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Research Report

Overexpressing neuroglobin improves functional recovery by inhibiting neuronal apoptosis after spinal cord injury

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ABSTRACT

The current study was performed to evaluate the mechanisms and therapeutic effects of overexpressing neuroglobin (Ngb) on spinal cord injury (SCI). Adeno-associated virus (AAV) was injected in the T12 section 7 days before SCI. Animals were randomly divided into four groups: a sham group, a vehicle group, an AAV-EGFP group and an AAV-Ngb group. Recovery of hind limb locomotor function was determined during the 3-week post operation period by the Basso, Beattie and Bresnahan locomotor rating scale. At 24 h after SCI and at the end of the study, the segments of spinal cord, centered with the lesion site were harvested for histopathological analysis. Immunofluorescence was performed using antibodies to recognize neuN in the lesion sections. At 24 h after SCI, the spinal cord tissue samples were removed to analyze tissue concentrations of superoxide dismutase (SOD) and malondialdehyde (MDA). Apoptotic cells were assessed using a terminal deoxynucleotidyl transferase, dUTP nick end labeling (TUNEL) kit. The expression of bcl-2, bax, cytochrome c, and cleaved caspase-3, were determined by Western blot assay and immunostaining analysis. The results showed that animals overexpressing Ngb had significantly greater recovery of locomotor function, less neuronal loss and fewer apoptotic cells. In addition, overexpressing Ngb significantly increased bcl-2 expression and SOD level, decreased bax expression, attenuated the release of cytochrome c from mitochondria to the cytosol fraction, and reduced the activity of caspase-3 and MDA level after SCI. These findings suggest, that overexpressing Ngb can significantly improve the recovery of locomotor function. This neuroprotective effect may be associated with the inhibition of neural apoptosis via the mitochondrial pathway.

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1. Introduction

Spinal cord injury (SCI) is a highly debilitating pathology that can often lead to devastating and catastrophic dysfunction (Di Paola et al., 2011). It results in negative physical and psychological effects on the individual and the family. The pathophysiology of SCI involves primary and secondary mechanisms of injury (Oyinbo, 2011). The primary injury is irreversible; whereas, the mechanisms of the secondary injury include ionic disturbances, vascular changes, generation of free radicals and free radical induced-lipid peroxidation, glutamate concentration, mitochondrial damage, and apoptosis (Blight, 2002). Apoptosis is a very important mechanism of secondary injury after SCI (Liu et al., 2011) that is triggered by a number of mechanisms. In recent years, many studies have focused on secondary injury, given the positive response to therapeutics using free radical scavengers or anti-apoptotic drugs.

Neuroglobin (Ngb) is expressed in neurons, retinal cells and some endocrine tissues and is present in all vertebrates (Brittain, 2012). Several studies have demonstrated that Ngb has a neuroprotective effect. Ngb overexpressing transgenic (Ngb-Tg) mice were more resistant to focal cerebral ischemia *via* a mechanism that reduced oxidative stress compared to wild-type mice (Wang et al., 2008). Overexpressing Ngb has been shown to be neuroprotective against hypoxia *in vitro*, in part by improving mitochondria function and decreasing oxidative stress (Liu et al., 2009). In addition, Shang et al. (Shang et al., 2012) demonstrated that overexpressing Ngb exerts significant neuroprotective effects after mechanical injury. Thus, Ngb may promote the treatment of reactive oxygen species (ROS)-related diseases (Li et al., 2011). The origin of Ngb's protective mechanism is in the intrinsic apoptotic pathway. Ngb has been shown to intervene in this process by modulating the release of mitochondrial cytochrome c (Brittain, 2012). In our recent study, we demonstrated that overexpressing Ngb had neuroprotective effects after SCI (Chen et al., 2012). However, the mechanisms underlying the neuroprotection induced by overexpressing Ngb are not fully understood.

In the present study, we determined the efficacy of overexpressing Ngb, by pretreating for 7 days prior to the onset of SCI. We also examined the expression of bax, bcl-2, cleaved-caspase-3, and cytochrome c following the overexpression of Ngb in the presence of apoptosis induced by traumatic SCI in order to determine the mechanism underlying the overexpression of Ngb.

2. Results

2.1. *AAV-Ngb increase Ngb expression in the spinal cord*

As shown in Fig. 1(C)–(E), the representative GFP signal in tissue section at ay 7 after AAV-EGFP injection and the transduction efficiency was more than 85%. In Fig. 1(E), (G) and (H), the level of Ngb expression was determined by Western blot analysis. There was no significant difference in the level of Ngb protein expression between the two groups prior to injection. However, the level of Ngb protein expression in the AAV-Ngb group increased significantly at day 3, 7, and 14 compared to the levels in the NS groups ($p<0.05$). The level of Ngb reached a peak level at day 7 and remained at this level through day 14.

2.2. *AAV-Ngb reduces motor disturbances after SCI*

The hind limb locomotor functions after SCI were observed in all groups. As shown in Fig. 2, the sham group initially showed a slight decrease in the BBB score, but showed full recovery by 3 days. The vehicle, AAV-EGFP, and AAV-Ngb groups initially showed a sharp decrease in the BBB score. Animals in the vehicle and AAV-EGFP groups showed only a partial recovery until 3 weeks after SCI. In contrast, overexpression of Ngb significantly ameliorated the hind limb locomotor disturbance.

