondrial pathway after spinal cord trauma [Current Zoology 57 (3): 340–349, 2011].

Keywords Nimodipine, Mitochondrial-pathway, Alleviation, Spinal cord injury, Apoptosis

Spinal cord injury (SCI) is a devastating experience that results in permanent neurological deficit. SCI leads to strong up-regulation of genes involved in transcription, inflammation and cell death (Proskuryakov et al., 2002; Bethea et al., 1998; Ahn et al., 2006). Researchers have found that apoptosis can lead to tissue damage after SCI in mice, monkeys and humans (Lou et al., 1998; Ahn et al., 2006; Barut et al., 2005; Colak et al., 2005; Crowe et al., 1997; Eldadah et al., 2000).

It is well known that mitochondria serve not only as the location of ATP production, but also play a key role in cell death. Many pro-apoptotic signals converge at the mitochondria and trigger a change in mitochondrial membrane permeability, resulting in several mitochondrial proteins crucial for apoptosis to be released into the cytoplasm. Pro-apoptotic proteins include the caspase activator cytochrome c (Liu et al., 1996), caspase coactivator Smac/Diablo (Verhagen et al., 2000; Du et al., 2000), caspase-9 (Colak et al., 2005; Susin et al., 1999a, 1999b; Yi et al., 2004), and apoptosis-inducing factors that are transported into the nucleus to induce DNA fragmentation (Zamzani et al., 1996). However, the process of pro-apoptotic factor release from mitochondria remains unclear. The central role played by the permeability transition pore (PTP) protein is complex (Narita et al., 1998; Zamzani and Kroemer, 2001). PTP mainly consists of a voltage-dependent channel in the outer mitochondrial membrane and an adenine nucleotide translocase in the inner mitochondrial membrane. Activation of this complex ion channel triggers increased permeability of the outer mitochondrial membrane for the passage of protein molecules and of the inner mitochondrial membrane for the passage of low molecular weight ions (Zamzani and Kroemer, 2001). The increase in inner mitochondrial membrane permeability depolarizes the mitochondrial membrane potential. Several studies suggest that pro-apoptotic bcl-2-like proteins such as bax interact with PTP to form a large pore that permits the release of pro-apoptotic proteins (Shimizu et al., 2000). Conversely, anti-apoptotic proteins such as bcl-2 prevent the opening of PTP (Li et al., 1996; Narita et al., 1998; Shimizu et al., 1998, 1999,
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2000; Marzo et al., 1998), which can also cause a flux of ions into the mitochondria, mediating swelling and eventually rupturing the outer mitochondrial membrane (Van der Heiden et al., 1997, Diaz-Prieto. et al., 2008). The hypothesis of pro-apoptotic factor release from mitochondria through the bcl-2/bax pathway in vitro is generally accepted by many scientists, although the viewpoint is equivocal in vivo.

It is unclear whether under in vivo conditions pro-apoptotic factors combine with PTP to induce a series of ion signals that evoke cell death through mitochondrial membrane depolarization. Impairments related to SCI include serious pathological cell death, and changes in calcium accumulation and extrusion typical for cerebral ischemia (Zhu et al., 2000). Although the kinetics of calcium signals evoked by membrane depolarization (Shmlgol and Kostyuk, 1995) remains unclear, research into calcium signals suggest that Ca\textsuperscript{2+} channel antagonists can deter the process of impairments induced by SCI. The present study was designed to assess the role of Ca\textsuperscript{2+} in apoptotic-induced functional deficits in a mouse model of SCI. The Ca\textsuperscript{2+} blocker nimodipine was used as the test agent to explore this possibility.

1 Materials and Methods

1.1 Materials and grouping

This study used 84 mice purchased from Nanjing Qinglongshan Experimental Animal Factory. The mice were reared in separate cages under environmental conditions including free diet, 12–12 h light-dark cycles and room temperature ranging from 23–25°C. All experiments were carried out in accordance with the National Institutes of Health guidelines. Mice weighing 25–30g were randomly divided into 3 groups (28 mice in each group): control group underwent sham injuries and SCI/nimodipine group underwent SCI before treatment with nimodipine.

