Adenovirus vector-mediated *in vivo* gene transfer of nuclear factor erythroid-2p45-related factor 2 promotes functional recovery following spinal cord contusion

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Abstract. The aim of the present study was to investigate whether nuclear factor erythroid 2p45-related factor 2 (Nrf2) overexpression by gene transfer may protect neurons/glial cells and the association between neurons/glial cells and axons in spinal cord injury (SCI). In the present study, Nrf2 recombinant adenovirus (Ad) vectors were constructed. The protein levels of Nrf2 in the nucleus and of the Nrf2-regulated gene products heme oxygenase-1 (HO-1) and NAD (P)H-quinone oxidoreductase-1 (NQO1), were detected using western blot analysis in PC12 cells following 48 h of transfection. Furthermore, the expression of Nrf2 was localized using an immunofluorescence experiment, and the expression of Nrf2, HO-1 and NQO1 were detected using an immunohistochemical experiment in the grey matter of spinal cord in rats. Post-injury motor behavior was assessed via the Basso, Beattie and Bresnahan (BBB) locomotor scale method. In PC12 cells, subsequent to Ad-Nrf2 transfection, nuclear Nrf2, HO-1 and NQO1 levels were significantly increased compared with the control ($P<0.01$). There was statistically significant changes in the PC12-Ad-Nrf2 group [Nrf2 (1.146±0.095), HO-1 (1.816±0.095) and NQO1 (1.421±0.138)] compared with the PC12-control group [Nrf2 (0.717±0.055), HO-1 (1.264±0.081) and NQO1 (0.921±0.088)] and PC12-Ad-green fluorescent protein group [Nrf2 (0.714±0.111), HO-1 (1.238±0.053) and NQO1 (0.987±0.045); $P<0.01$]. The BBB scores of the rats indicated that they had improved functional recovery following the local injection of Ad-Nrf2. On the third day following the operation, BBB scores in the adenovirus groups (0.167±0.408) were significantly decreased compared with the SCI group (1±0.894; $P<0.05$). In the injured section of the spinal cord in the rats, the number of positive cells expressing Nrf2, HO-1 and NQO1 were raised compared with the control and SCI groups, indicating that the adenovirus vector-mediated gene transfer of Nrf2 promotes functional recovery following spinal cord contusion in rats.

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Introduction

Secondary injury serves a key function in the outcome of patients with spinal cord injury (SCI) (1). It is important to reduce the apoptosis or necrosis of neurons, and maintain the links between neurons/glial cells and axons in SCI (2,3). The mechanism of secondary injury is complex (4). Numerous studies have identified that microvascular perfusion changes, free radical production and lipid peroxidation, necrosis and apoptotic cell death and the dysregulation of ionic homeostasis are able to promote secondary injury following SCI (5-8). Previous studies have attempted to identify a desirable target which is able to interrupt multi-mechanisms underlying secondary injury.

Nuclear factor erythroid 2p45-related factor 2 (Nrf2) is a member of the Cap 'n' Collar basic-leucine-zipper family of transcription factors (9). Under numerous stimuli, Nrf2 translocates from the cytoplasm to the nucleus (10) and sequentially binds to antioxidant response element (ARE) (11). ARE is a promoter element commonly identified in protective genes and its products are involved in reducing oxidative stress, inflammatory damage and reducing the accumulation of toxic metabolites (12). Nrf2 transactivates the expression of a number of cytoprotective enzymes by binding to ARE motifs, including heme oxygenase-1 (HO-1) and NAD(P)H-quinone oxidoreductase-1 (NQO1). These products regulated by the Nrf2 gene, in order to protect the cell from oxidative or xenobiotic damage (13-16).

It has been noted that ~7% of the normal number of axons below the injury level are required to mediate meaningful distal neurologic function (17,18). In order to maintain the necessary neurologic functions, 1.4-12% of the total number of axons across the spinal cord injury site are required (19-21). Therefore, even small increases in neuroprotection may affect functionally relevant neurologic recovery and thus is

important for SCI patients (22). However, neurons have low antioxidant abilities and are highly sensitive to oxidative stress, therefore, increased levels of reactive oxygen species (ROS) easily induces neuron damage (23,24). The Nrf2-ARE pathway in central nervous system (CNS) injury serves a protective function (25). It has been proven that Nrf2 serves pivotal functions in the cell, which may defend against the oxidative stress of traumatic brain injury (TBI) in rats or mice, and decrease the severity of neurological deficit. On the contrary, Nrf2 knockout increased the severity of TBI, even with the use of an Nrf2 inducer (26,27). Furthermore, the disruption of Nrf2 may upregulate the activity of nuclear factor-κβ and proinflammatory cytokines following TBI or SCI in mice (28,29). The aim of the present study was to investigate whether Nrf2 gene transfer overexpression can protect neurons/glial cells, and the association between neurons/glial cells and axons during SCI.