2.3. *AAV-Ngb increases neuronal survival after SCI*

Neuron survival was examined by an immunofluorescence technique using a specific antibody for neuron (neuN) and TUNEL analysis (Fig. 3). Qualitatively, neuron density was decreased in the lesion sections of the vehicle group, the AAV-EGFP group, and the AAV-Ngb group when compared to the corresponding sections of the sham group. However, AAV-Ngb significantly attenuated the decrease of neuron survival compared to vehicle animals. There was no difference between the apoptotic index (AI=(the number of the TUNEL-positive cells)/(the total number of the nucleated cells)) in the vehicle group and the AAV-EGFP group. The number of TUNEL-positive cells in the AAV-Ngb group was significantly decreased compared to the vehicle group and the AAV-EGFP group.

2.4. *AAV-Ngb attenuates oxidative damages after SCI*

MDA and SOD were used for biochemical evaluation. Fig. 4 shows the effects of overexpressing Ngb on the levels of SOD (A) and MDA (B). Values are expressed as mean±SD; $n=4$ for each group. Statistical analysis was performed using a one-way ANOVA with Tukey's test ($^*p<0.05$ compared to the vehicle group). In the spinal cord tissues, the MDA levels were significantly higher in the vehicle, AAV-EGFP and AAV-Ngb groups than the sham group. At the same time the SOD levels were significantly lower in the vehicle, AAV-EGFP, and AAV-Ngb groups compared to the sham group. However, overexpressing Ngb prevented the elevation of MDA levels and the attenuation of SOD levels.

2.5. *The effects of AAV-Ngb on bcl-2 and bax expression after SCI*

In order to further quantitatively assess the effects of overexpressing Ngb on the activation of apoptotic pathways, immunostaining and Western blot analysis were carried out to measure the levels of expression of apoptosis regulatory proteins, such as bcl-2 (anti-apoptotic) and Bax (pro-apoptotic), in the sham, vehicle, AAV-EGFP, and AAV-Ngb treated animals (Fig. 5, $^*p<0.05$ compared to the sham group). A significant decrease in bcl-2 expression and an increase in bax expression were observed in the vehicle, AAV-EGFP

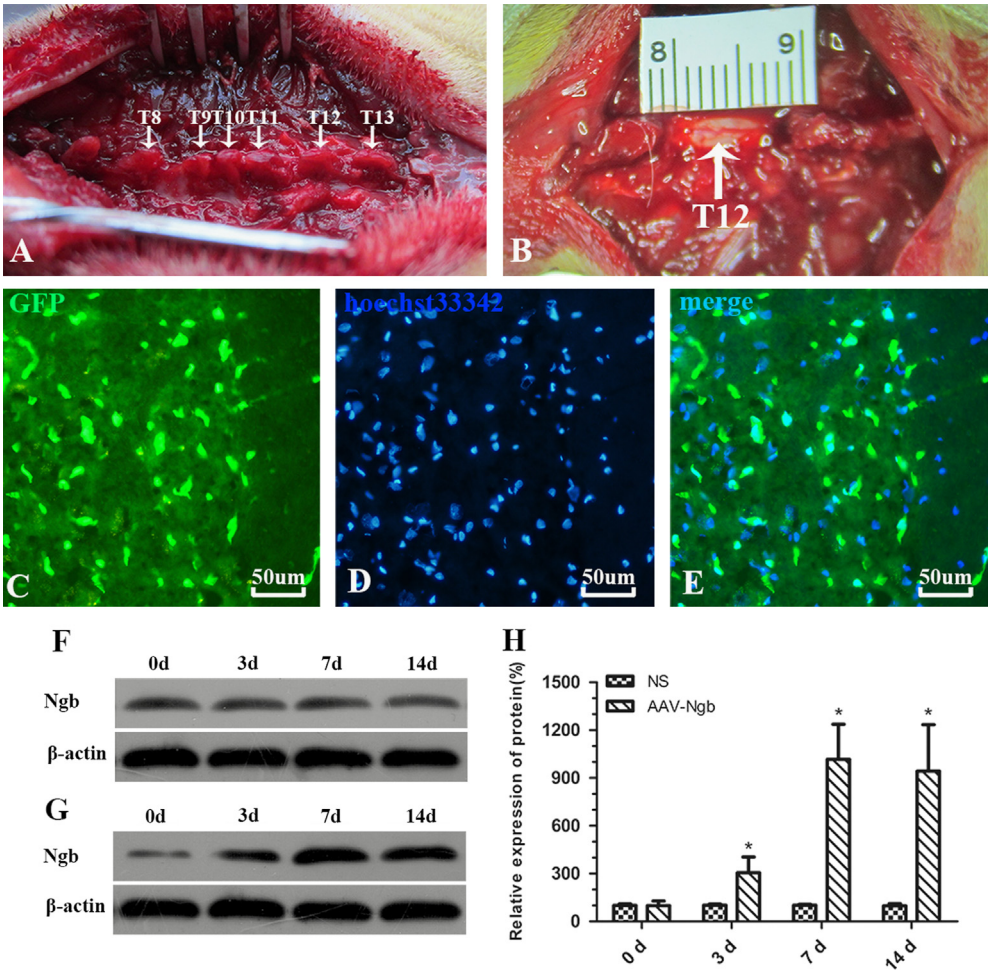

Fig. 1 – The examination of Ngb protein in the spinal cord tissues. The AAV injective and SCI position was at T12 (A) and (B). The GFP signal in tissue section at ay 7 after AAV-EGFP injection and the transduction efficiency was measured under the fluorescence microscope (C)–(E). Ngb and β-actin were assessed by Western blot assay in the spinal cord obtained from the NS group (F) and the AAV-Ngb group (G) at four different time points. A representative blot is shown; the densitometric analysis of protein bands of all animals is reported in panel H ($n \geq 3$ for each group, $^{*}p < 0.05$ compared to the NS group).

and AAV-Ngb groups compared to the sham group. However, treatment with AAV-Ngb could attenuate the increased expression of bax and the decreased expression of bcl-2.