1.2 Preparation of animal models

In this study, we improved upon Nystrom’s method (Nystrom et al., 1988) in the establishment of a SCI model. Animals were anesthetized with chloral hydrate (4 mg/kg, IP). After anesthesia the animals were prepared for SCI. The spinal processes from Th7-Th9 were exposed for all three groups. Mice in the SCI and SCI/nimodipine groups then had a 30g weight drop pressed against the spinal cord for five minutes (30 g \times 5 min). SCI success was confirmed by quick jerks of the hind limbs observed in trauma-surgery animals. Animals in the SCI/nimodipine group then received a subcutaneous injection of 1 mg/kg nimodipine (Richard et al., 1981) 30 min after trauma. All subject groups were reared under optimal environmental conditions.

Mice were euthanized by cervical vertebra dislocation at 8 h, 1 d, 3 d and 7 d after trauma, and spinal cord samples were collected and processed.

1.3 Material selection and slice preparations

Animals were anesthetized and then perfused with 4% paraformaldehyde at set periods after SCI. Spinal segments Th7-Th9 were excised and fixed in 4% paraformaldehyde at set periods after SCI. Spinal segments Th7-Th9 were excised and fixed in 4% paraformaldehyde at set periods after SCI. Spinal cord segments Th7-Th9 were collected from animals in all groups and digested by 0.25% trypsin–0.02% EDTA (Gibco Laboratories, USA). After suspension, the dissociated cells were rinsed with PBS and filtered through nylon net (400 holes). The cells were diluted to (1–5) \times 10^6 cell/ml, fixed with 70% ethanol (4°C) for 1–2 h and rinsed with PBS (5 min \times 3 rinses). The cells were analyzed with FCM after being stained with 1 ml propidium iodide (including RNAase) for 30 min. Laser wave-length was 488 nm and the wave-length of emitted light was beyond 630 nm. A histogram was used to assay the red intensity of propidium iodide. The software Cell Quest and Modfit LF (Cai, et al., 2005) were used to assay the phase (G0/G1, S and G2/M) and apoptosis of 10 000 cells. The number of apoptosis cells were counted and expressed with blue wave peak.
1.4.3 Measurement of mitochondrial transmembrane potential (ΔΨm) The possible role of the mitochondrial-dependent apoptosis pathway was further examined by rhodamine 123 (Rh123), a positively charged probe that accumulates in the mitochondria dependent on transmembrane potential (Baraccaa et al., 2003; Pithon-Curi et al., 2003). Flow cytometric analysis of mitochondrial activity was attained using Rh123. The suspended spinal cord cells were then incubated with 10 mg/ml Rh123 at 37°C for 30 min and digested by 0.25% trypsin-0.02% EDTA and washed with ice-cold PBS. Finally, the cells were washed and re-suspended in 0.5 ml PBS before measuring fluorescence was using flow cytometry (Becton Dickinson).

1.4.4 Caspase-3, bax, and bcl-2 expression detected by immunohistochemistry Tissue samples fixed in phosphate-buffered 10% formalin and embedded in paraffin wax were serially sectioned (5 μm), and mounted on APES-coated slides (Boster, Wuhan, China). These slides were dewaxed, washed in citrate buffer (pH 6.0; 10 min), blocked in 10% normal goat serum/4% BSA (overnight at 4°C) and then incubated (1 h at room temperature) with mouse monoclonal anticaspase-3 (BA0588, Boster, Wuhan, China), anti-bax (BA0412, Boster, Wuhan, China) and anti-bcl-2 (BA0315, Boster, Wuhan, China). Monoclonal antibodies and antiserum were diluted in antibody diluent according to the instructions in ABC kits (Boster, Wuhan, China). After rinsing in 0.05 ml PBS, sections were incubated for 1 h with biotinylated sheep anti-mouse IgG antibodies diluted 1:500 in antibody diluent. After washing with PBS and subsequent incubation for 1 h, Streptavidin peroxidase complex was applied. After washing again, a peroxidase reaction was undertaken in DAB solution containing 0.01% of H2O2 in Tris-HCl buffer (50 mM, pH 7.6), and was subsequently washed with distilled water. After light counterstaining with Mayer’s hematoxylin, the sections were dehydrated and coverslipped. Photomicrographs were taken with a Leica DM 2500 microscope. The dark-brown positive cells in sections were counted using 400× magnification (10× ocular and 40× objective). The average number of positive cells from 20 randomly selected fields of different groups were determined and used in data evaluation.