Materials and methods

Experimental overview. Gene transfer has been widely used for experimental research (30). However, to the best of our knowledge, Nrf2 gene transfer to TBI and SCI have not been reported. In the present study, Nrf2 recombinant adenovirus vectors were constructed that were then transfected into PC12 cells and locally injected into SCI in rats. The protein levels of Nrf2 in the nucleus and the Nrf2-regulated gene expression of HO-1 and NQO1 were detected using western blot analysis in PC12 cells following 48 h of transfection. Furthermore, the expression of Nrf2 was localized by using immunofluorescence and the expression of Nrf2, HO-1 and NQO1 were detected using immunohistochemistry in the grey matter of the spinal cord in rats. Post-injury motor behavior was assessed via the Basso, Beattie and Bresnahan (BBB) locomotor scale method.

Cell line. PC12 cell line (a neuron model) was provided by Department of Neurology of the First Affiliated Hospital of Chongqing Medical University (Chongqing, China) and cultured in RPMI 1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) with 10% calf serum (HyClone; GE Healthcare Life Sciences), 5% horse serum (HyClone; GE Healthcare Life Sciences), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured in a humidified atmosphere incubator at 5% CO_2 and 37˚C, and the medium was changed every other day.

Constructing recombinant adenoviral vectors. The adenovirus shuttle plasmids pAV-MCMV-green fluorescent protein (GFP)-Nrf2 and pAV-MCMV-GFP were purchased from Microbix Biosystems (Mississauga, ON, Canada). Recombinant adenoviral vectors were generated by using the Admax Cre-lox system (Microbix Biosystems, Inc.). The adenovirus was propagated in 293 cells (Health Science Research Resources Bank Osaka, Japan). and purified by $CsCl_2$ density gradient centrifugation (40,000 x g, 2 h, 4˚C). Virus titers were determined using plaque assays. For PC12 cell transfection, 2 ml PC12 cells ($1x10^5$/ml) supernatant was incubated at 37˚C in 6-well plates overnight. Then they were infected for 20 min at 37˚C with viral supernatant containing vectors at a multiplicity of infection of 100 in the presence of 8 μg/ml polybrene. PC12 cells were divided into three groups: PC12-Control (no virus infection), PC12-Ad-Nrf2 group (Ad-Nrf2 infection) and PC12-Ad-GFP group (Ad-GFP infection).

Animal preparation. A total of 100 adult (5 weeks) male and female Sprague-Dawley rats of Specific-pathogen free (SPF) (180-250 g; Chongqing Medical University, Chongqing, China) were housed under a room temperature and humidity (24˚C and 50%) on a 12 h light-dark cycle with *ad libitum* access to food and water. All experimental procedures were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23, revised 1996), and the number of animals used and their suffering were minimized. Ethical approval was provided by the Yongchuan Hospital of Chongqing Medical University (Chongqing, China). Using a random number table, rats were divided into: i) a Sham-operated group (n=25), ii) a SCI group (n=25), iii) an Ad-Nrf2 group (n=25), and iv) an Ad-GFP group (n=25). On the 1st, 3rd, 7th, 14th and 28th day subsequent to surgery, 5 surviving rats were selected from each group for further experimental study.

The extradural compression of the modified Allen's method was used to produce the SCI animal model (31). Briefly; rats were anesthetized intraperitoneally with chloral hydrate (300 mg/kg) and underwent a laminectomy to expose the dorsal portion of spinal cord from T8 to T10 levels. Moderate or severe contusion injury was performed with a weight-drop device by dropping a 10 g rod (3 mm in diameter, 5 cm in height with injury pulse 10x5 gcf). Within 30 min following the injury, 1 μl adenoviral vector, diluted to $5x10^{10}$ pfu/ml prior to use, was injected into the 2 mm spinal cord stump from the wound site vertically into each stump at a depth of 0.8 mm using a Hamilton micro-injector. The injection rate was slow in order to minimize damage to the spinal cord. Each animal was injected with a total of $5x10^8$ pfu viruses. A constant body temperature was maintained with an overhead heating lamp during the experiment. In the Sham-operated group, the rats underwent the same laminectomy procedure but no trauma was produced. The GFP expression of PC12 cells were detected at 48 h after virus transfection with fluorescence microscopy.