2.6. *AAV-Ngb attenuates the release of cytochrome c after SCI*

The levels of cytochrome c expression in the cytosol or mitochondrial fraction from the lesion tissues of sham, vehicle, AAV-EGFP and AAV-Ngb groups were determined by Western blot assay. The expression of cytochrome c increased in the cytosolic fraction and decreased in the mitochondrial fraction after T12 SCI (Fig. 6(A)). The release of cytochrome c from mitochondria into the cytosol after SCI was antagonized by overexpressing Ngb (Fig. 6(B)).

2.7. *AAV-Ngb attenuates active caspase-3 expression after SCI*

In the present study, the levels of activated caspase-3 were determined by immunofluorescence assays (Fig. 7(A)–(E)) and by Western blot detection of a 17/19-kDa fragment (active form) (Fig. 7(F) and (G)) for the different treatment groups. Overexpressing Ngb decreased the level of active caspase-3 protein to a greater extent compared to the vehicle treated group.

3. Discussion

This present study demonstrates that overexpression of Ngb at the lesion of the spinal cord had protective effects after SCI. First, we demonstrated that the intracellular levels of Ngb in adult spinal neurons were elevated by transduction with AAV-Ngb. Second, we found that overexpressing Ngb promoted functional recovery after SCI. Third, three weeks after SCI, the number of neuN positive cells in AAV-Ngb treated rats was significantly increased compared to vehicle treated or AAV-EGFP treated rats. Fourth, we found that overexpressing Ngb resulted in an increase in bcl-2, a decrease in bax and activated caspase-3, and the release of

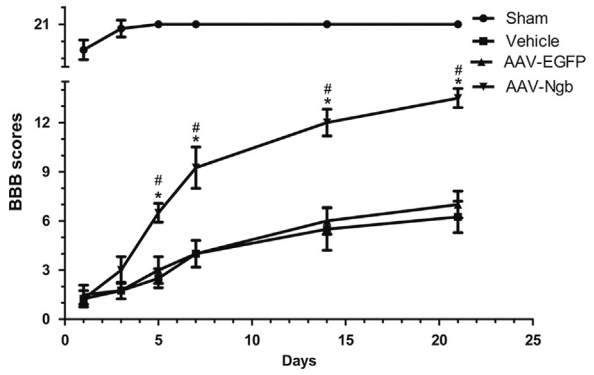

Fig. 2 – Effects of overexpressing Ngb on hind limb locomotor disturbances after compression SCI. The degree of motor disturbance was assessed at 1, 3, 5, 7, 14, and 21 days after SCI by BBB criteria. Data are presented as means ± SD ($n=4$, each). $^{*}p<0.05$ compared to the vehicle group and $^{\#}p<0.05$ compared to the AAV-EGFP group.

cytochrome c from mitochondria to the cytoplasm compared to vehicle treated or AAV-EGFP treated rats.

Over the last few years, a large number of studies have demonstrated that gene therapies for spinal cord injuries have great potential. Gene transfer, using viral vectors with specific genes for therapeutic purposes, has been particularly successful for the treatment of SCI (Chen et al., 2007; Fortun et al., 2009; Kwon et al., 2007; Nakajima et al., 2010; Parikh et al., 2011; Zhang et al., 2007; Zhao et al., 2012). AAV vectors are prospective viral systems for gene delivery in the spinal cord because they not only can achieve sustained, long-tern specific gene expression for the treatment of SCI, but also can transduce post-mitotic cells, such as neurons and astrocytes (Klein et al., 2008; Snyder et al., 2011). Therefore, we used an AAV vector serotype 9 to deliver Ngb gene to the cord of the T12 segment *in vivo*. Over-expressing Ngb in the injected site peaked at 7 days after AAV-Ngb was injected. Thus, in this study, we injected the AAV-Ngb 7 days before SCI. Ngb plays a significant role in protecting the brain from hypoxic and/or ischemic injury (Khan et al., 2006; Sun