1.4.5 Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) Trizol reagent (Invitrogen Co. Ltd.) was used to extract total RNA the above samples. An RT reaction was carried out according to instructions in the RT reaction kit (Promega Co. Ltd.). We investigated the expression of caspase-3 mRNA, Bcl-2 mRNA and Bax mRNA in tissue samples. PCR primers and PCR condition are listed in Table 1.

A pair of primers (upstream: 5’-GCAATGCTGGTGTT CATGGTGG-3’, downstream: 5’-GTCGTAACCACAG GCAATTGTGATGG-3’) were used to amplify the β-actin gene as an internal standard.

1.5 Statistical analysis The data were expressed as mean ± SD, and analyzed using Two-Way ANOVA and one-way ANOVA analysis of variance. Data were transformed to ensure homogeneity of variance. LSD’s multiple comparisons were used to identify differences between groups at the 5% and 1% significance level. Statistical tests were performed using SPSS version 17.0.

2 Results

2.1 Motor score assessment and histological observation

2.1.1 Inclined plane test results Inclined plane test results are summarized in Table 2. Inclined plane test scores after spinal injury declined significantly in SCI and SCI/nimodipine groups (two-way ANOVA, F2,78= 560.26, P <0.001), before increasing gradually (two-way ANOVA analysis of variance, F3,78=8.92, P <0.001). The inclined plane angle at 7 d after trauma ranged from 34–38° in the SCI group and from 45–50° in the SCI/nimodipine group. The means for the SCI/nimodipine group were significantly higher than those for the SCI group (One-Way ANOVA, F2,18=410.14, P <0.001).

Table 1 Semi-quantitative RT-PCR of genes, GenBank accessions, primer sequences, predicted size of PCR products and annealing temperatures

<table>
<thead>
<tr>
<th>GenBank accessions</th>
<th>Amplified gene</th>
<th>Primer sequences</th>
<th>Size of predicted PCR products</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_009810</td>
<td>caspase-3</td>
<td>upstream: agcttcttcagaggcgacta downstream: ggcacaaatcaaggagttct</td>
<td>381bp</td>
<td>58.5°C</td>
</tr>
<tr>
<td>NM_007527</td>
<td>bax</td>
<td>Upstream: cgaagtgtcctccgaagatt Downstream: atgtggagccaggggagag</td>
<td>381bp</td>
<td>62.9°C</td>
</tr>
<tr>
<td>NM_009743</td>
<td>bcl-2</td>
<td>Upstream: gtagcggagacgttcat Downstream: etgtgcattgttctcag</td>
<td>302bp</td>
<td>60.9°C</td>
</tr>
</tbody>
</table>
2.1.2 Motor function results  Motor scores after spinal injury displayed similar changes to the inclined plane test results (Table 3). Motor scores declined significantly in the SCI and SCI/nimodipine groups when compared to the control group (Two-Way ANOVA analysis of variance, $F_{2,18}=2887.14$, $P<0.001$). Motor scores began to increase in the SCI and SCI/nimodipine groups (Two-Way ANOVA analysis of variance, $F_{2,78}=8.92$, $P<0.001$) 7 d post-trauma. Scores in the SCI/nimodipine group were significantly higher than those in the SCI group ($F_{2,18}=628.42$, $P<0.001$).

2.1.3 HE Staining  The injured segments of myeloid structure began to develop approximately 1 d after injury, and many hemorrhagic foci were found in the gray matter. Neurons in the anterior horn survived in the SCI group but displayed pycnosis. Serious myeloid damage was observed 3 d after injury. Only a small amount of neurons, mostly distributed in the posterior horn, were preserved in the gray matter. Swollen axons and vacuoles were observed. The range of injury did not persist after 7 d and the injured segments became narrower. There were still some normal structures in white matter areas, but cavitation and demyelination were less prominent in SCI/nimodipine group.