Western blot analysis. The proteins were extracted using a commercial kit according to the manufacturer's protocol (NBP2-37853, Novus Biologicals), and the protein expression of Nrf2, HO-1 and NQO1 in a neuron model (PC12 cells) were analyzed using western blot analysis. Briefly, 50 μg of protein extracts determined by Pierce BCA Protein Assay Kit (23227, Thermo Fisher Scientific, Inc.) were separated on 15% SDS-PAGE and then were electrotransferred to a polyvinylidene difluoride filter (PVDF, EMD Millipore, Billerica, MA, USA). The wet electroblotting (using Mini Trans-Blot Module, 1703935, Bio-Rad Laboratories, Inc.) was performed at constant voltage (20 V) for 1 h at 4˚C with CAPS based transfer buffer (10 mM CAPS, pH 11, 10% methanol) Then the membranes were blocked with 5% nonfat milk for 1 h at room temperature and incubated with primary antibodies anti-Nrf2 (ab89443, 1:500; Abcam), anti-HO-1 (CL5275, 1:500; Abcam), anti-NQO1 antibodies (ab28947, 1:500; Abcam) and anti β-actin (mAbcam 8226, 1:2,000; Abcam) overnight at 4˚C,

followed by anti-goat immunoglobulin G horseradish peroxidase-conjugated secondary antibodies (sc-2354, 1:5,000; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. Following rinsing with a buffer, the protein bands were visualized using an enhanced chemiluminescence kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Film signals were digitally quantified by internal control β-actin using Quantity 4.6.2 software (Bio-Rad Laboratories, Inc.).

BBB locomotor scale. The BBB locomotor scale method was used to assess the hind limb functional improvement of treated animals with spinal cord contusion (32). At 1, 3, 7, 14 and 28 days subsequent to surgery, behavioral analysis was performed based on movement of the hind limb, weight support, forelimb-hind limb coordination and trunk stability.

GFP detection. All animals were perfused transcardially with 4% paraformaldehyde. The cords, ~2 cm in length with the contused site at the center of the sample, were segmented. The parasagittal ice-frozen spinal cord sections (~500 μm away from the sagittal plane, with a thickness of 10 μm) were prepared to detect GFP expression with fluorescent microscopy.

Immunohistochemistry. Five samples from each group on the 3rd postoperative day were fixed with 4% paraformaldehyde for 48 h at room temperature, embedded in paraffin and cross-sections (thickness, 10 μm) were prepared. After deparaffinization and dehydration, sections were boiled in Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA Solution, 0.05% Tween-20, pH 9.0) for antigen unmasking, followed by extensive washing with PBS. Sections were subsequently incubated with 3% H_2O_2 for 10 min, and then rinsed with PBS for three times. Sections were blocked with 2% goat serum albumin in PBS for 20 min and incubated with mouse anti-Nrf2, anti-HO-1 and anti-NQO1 antibodies (all at a dilution of 1:100) overnight at 4˚C. Sections incubated without a primary antibody were used as negative controls. After washing 3 times with PBS, sections were incubated with Goat anti-Mouse IgG Secondary Antibody [HRP (Horseradish Peroxidase), HAF007, 1;1,000, Novus Biologicals] for 10 min at 37˚C. After washing with PBS, peroxidase was stained with Mouse specific HRP/DAB (ABC) Detection IHC kit (ab64259; Abcam) and viewed under a light microscope (magnification, x400,; Olympus BX50; Olympus Corporation).The positive cells were counted in five different fields of view in the gray matter in five random sections of each rat. All images were captured using Sim PCI 6.0 (Compix Media, Inc.).

Statistical analysis. Data were analyzed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) and were presented as the mean ± standard error of the mean. Statistical differences were measured using a one-way analysis of variance and Bonferroni's test to compare the differences between groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PC12 cell data. GFP expression in PC12 cells was observed 48 h subsequent to adenovirus transfection. It is likely that Nrf2 is able to affect PC12 proliferation and axonal growth, so the morphology is different between Fig. 1A-C and between Fig. 1D-F (33). The expression of GFP following Ad-Nrf2 transfection was mainly located in the nucleus, and its expression in the cytoplasm was observed following Ad-GFP transfection (Fig. 1A-F).