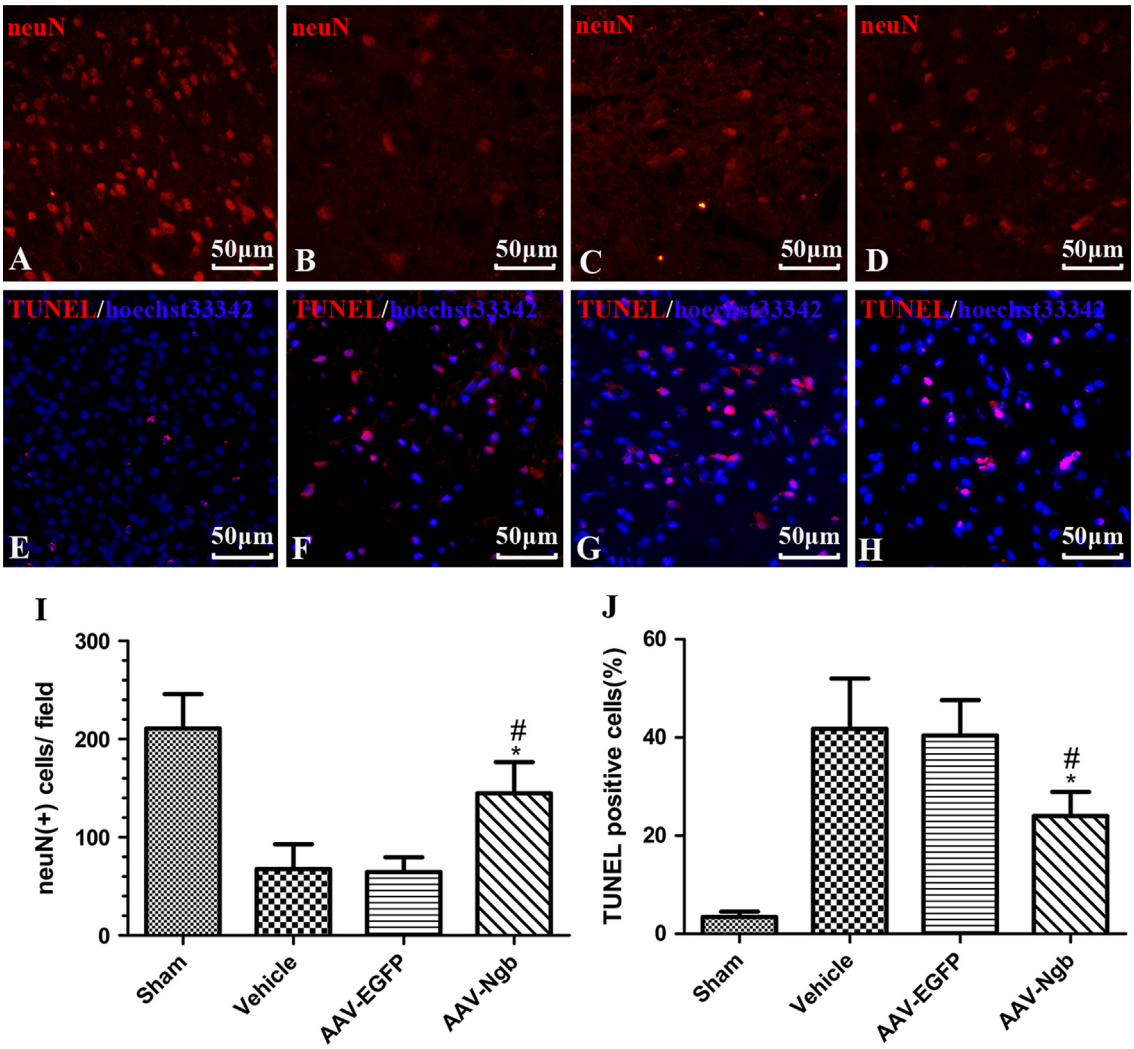

Fig. 3 – The effect of AAV-Ngb on the survival of neurons. The density of neurons at day 21 after SCI in the sham (A), vehicle (B), AAV-EGFP (C), and AAV-Ngb (D) groups was determined by immunofluorescent labeling with anti-neuN antibody. The TUNEL positive cells at 24 h after SCI in the sham (E), vehicle (F), AAV-EGFP (G), and AAV-Ngb (H) groups were measured by a TUNEL kit. (I) and (J) Values are presented as mean ± SD. $^{*}p<0.05$ compared to the vehicle group and $^{\#}p<0.05$ compared to the AAV-EGFP group.

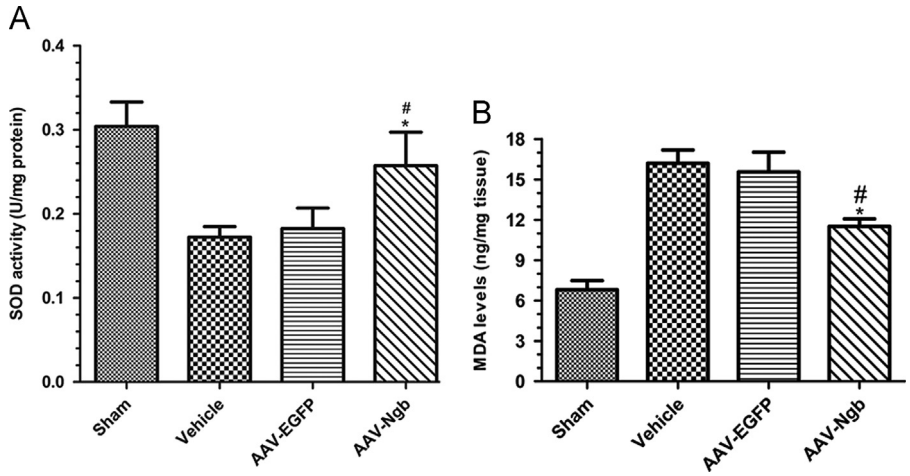

Fig. 4 – The effect of overexpressing Ngb on the levels of SOD (A) and MDA (B). Values are expressed as mean ± SD; $n=4$ for each group. Statistical analysis was performed using a one-way ANOVA with Tukey's test. $^{*}p<0.05$ compared to the vehicle group and $^{\#}p<0.05$ compared to the AAV-EGFP group.