2.2 The cell cycle and mitochondrial membrane potential detection

2.2.1 The cell cycle and apoptosis after SCI injury

The proportion of early apoptotic cells and late apoptosis/necrosis cells increased significantly to their maximum levels of 55.35% for the SCI group and 45.33% for the SCI/nimodipine group 3 d after surgery (Fig 1). However, the rate of cell apoptosis in the SCI/

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Table 2  Inclined plane test results

<table>
<thead>
<tr>
<th>Group</th>
<th>8 h</th>
<th>24 h</th>
<th>72 h</th>
<th>168 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>65.4±2.3</td>
<td>63.5±3.1</td>
<td>65.5±2.1</td>
<td>64.5±1.3</td>
</tr>
<tr>
<td>SCI</td>
<td>33.5±1.5**</td>
<td>38±2.5**</td>
<td>27.5±2.1**</td>
<td>36±1.7**</td>
</tr>
<tr>
<td>SCI/nimodipine</td>
<td>35.5±2.8**</td>
<td>37±2.1**</td>
<td>38.5±1.5***</td>
<td>47.5±2.5***</td>
</tr>
</tbody>
</table>

Data expressed as mean±SD, n=7. Asterisks indicate level of significant differences between members of treatment and control pair within a column (**, $P<0.001$), # indicate level of significant differences between members of treatment with SCI and SCI/nimodipine within a column (# # #, $P<0.001$). One-Way ANOVA ($F_{2,18}=437.25; 4 h$, $F_{2,18}=235.10; 72 h$, $F_{2,18}=704.60; 168 h$, $F_{2,18}=410.14$) followed by LSD Post Hoc Test.

Table 3  Motor scores

<table>
<thead>
<tr>
<th>Group</th>
<th>8 h</th>
<th>24 h</th>
<th>72 h</th>
<th>168 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5±0</td>
<td>5±0</td>
<td>5±0</td>
<td>5±0</td>
</tr>
<tr>
<td>SCI</td>
<td>1.1±0.2**</td>
<td>1.2±0.3***</td>
<td>1.2±0.3**</td>
<td>1.4±0.5**</td>
</tr>
<tr>
<td>SCI/nimodipine</td>
<td>1.1±0.2**</td>
<td>1.2±0.3**</td>
<td>1.4±0.5**</td>
<td>1.7±0.3***</td>
</tr>
</tbody>
</table>

Values are mean±SD, Asterisks indicate level of significant differences between members of treatment and control pair within a column (**, $P<0.001$), # indicates level of significant differences between members of treatment with SCI and SCI/nimodipine within a column (#, $P<0.05$). One-Way ANOVA ($F_{2,18}=2549.02; 24 h$, $F_{2,18}=1082.43; 72 h$, $F_{2,18}=404.40; 168 h$, $F_{2,18}=628.43$) followed by LSD Post Hoc Test.

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![Fig. 1 The ratio of apoptosis in different groups](image)

Data are expressed as mean ± SD. Apoptosis ratio of the control group (<0.05%) cannot be displayed in Fig. 1 because it is far less than that of the treatment group. Asterisks indicate level of significant differences between members of treatment and control pair, # indicates level of significant differences between members of treatment with SCI and SCI/nimodipine, ***, $P<0.001$ (One-Way ANOVA, LSD multiple comparisons: 8 h, $F_{2,18}=766.08; 24 h$, $F_{2,18}=810.76; 72 h$, $F_{2,18}=1002.01; 168 h$, $F_{2,18}=365.99$)
nimodipine group was significantly lower than in the SCI group after 1 d (F2,18=366.95, P <0.001). In the SCI and SCI/ nimodipine groups, the percentage of cells at G0/G1 (Table 4) phase after SCI injury decreased significantly compared to the percentage before injury (Two-Way ANOVA analysis of variance, F2, 78=61.09, P <0.001), whereas the percentage of cells in G2/M (Table 4) phases significantly increased (F2, 78=28.87, P <0.001). The S and G2/M subpopulation percentage for the SCI and SCI/ nimodipine groups increased significantly 3 d after trauma (Table 4), while the G0/G1 proportion decreased significantly (F2, 18=8812.19, P <0.001; F2, 18=684.72, P < 0.001). Our results show that there was a slight arrest in cell cycle at the G2/M phase after SCI injury. The proportion of G2/G1 cells did not change significantly (Table 4).