Western blot analysis was performed to evaluate the protein levels of nuclear Nrf2 (57 kDa), HO-1 (32 kDa) and NQO1 (30 kDa) in whole cells. Subsequent to Ad-Nrf2 transfection, nuclear Nrf2, HO-1 and NQO1 were significantly increased compared with the control ($P<0.01$; Fig. 1G-I). There was statistically significant changes in the PC12-Ad-Nrf2 group [Nrf2 (1.146±0.095), HO-1 (1.816±0.095) and NQO1 (1.421±0.138)] compared with the PC12-Control [Nrf2 (0.717±0.055), HO-1 (1.264±0.081) and NQO1 (0.921±0.088)] and PC12-Ad-GFP group [Nrf2 (0.714±0.111), HO-1 (1.238±0.053) and NQO1 (0.987±0.045); ($P<0.01$)].

BBB Locomotor scale results. The animal behavioral analysis was performed at 1, 3, 7, 14 and 28 days following the operation. In the Sham-operated group, BBB scores between day 1 and day 28 did not decrease significantly. On the 3rd day following the operation, BBB scores in the Ad-Nrf2 group (0.167±0.408) exhibited a significantly decreased comparing with the SCI group (1±0.894; $P<0.05$). On the 7th day following the operation, the BBB scores in the Ad-Nrf2 group (2.333±0.516) were significantly increased compared with the Ad-GFP group (0.9±0.21; $P<0.05$). On the 14th and 28th day, there was a statistically significant increase in the Ad-Nrf2 group (day 14, 9.833±1.17; day 28, 14±2.608) compared with the SCI group (day 14, 8±1.265; day 28, 11.167±1.901) and the Ad-GFP group (day 14, 7.167±1.17; day 28, 10.333±1.633; $P<0.05$; Fig. 2).

Gene transfer efficacy. To identify the efficacy of the gene transfer of Ad-Nrf2 and Ad-GFP, the ice-frozen parasagittal sections of the spinal cords, ~2 cm in length with the contused site at the center of the sample, were detected using a fluorescence microscope. Green fluorescence was detected in the spinal cords, primarily near the injected sections, on the 1st day following SCI. The prevalence of the fluorescence expression was observed on the 3rd day following SCI and the contused parts also exhibited GFP expression. From that point onwards, the fluorescence expression decreased gradually (Fig. 3A-E).

Immunohistochemical study. The expression levels of Nrf2, HO-1 and NQO1 in grey matter were localized and analyzed using an immunohistochemical experiment on the 3rd day following the operation. Very few cells were positive for Nrf2, HO-1 and NQO1 in the Sham-operated group. Nrf2, HO-1 and NQO1 immunoreactivity was present in neurons and glial cells following SCI. In the Ad-Nrf2 group, the positive neurons and glial cells for Nrf2, HO-1 and NQO1 were significantly increased compared with the control and SCI groups ($P<0.01$; Fig. 4). It should be noted that due to the poor homogeneity of spinal cord tissue, the tissue specimens from different rats ought to be treated carefully at the time of fixing and the section orientated to reduce the morphological effects.