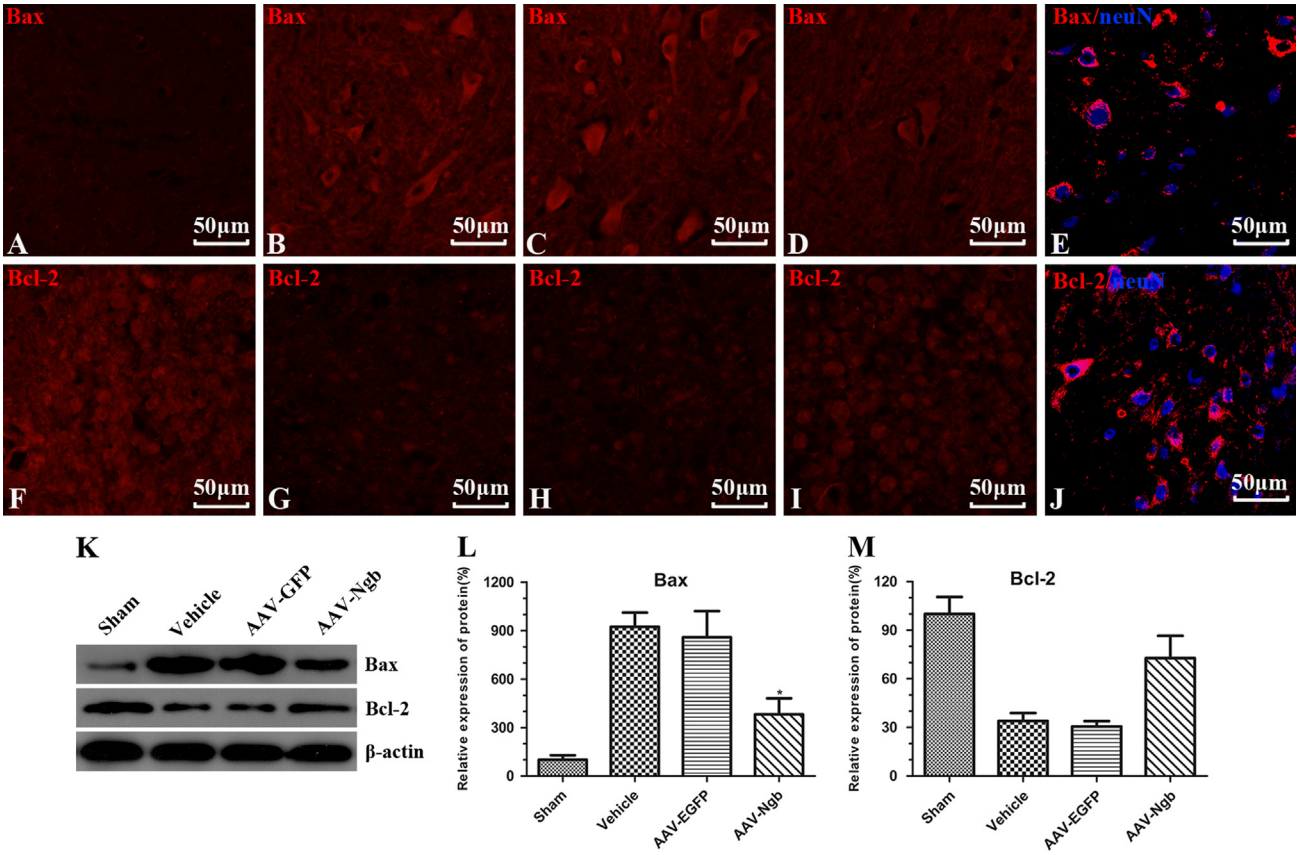

Fig. 5 – Effects of overexpressing Ngb on bax and bcl-2 levels of expression after SCI. In the sham group, there was no positive staining for bax in the lesion section (A). An increase in bax expression was found in the vehicle group (B). AAV-Ngb significantly inhibited the SCI-induced increase in bax expression (D), whereas AAV-EGFP (C) did not have this effect. However, positive staining for bcl-2 was observed in the lesions of sham-operated rats (F). There was less staining for bcl-2 observed after SCI (G). The bcl-2 expression in the AAV-EGFP group was similar to the vehicle group (H). Overexpressing Ngb significantly prevented the decrease of bcl-2 expression after SCI (I). And the Bcl-2 (red) and Bax (red) signals are most within neurons (blue) (E) and (J). Immunoblots (K) showed bax expression increased and bcl-2 expression decreased in the lesion tissues from SCI. However, overexpressing Ngb significantly increased the bcl-2 expression and reduced the bax expression. In panel (L) and (M), the levels of bax and bcl-2 were normalized to total actin and then expressed as a relative percentage compared to the sham group.

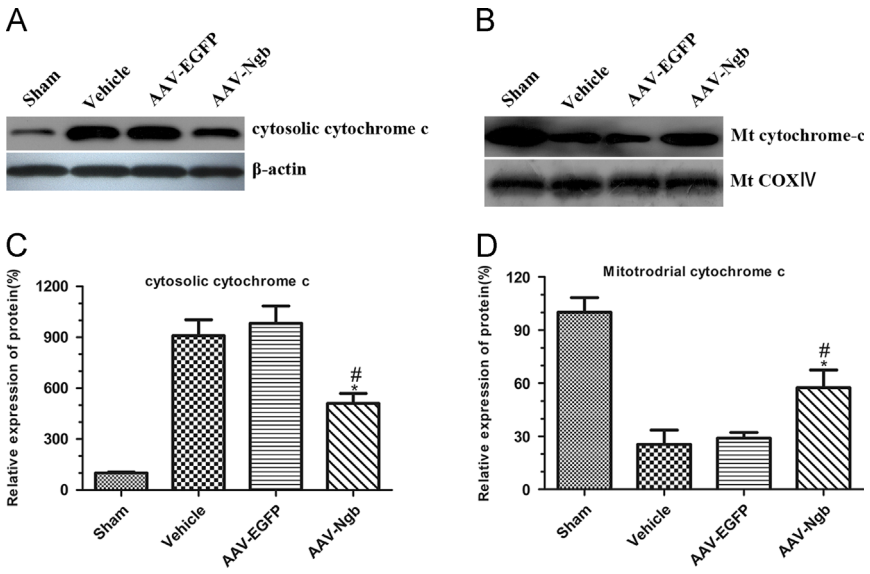

Fig. 6 – Effects of overexpressing Ngb on the release of cytochrome c. Cytochrome c oxidase (COX) was used as a control of the mitochondrial fraction and actin was used as a control of the cytosolic fraction. (A) and (B) The spinal cord tissue lysates of the sham, vehicle, AAV-EGFP and AAV-Ngb groups were used for immunoblotting. (C) and (D) Values are mean ± SD, $n \geq 3$ animals per group. $^{*}p < 0.05$ compared to the vehicle group, and $^{\#}p < 0.05$ compared to the AAV-EGFP group.