2.2.2 Mitochondrial membrane potential after SCI injury Loss of mitochondrial transmembrane potential (ΔΨm) diminishes the mitochondria’s ability to accumulate fluorochrome Rh123. Fig. 2 shows that cell recovery after SCI injury was dramatically reduced during uptake of Rh123. The mitochondrial transmembrane potential decreased immediately after SCI treatment and reached its lowest value 3 d after SCI (F3, 78=32.25, P <0.001). The average Rhodamine123 fluorescence intensity (percentage of the control group) plotted as a function of time after SCI treatment. Data are expressed as mean±SD. Asterisks indicate level of significant differences between members of treatment and control pair, # indicates level of significant differences between members of treatment with SCI and SCI/ nimodipine. ***, P<0.001; ###, P<0.001 (One-Way ANOVA, LSD multiple comparisons: 8 h, F2, 18=273.15; 24 h, F2, 18=99.62; 72 h, F2, 18=914.35; 168 h, F2, 18=102.86).

2.3 bcl-2/bax and caspase-3 expression ratio post-trauma

2.3.1 Immunohistochemistry and quantification of apoptosis The cytoplasm of positive cells was stained yellow-brown. Immunohistochemistry staining identified several caspase-3, bax and bcl-2 apoptotic cells in the sham-operated control animals (Table 5). Positive cells were observed in both the gray and white matter in the SCI group 8 h after injury. Staining in the gray matter was more intense than in other regions, especially at the periphery of the damaged site where the injured tissue merged and surrounded the healthy tissue (Table 5). The number of caspase-3 positive cells 1 d post surgery was 183±8.5, which then increased to 194.6±11.33 d post surgery, and finally decreased to 190±8.9 7 d post surgery (Table 5). There were statistically significant differences in cell counts between the sham-operated control groups and the two SCI groups (F2, 78=1678.21, P < 0.001). The expression of bcl-2 decreased from the highest level (64.2±7.1) 8 h after injury in SCI group to the lowest level 3 d after injury (34.6±5.9), before increasing 7 d after injury (104.2±10.4).

Positive cells were found at the lesion center and in a distant location in the SCI/nimodipine group 1 d post-trauma (Table 5). The mean number of caspase-3-positive cells in the SCI/nimodipine group was 157.4±14.2 at 3 d post-injury, which was significantly lower than the mean for the SCI group at the same time point (F3, 78=622.5, P < 0.001). The mean number of positive cells in SCI/nimodipine group was 138.2±7.47 d post injury, which was also significantly lower than of the SCI group (F2, 18=1389.28, P < 0.001). Similarly, the number of bax positive cells in the SCI/ nimodipine group was significantly lower than for the SCI group at both 3 d (F2, 18=945.15, P < 0.001) and 7 d (F2, 18=115.89, P < 0.001) (Table 5).

The number of bcl-2-positive cells in the SCI/ nimodipine group at 8 h post-injury was 81.2±5.7, 3 d was 55.2±8.3 and 7 d was 132±8.4. There were statistically significant differences between the trauma-only

![Fig. 2 The mitochondrial membrane potential (ΔΨm) across groups](image-url)
### Table 4  Cell proportions at G0/G1, G2/M, G2/G1 and S phases in different groups

<table>
<thead>
<tr>
<th>Group</th>
<th>G0/G1 cells (%)</th>
<th>G2/M cells (%)</th>
<th>G2/G1 cells (%)</th>
<th>S cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>SCI</td>
<td>SCI/nimodipine</td>
<td>Control</td>
</tr>
<tr>
<td>8 h</td>
<td>98.4±0.23</td>
<td>97.1±0.14***</td>
<td>96.88±0.18***</td>
<td>2.19±0.01</td>
</tr>
<tr>
<td>1 d</td>
<td>98.05±0.34</td>
<td>97.46±0.34**</td>
<td>97.46±0.24***</td>
<td>1.57±0.03</td>
</tr>
<tr>
<td>3 d</td>
<td>99.1±0.41</td>
<td>95.88±0.18***</td>
<td>96±0.32***</td>
<td>0.45±0.04</td>
</tr>
<tr>
<td>7 d</td>
<td>98.72±0.28</td>
<td>97.42±0.41***</td>
<td>98.61±0.37##</td>
<td>0.33±0.03</td>
</tr>
</tbody>
</table>

Data expressed as mean±SD. Asterisks indicate level of significant differences between members of treatment and control pair (*, \(P<0.05\); **, \(P<0.01\); ***, \(P<0.001\)), # indicates level of significant differences between members of treatment with SCI and SCI/nimodipine (##, \(P<0.01\); ###, \(P<0.001\)).