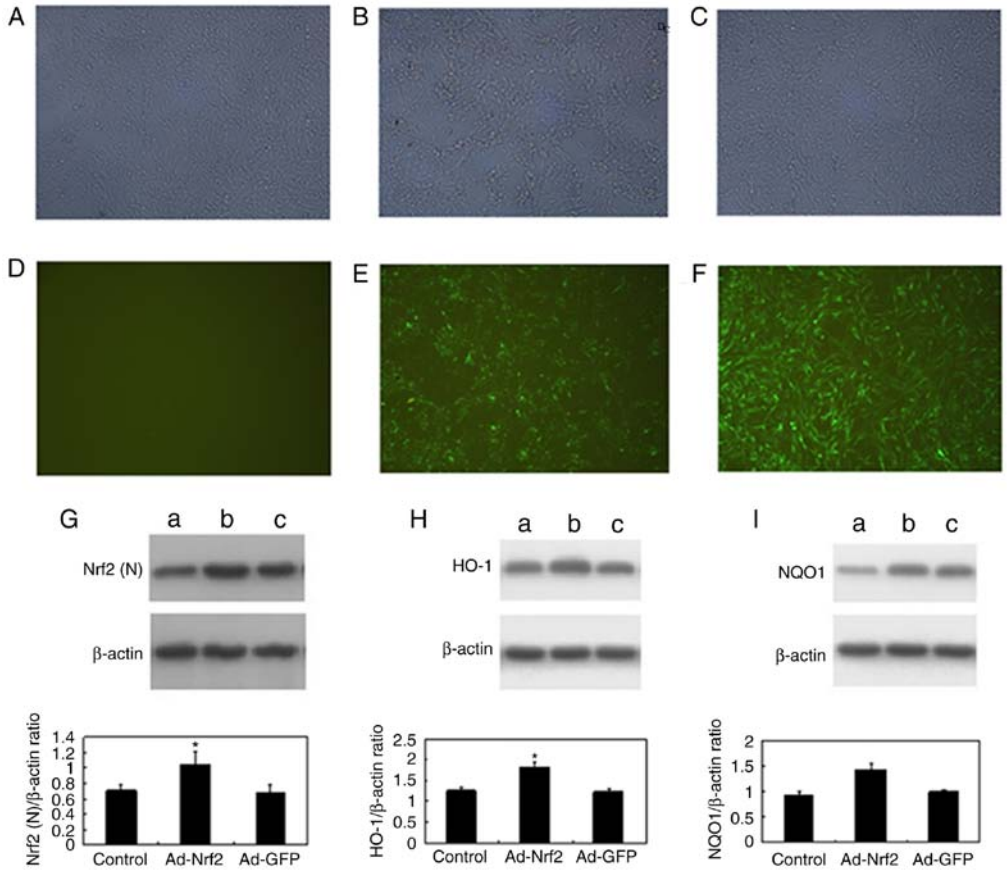

Figure 1. Expression of GFP in cytoplasm 48 h after recombinant adenovirus infection. Expression of GFP in the (A) PC12-Control, (B) PC12-Ad-Nrf2 and (C) PC12-Ad-GFP groups captured by light microscopy. Expression of GFP in the (D) PC12-Control, (E) PC12-Ad-Nrf2 and (F) PC12-Ad-GFP groups captured by fluorescent microscopy. Magnification, x200. Subsequent to Ad-Nrf2 transfection, western blot analysis was used to examine the levels of (G) nuclear Nrf2, (H) HO-1 and (I) NQO1 in the PC12-Control, PC12-Ad-Nrf2 and PC12-Ad-GFP groups. Bars represented the ratio of the β-actin value (mean ± standard error of the mean). *P<0.01 vs. control group. GFP, green fluorescent protein; Ad, adenovirus; Nrf2, nuclear factor erythroid 2p45-related factor 2; HO-1, heme oxygenase-1; NQO1, NAD(P)H: quinone oxidoreductase-1.