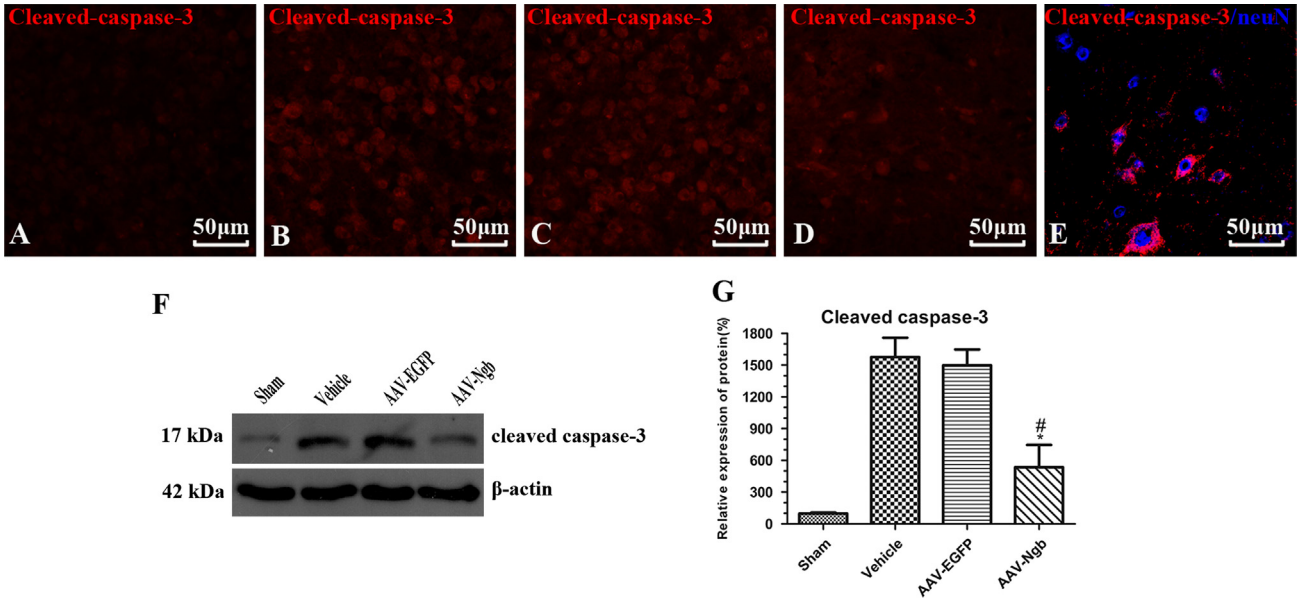

Fig. 7 – Effects of overexpressing Ngb on caspase-3 activation by immunostaining and Western blot assay. Immunostaining of spinal cord tissues with active caspase-3 in the sham (A), vehicle (B), AAV-EGFP (C), and AAV-Ngb (D) groups at 24 h after SCI. And the cleaved caspases-3 (red) signals are most within neurons (blue) (E). (F) The expression level of active caspase-3 protein was examined by western blot. Actin expression was detected as a control. The densitometry of each band was showed in panel G. $^{*}p < 0.05$ compared to the vehicle group. $^{\#}p < 0.05$ compared to the AAV-EGFP group.

et al., 2003), and also from traumatic brain injury (Shang et al., 2012). Here, we assessed the therapeutic effects of overexpressing Ngb in the SCI. Results showed that overexpressing Ngb promoted function recovery after SCI.

Apoptosis, the process of programmed cell death, is considered a vital component of various processes, including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development, and chemical-induced cell death (Elmore, 2007). Apoptosis is one of the mechanisms of secondary injury in SCI (Yin et al., 2012). In the present study, we demonstrated that overexpressing Ngb can attenuate apoptosis of the injured cord. Thus, the inhibitory effect of overexpressing Ngb on apoptosis implied that inhibition of apoptosis contributes to the mechanism by which overexpressing Ngb restrains the extent of secondary injury.

The increased production of ROS, which induces lipid peroxidation, is one of the most important factors that contribute to spinal cord motor neuron death following SCI (Hall, 1993; Xu et al., 2005). SOD enzyme activity and MDA are widely used as indexes of oxidative damage (Liu et al., 2011). SCI significantly increased MDA levels and also decreased superoxide dismutase (SOD) enzyme activity in spinal cord tissue (Kalayci et al., 2005). Previous reports stated that Ngb expression enhances the response to oxidative stress and overexpression of Ngb can decrease ROS production (Li et al., 2008; Nayak et al., 2009). The results in this study indicated that overexpressing Ngb could notably attenuate the decrease in SOD enzyme activity and the increase in MDA levels due to its ability to scavenge ROS.