One-Way ANOVA, LSD multiple comparisons. (G0/G1 phase: 8h, \(F_{2,18}=228.00\); 24h, \(F_{2,18}=454.94\); 168h, \(F_{2,18}=41.84\). G2/M phase: 8h, \(F_{2,18}=1168.92\); 24h, \(F_{2,18}=1513.79\); 168h, \(F_{2,18}=1025.29\). G2/G1 phase: 8h, \(F_{2,18}=1.80\); 24h, \(F_{2,18}=16.70\); 168h, \(F_{2,18}=110.41\). S phase: 8h, \(F_{2,18}=2.90\); 24h, \(F_{2,18}=3007.92\); 168h, \(F_{2,18}=8812.19\); 168h, \(F_{2,18}=214.23\).

### Table 5  number of immunohistochemical positive cells in different groups using three kinds of antibody

<table>
<thead>
<tr>
<th>Group</th>
<th>caspase-3</th>
<th>bax</th>
<th>bcl-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>SCI</td>
<td>SCI/nimodipine</td>
</tr>
<tr>
<td>8 h</td>
<td>8.3±0.3</td>
<td>153.6±8.3***</td>
<td>141.2±5.8***</td>
</tr>
<tr>
<td>1 d</td>
<td>9±0.6</td>
<td>183±8.5***</td>
<td>172±8.9***</td>
</tr>
<tr>
<td>3 d</td>
<td>8.7±0.3</td>
<td>194.6±11.3***</td>
<td>157±14.2***</td>
</tr>
<tr>
<td>7 d</td>
<td>9±0.6</td>
<td>190±8.9***</td>
<td>138.2±7.4***</td>
</tr>
</tbody>
</table>

The data are expressed as mean±SD. Asterisks indicate level of significant differences between members of treatment and control pair within a row (**, \(P<0.001\)), # indicates level of significant differences between members of treatment with SCI and SCI/nimodipine within a row (##, \(P=0.01\); ###, \(P=0.001\)).

One-Way ANOVA (caspase-3: 8 h, \(F_{2,18}=1351.24\); 24h, \(F_{2,18}=1347.10\); 168h, \(F_{2,18}=1389.28\). bax: 8 h, \(F_{2,18}=98.86\); 24h, \(F_{2,18}=68.34\); 168h, \(F_{2,18}=945.15\). bcl-2: 8 h, \(F_{2,18}=305.65\); 24h, \(F_{2,18}=69.01\); 168h, \(F_{2,18}=85.09\); 168h, \(F_{2,18}=444.33\) followed by LSD Post Hoc Test.
group and nimodipine-treated group (8 h: $F_{2,18}=305.65$, $P < 0.001$; 3 d: $F_{2,18}=85.08$, $P < 0.001$; 7 d: $F_{2,18}=444.32$, $P < 0.001$) (Table 5).

### 2.3.2 bcl-2/bax mRNA and caspase-3 mRNA ratio post-trauma
Mitochondrial membrane depolarization induces mitochondrial PTP opening, in which the bcl-2 family (Fig. 3) plays a regulatory role. RT-PCR assay showed that the amount of bax (Fig. 4) increased markedly at the after injury, with a 20% increase at 8 h, 410% increase at 1 d and 900% increase at 3 d. At 7 d post injury the levels decreased dramatically. The bax mRNA expression level in each subject of SCI/nimodipine group was remarkably lower than any individual of SCI group at all time points (8 h: $F_{2,18}=45.9$, $P < 0.001$; 1 d: $F_{2,18}=101.41$, $P < 0.001$; 3 d: $F_{2,18}=15.16$, $P < 0.001$).