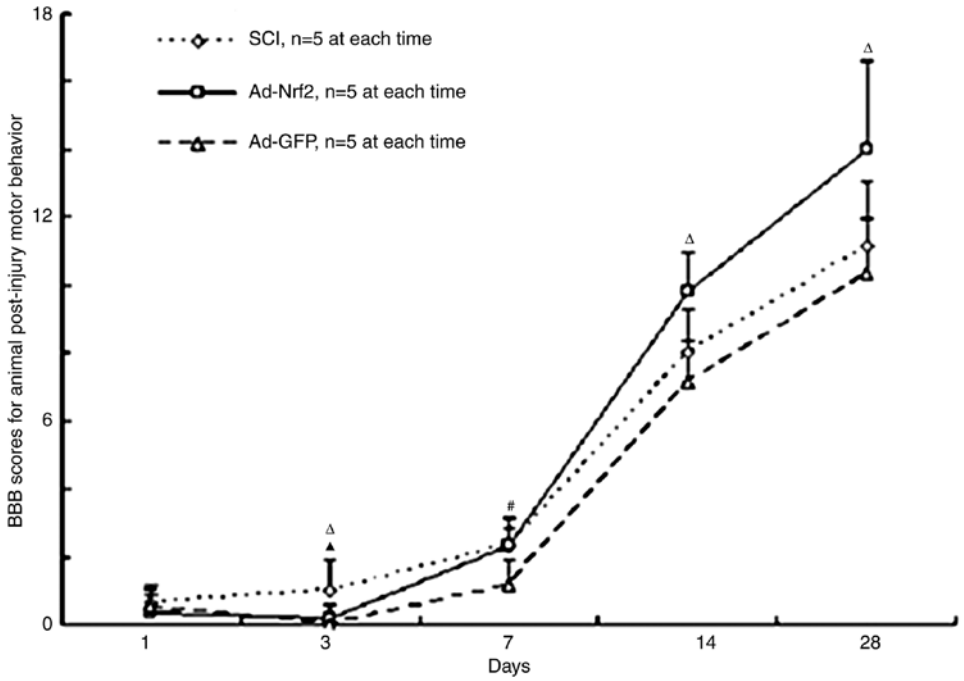

Figure 2. BBB scores of post-injury motor behavior for the SCI group, the SCI rats injected with Ad-Nrf2 (Ad-Nrf2 group) and the SCI rats injected with Ad-GFP (Ad-GFP group). n=5, mean ± standard error of the mean. *P<0.05, SCI group vs. the Ad-Nrf2 and Ad-GFP groups; #P<0.05, SCI and Ad-Nrf2 groups vs. the Ad-GFP group; ΔP<0.05, Ad-Nrf2 group vs. the SCI and Ad-GFP groups. SCI, spinal cord injury; Nrf2, nuclear factor erythroid 2p45-related factor 2; BBB, the Basso, Beattie and Bresnahan locomotor scale; GFP, green fluorescent protein; Ad, adenovirus.

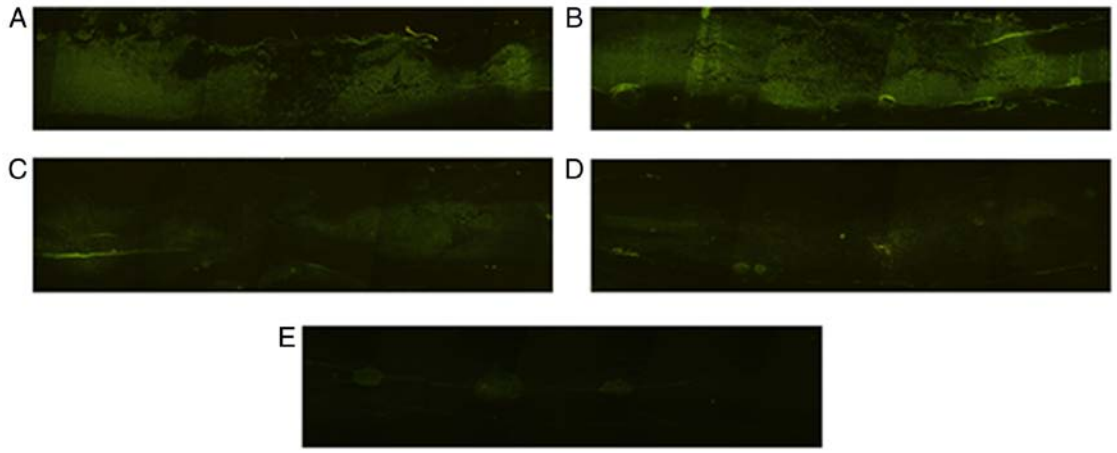

Figure 3. GFP expression in the parasagittal sections of rat spinal cords transfected with Adenovirus-nuclear factor erythroid 2p45-related factor 2 at different time points (n=5) following surgery (magnification, x4). The green fluorescence in the spinal cords was observed on (A) day 1, (B) day 3, (C) day 7, (D) day 14 and (E) day 28. Each picture was connected with a different section of the entire spinal cord section. GFP, green fluorescent protein.