Intracellular ROS production is consistent with mitochondrial damage (Kim et al., 2004). The previous findings strongly suggest that the increased production of ROS is an early and likely causal event that contributes to spinal cord motor neuron death following SCI (Xu et al., 2005). Excessive production of ROS may cause mitochondrial dysfunction and stimulate cytochrome c release, resulting in cell apoptosis (Orrenius, 2007). In the present study, we found that overexpressing Ngb attenuated the increase in MDA and the decrease in SOD enzyme activity after SCI. Therefore, we suggest that the antioxidant effect of overexpressing Ngb contributes to its mitochondrial protection.

In the mitochondrial apoptosis pathway, mitochondria open the channels in the outer membrane, thereby releasing cytochrome c from the inter membrane space of the mitochondria into the cytosol. Cytochrome c then activates caspases by binding to Apaf-1 and inducing it to associate with procaspase-9, thereby triggering caspase-9 activation and initiating the proteolytic cascade that culminates in apoptosis (Green and Reed, 1998). The permeability of the outer membrane of mitochondria and the release of cytochrome c are regulated by some proteins. Among these proteins, bax and bcl-2 play a most important role in the apoptosis pathway related to mitochondria. The pro-apoptosis factor bax is a nuclear-encoded protein residing in the cytosol that adopts a globular alpha-helical structure, seemingly as monomers. Following a variety of stress signals, it converts into pore-forming proteins by changing conformation and assembling into oligomeric complexes in the mitochondrial outer membrane. Cytochrome c from the mitochondrial inter membrane space then empties into the cytosol (Westphal et al., 2011). Release of cytochrome c triggers active caspase-3 and ultimately induces cell apoptosis. On the contrary, the anti-apoptosis factor bcl-2 presents in the outer mitochondrial membrane which blocks cytochrome c release from mitochondria to the cytosol (Wu et al., 2009). Recent studies demonstrated that bcl-2 restrains apoptosis through regulating an antioxidant pathway (Luna-Lopez et al., 2013) and inhibits neural death by reducing the generation of ROS (Kane et al., 1993). In this study, we demonstrated that SCI down-regulated the expression of bcl-2 protein and up-regulated the expression of bax protein, whereas AAV-Ngb blocked these effects. Thus, we suggest that the neuroprotective effects of AAV-Ngb are associated with the reduction in ROS production and the maintenance of mitochondrial function.

In summary, our present observations suggest that over-expressing Ngb is an important treatment against SCI. The mechanism may be related to the maintenance of mitochondrial function by reducing SCI-induced ROS production and inhibiting the reduction of bcl-2 expression and the increase in bax and active caspase-3 expression induced by SCI.

4. Materials and methods

4.1. *Production of AAV-Ngb and injection*

The synthesis of recombinant adeno-associated virus (AAV) vector particles involved inserting the rat Ngb cDNA from a recombinant vector carrying the Ngb gene (yrgene, Hunan, China) in the plasmid pAOV-CMV-EGFP (neuronbiotech, Shanghai, China). As a negative control, an adenoviral vector expressing enhanced green fluorescent protein (EGFP) of the cytomegalovirus (CMV) promoter was constructed. pAOV-CMV-EGFP or pAOV-CMV-EGFP-Ngb plasmid and the helper plasmid pAAV-RC were propagated in HEK293 cells by calcium phosphate. The viruses were purified using CsCl density gradient dialysis. The titer of the viruses was then determined by quantitative polymerase chain reaction (qPCR).

The expression levels of Ngb at 0d, 3d, 7d, and 14d after injecting AAV-Ngb or NS were tested by Western blot. Eight rats ($n=4$ per group, AAV-Ngb group and NS group) were used in a preliminary experiment to determine the injection time of AAV before SCI. In this pilot study, the rats were anesthetized with chloral hydrate (500 mg/kg) and a laminectomy was performed at the T12 level exposing the cord. In the AAV-Ngb group, 3 μl of AAV-Ngb at a concentration of 3×10^{12} vg/ml was injected in the cord at T12 using a 5 μl micro-syringe with a 33-gauge needle (Hamilton, Reno, NV, USA) at a rate of 0.2 μl/min. In the NS group, 3 μl of NS was injected in the cord at T12. The needle was left for 5 min after injection and then slowly withdrawn. The muscles and skin were then sutured. We determined that high levels of Ngb expression occurred at seven days following the injection. Therefore, a seven day protocol was used in all experiments.

4.2. *Spinal cord injury and experimental groups*

Adult male Sprague-Dawley rats (280–320 g) were used in this study. Experimental procedures complied with the Guide for the Use of Laboratory Animals and were approved by the Animal Experimentation Ethics Committee of the Fujian Medical University.

Spinal cord compression at T12 was performed following a previously established static compression model (Hall et al., 2012; Wang et al., 2011). Briefly, the skin and muscle were incised and a laminectomy was performed at T12 (Fig. 1(A) and (B)). The compression was applied by suspending the base of a compression platform (area 2×5 mm^2) onto the exposed cord. A weight of 50 g was applied statically to the platform for exactly 5 min. After removing the platform, the muscles and skins were sutured.

One hundred and twenty rats were divided into four groups (30 rats per group) in the following experiments. Rats in the sham group underwent a laminectomy only. Rats in the vehicle group received an injection of 3 μl NS, 7 days before SCI. Rats in the AAV-EGFP group received an injection

of 3 μl AAV-EGFP, 7 days before SCI. Rats in the AAV-Ngb group received an injection of 3 μl AAV-Ngb, 7 days before SCI. The AAV-EGFP, AAV-Ngb, and NS groups were injected into the cord at T12 using the method (Hall et al., 2012; Wang et al., 2011) described above.