In contrast, the amount of bcl-2 expression in SCI group declined drastically to between 25%–50% of original levels. The bcl-2 expression level in the SCI/nimodipine group was higher than for the SCI group (Fig. 4). As a result, the ratio of bcl-2/bax (Fig. 5) in SCI/nimodipine group 8 h post-injury was was 23% of the control group, and then gradually decreased with the prolonged recovery time. The ratio at 7 d recovered to the 8 h value. The ratio of bcl-2/bax in the SCI/nimodipine group was remarkably higher than that in the SCI group (8 h, $F_{2,18}=1311.06$, $P < 0.001$; 1 d, $F_{2,18}=2717.53$, $P < 0.001$; 3d, $F_{2,18}=1881.6$, $P < 0.001$; 7 d, $F_{2,18}=1200.83$, $P < 0.001$).

The amplified products of approximately $\beta$-actin (498 bp) and caspase-3 (381 bp) were similar in size as expected (Fig. 6). Caspase-3 mRNA levels in the spinal

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**Fig. 3** Electrophoresis photo and expression of mRNA of relative quantity RT-PCR for bcl-2 at different time-periods
Data expressed as mean±SD, Asterisks indicate level of significant differences between members of treatment and control pair, # indicates level of significant differences between members of treatment with SCI and SCI/nimodipine. ***, $P<0.001$; **, $P<0.05$ (One-Way ANOVA, LSD multiple comparisons: 8 h, $F_{2,18}=242.96$; 24 h, $F_{2,18}=287.24$; 72 h, $F_{2,18}=161.40$; 168 h, $F_{2,18}=169.20$)

**Fig. 4** Electrophoresis photo and expression of mRNA of relative quantity RT-PCR for bax at different time-periods
Data expressed as mean±SD, Asterisks indicate level of significant differences between members of treatment and control pair, # indicates level of significant differences between members of treatment with SCI and SCI/nimodipine. ***, $P<0.001$; ***, $P<0.001$ (One-Way ANOVA, LSD multiple comparisons: 8 h, $F_{2,18}=1.5$; 24 h, $F_{2,18}=45.90$; 72 h, $F_{2,18}=101.41$; 168 h, $F_{2,18}=15.16$)
cord began to rise at 8 h after injury, with significant differences between the levels for the control and SCI groups at all time points after surgery. Both groups reached a peak after 3 d, but the levels decreased and returned to normal after 14 d. The expression of caspase-3 mRNA at 1 d, 3 d and 7 d of post-injury in the SCI/nimodipine group was significantly lower than those in the SCI group at the corresponding time point (1 d, \( F_2,18=59.83, P < 0.001 \); 3 d, \( F_2,18=98.23, P < 0.001 \); 7 d, \( F_2,18=94.83, P < 0.001 \)).

3 Discussion

3.1 SCI decreases spinal cord cell viability and induces apoptosis through the mitochondria pathway

A series of secondary changes occur in local spinal cord tissue after SCI. Secondary injury is an active regulatory process at the cellular and molecular levels, which can last for hours and can be reversed and controlled (Casha et al., 2001; Takahashi et al., 1999; Ramer et al., 2000). In this study, we found that mitochondria are directly involved in the stress cellular response due to the interaction between mitochondrial membrane potential changes and apoptosis. Cell apoptosis reached a peak at 3 d post injury. We found that the induction of cell death was associated with the loss of mitochondrial transmembrane potential and mitochondria dysfunction. Intra-mitochondrial contents release is controlled by mitochondrial PTP. The opening of PTP, which is located in the mitochondrial inner membrane, is responsible for the onset of mitochondrial

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**Fig. 5** The ratio of bcl-2/bax mRNA at different times

Data expressed as mean ± SD. Asterisks indicate level of significant differences between members of treatment and control pair, # indicates level of significant differences between members of treatment with SCI and SCI/nimodipine. ***, P<0.001; **, P<0.01; *, P<0.05; #, P<0.05; ##, P<0.01; ###, P<0.001 (One-Way ANOVA, LSD multiple comparisons: 8 h, \( F_2,18=1311.06 \); 24 h, \( F_2,18=2717.53 \); 72 h, \( F_2,18=1881.61 \); 168 h, \( F_2,18=1200.83 \)).

**Fig. 6** Electrophoresis photo and the expression of mRNA of relative quantity RT-PCR for caspase-3 at different time-periods

Data expressed as mean ± SD. Asterisks indicate level of significant differences between members of treatment and control pair, # indicates level of significant differences between members of treatment with SCI and SCI/nimodipine. ***, P<0.001; **, P<0.01; *, P<0.05; #, P<0.05; ##, P<0.01; ###, P<0.001 (One-Way ANOVA, LSD multiple comparisons: 8 h, \( F_2,18=59.83 \); 24 h, \( F_2,18=65.81 \); 72 h, \( F_2,18=98.29 \); 168 h, \( F_2,18=94.83 \)).
permeability transition (MPT). The rapid change of permeability associated with MPT causes membrane depolarization, uncoupling of oxidative phosphorylation, release of intramitochondrial ions and metabolic intermediates, and large-amplitude mitochondrial swelling (Mazur et al., 2002). Once PTP is opened, the disruption of the ΔΨm of the inner mitochondrial membrane leads to cytochrome c release, which combines with apoptosis protease activating factor 1, dATP, and procaspase-9 to form an apoptosome (Kroemer et al., 1998; Van der Heiden and Thrompson, 1999; Lemasters et al., 1998). Furthermore, procaspase-9 generates a holoenzyme capable of activating caspase-3 and caspase-7. Mitochondrial membrane potential changes (ΔΨm), and changes to the levels of caspase-3 mRNA were detected after SCI, while both the maximal apoptosis rate and the maximal late apoptosis/necrosis rate occurred at 3 d post-injury. We suggest that injury-induced mitochondria dysfunction is an early event in vivo, while significant apoptosis and DNA fragmentation were later events.

Our study showed that SCI leads to DNA damage and DNA fragmentation which further induced G2/M arrest. G1/S and G2/M checkpoints prevent the acquisition of multiple genetic changes by ensuring that DNA is repaired before replication in S phase and properly segregated during the M phase. Both DNA damage and incomplete DNA repair induce cell arrest at the G2/M checkpoint control to prevent possible mutations, translocations, or chromosome loss (Bose et al., 2005). In summary, it is suggested that mild SCI decreases cell viability and induces spinal cord cell apoptosis by a mitochondria-triggered apoptosis/necrosis pathway, which further induces G2/M arrest through DNA damage.

3.2 Nimodipine can alleviate SCI impairments induced by mitochondria-triggered apoptosis pathway through up-regulation of the bcl-2/bax ratio

The Ca²⁺ channel antagonist nimodipine may be a promising way to treat SCI. Here, we suggest that the inhibition of Ca²⁺ flux by nimodipine can efficiently alleviate apoptotic cell death and help maintain cell viability. Zhu et al. (2000) used the distinct models of apoptosis and necrosis to investigate the effects of mitochondrial Ca²⁺(CaM) homeostasis in the regulation of cell death in neurolastoma cells as well as cardiac myocytes. They found that Ca²⁺ homeostasis can regulate apoptosis and necrosis. Shimogol and Kostyuk (1995) showed that hyperglyceremia and surplus Ca²⁺ extrusion play a role in calcium signal transmission, and nimodipine was an effective drug to stimulate spontaneous cell activity. Our study helps verify the therapeutic effect of nimodipine for functional recovery from spinal cord injury proposed by previous researchers.

Our findings showed that the SCI-treated groups injected with nimodipine rehabilitated better than the trauma-only groups. The ratios of apoptosis, caspase-3 mRNA and bax mRNA expression were lower than those in the trauma groups, and the mitochondrial membrane potential and bcl-2 mRNA expression were up-regulated by nimodipine. We deduce that nimodipine alleviates PTP opening through regulation of the bcl-2/bax ratio. Results from the inclined plane test and the motor score assessment for nimodipine group support this deduction. These findings combinedly indicate that inhibition of calcium flux by nimodipine can lead to lower levels of apoptosis and tissue damage after SCI, and that this process may be mediated through mitochondrial pathways.

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