4.3. Behavioral assessment

The Basso, Beattie, Bresnahan Locomotor Rating Scale (BBB) (Basso et al., 1996) was used to test the restoration of motor function after SCI at T12. Two individuals who were blind to treatment performed all behavioral testing at pre-injury and 1, 3, 5, 7, 14, and 21 days post-injury.

4.4. Collection of spinal cord tissues

At the designated time points (day 1 and 21) after SCI, the animals were anesthetized with chloral hydrate (500 mg/kg) and intracardially perfused with PBS followed by 4% paraformaldehyde in PBS. A 2-cm section which centered at the lesion site of the spinal cord was removed and post fixed overnight by immersion in the same fixative. The tissue was placed in 30% sucrose in PBS for 48 h. The segment was then embedded and cut into 10 um thick transverse (day 1 and 21) sections using a cryostat microtome (Thermo Fisher Scientific). Serial sections (10 μm thickness) were collected every 100 μm.

4.5. Measurement of SOD and MDA enzyme activities

The tissue samples were homogenized and used to measure the level of SOD and MDA. The SOD activity was measured with a superoxide dismutase assay kit (Jiancheng Bioengineering) and the activity was expressed in U/mg protein. Briefly, the reagents were added in 0.1 ml tissue homogenate, mixed with vortex shaker, and bathed at 37 °C for 40 min. After cooling, they were mixed with a chromogenic agent and placed stationarily at room temperature for 10 min. And then the absorbance of supernatant was read at 550 nm. Lipid peroxidation in the spinal cord samples was estimated by tissue MDA concentration. The MDA levels were determined by the commercial assay kit produced by Jiancheng Bioengineering in China and the activity was expressed as nmol/mg protein. In the test reaction, the reagents were added in 0.1 ml tissue homogenate and mixed with vortex shaker, bath at 95 °C for 40 min. After cooling, the precipitate was pelleted by centrifuging at 4000 rpm for 10 min and the absorbance of supernatant was read at 532 nm.

4.6. Histological staining and immunohistochemistry

The spinal cord sections from the compression epicenter were incubated independently with antibodies against NeuN (1:100, Chemicon, USA), bax (1:20, Abcam, England), bcl-2 (1:50, Abcam, England), and cleaved caspase-3 (1:400, CST, USA) overnight at 4 °C. The sections were then incubated with donkey anti-mouse or rabbit antibody conjugated with Alexa Flour 568/fluorescein-conjugated antibody, anti-mouse antibody conjugated with Alexa Flour 350/fluorescein-conjugated antibody or hoechst33342 (2.5 μg/ml, sigma, USA) for 1 h at room temperature. The fluorescence images were examined under a fluorescence microscope (IX71, Olympus, Tokyo, Japan). The immunostaining procedure and acquisition of images were performed under the same experimental conditions and at the same time for each of the different groups.

4.7. TUNEL assay

A commercially available one-step TUNEL apoptosis assay kit (Beyotime Institute of Biotechnology,) was used to label the DNA strand break according to the manufacturer's instruction. Briefly, the tissues were rinsed with PBS, then permeabilized by 0.1% Triton X-100 at 4 °C, and then washed with PBS. Sections were immersed in 50 μl of TUNEL reaction mixture and incubated in a humid atmosphere in the dark at 37 °C for 1 h. Finally, hoechst33342 (2.5 μg/ml, Sigma) was added for 30 min at room temperature. The positive cells were imaged under an Olympus microscope using 540-nm excitation and 560-nm emission.

4.8. Preparation of mitochondrial, cytosolic fractions and total cell lysates

Spinal cord segments with lesions were harvested at 24 h after SCI. Cytosolic and mitochondrial fractions were prepared according to the manufacturer's protocol by using a commercially available cytosol/mitochondria fractionation kit (Beyotime, China). The cellular proteins were extracted by RIPA buffer contained with 1 mM PMSF and centrifuged at 12,000 rpm for 30 min. Protein concentrations were determined by the BCA protein assay.

4.9. Western blot analysis

Proteins were separated on SDS-PAGE gels and then transferred to a PVDF membrane. The membrane was blocked in 5% nonfat milk in TBS-T buffer for 1 h and then incubated with rabbit anti-bcl-2 (1:500, Abcam, Cambridge, USA), rabbit anti-bax (1:500, Abcam, Cambridge, USA), rabbit anti-cleaved-caspase-3 (1:500, Cell Signaling Technology, Danvers, USA), rabbit anti-cytochrome c (1:500, Abcam, Cambridge, USA), rabbit anti-Ngb (1:400, Santa Cruz Biotechnology, Dallas, USA), rabbit anti-COX (1:400, Santa Cruz Biotechnology, Dallas, USA), or rabbit anti-β-actin (1:400, Cell Signaling Technology, Danvers, USA) in TBS-T for 12 h at 4 °C. After this step, the membrane was incubated with HRP-conjugated secondary antibody in TBS-T for 1 h at room temperature. The membrane was incubated with enhanced chemiluminescent (ECL) reagent (Amersham, Piscataway, USA) and exposed to films in a dark room for 5–10 min. The optical density (OD) was determined using Gel-Pro analyzer software (Media Cybernetics, Washington, USA).

4.10. Statistical analysis

All statistical analyses were performed using SPSS 19.0. The data were presented as mean ± SD. One-way or two-way analysis of variance was used to compare the experimental groups. Differences at $p<0.05$ were considered statistically significant.

Acknowledgments

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