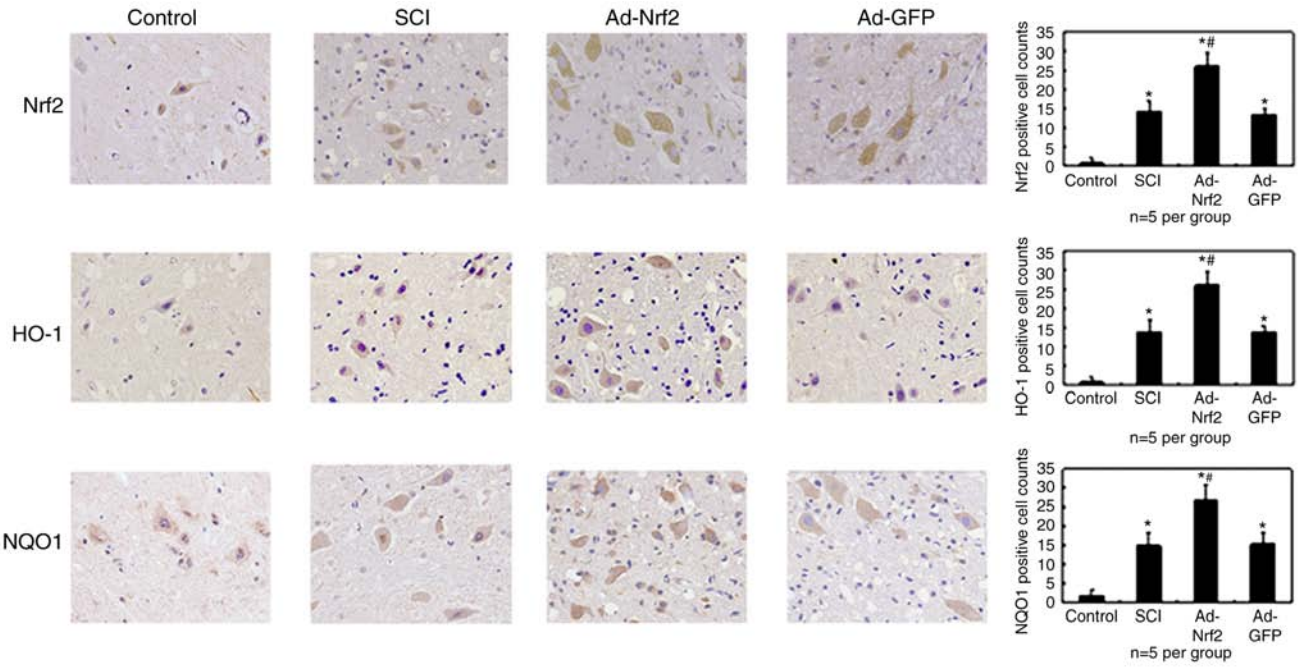

Figure 4. Immunohistochemical staining for Nrf2, HO-1 and NQO1 in the grey matter of the rats on the 3rd day following the operation (magnification, x400). Significantly increased Nrf2, HO-1 and NQO1 immunoreactivities were present in neurons/glial cells. In the Ad-Nrf2 group, the number of positive cells increased significantly vs. the SCI and Ad-DFP groups. Bars (mean ± standard error of the mean, n=5 in each group) presented the number of the positive glial cells following SCI. *P<0.01 vs. the control group; $^{\#}$P<0.01 vs. the SCI and Ad-GFP groups. GFP, green fluorescent protein; Ad, adenovirus; Nrf2, nuclear factor erythroid 2p45-related factor 2; HO-1, heme oxygenase-1; NQO1, NAD(P)H: quinone oxidoreductase-1; SCI, spinal cord injury.

Discussion

The leucine zipper transcription factor Nrf2 is a major component of ARE-driven gene expression (33). Oligonucleotide microarray analysis has indicated that Nrf2 is necessary in combating electrophiles and ROS (23,34-37). It serves a key function in protecting cells from oxidative stress. Nrf2 may protect the liver from acetaminophen-induced injury (38) and the lung from butylated hydroxytoluene-induced toxicity (39). Cho *et al* (40) demonstrated that disruption of Nrf2 significantly enhanced pulmonary sensitivity and responsivity to hyperoxic challenge. Compared with $Nrf2^{+/+}$ astrocytes, one previous study confirmed that $Nrf2^{-/-}$ primary astrocytes are more susceptible to oxidative stress and inflammation (37). $Nrf2^{+/+}$ astrocytes pretreated with t-butylhydroquinone induce Nrf2 nuclear translocation, resulting in the coordinated upregulation of ARE-driven genes and attenuation of H_2O_2 and platelet-activating factor-induced cell death (37).

PC12 cells (adrenal pheochromocytoma) was originally isolated from tumors in the rat adrenal medulla in 1976 (41). They resemble the phenotype of sympathetic ganglion neurons upon differentiation with nerve growth factor (NGF) and may be subcultured indefinitely. The PC12 cell line is traceable to a pheochromocytoma from the rat adrenal medulla (42-45). It has been used as the classical neuronal cell model due to its ability to acquire the features of sympathetic neurons (46,47).

PC12 cells have been used to investigate the cellular mechanisms by which prion protein fragments cause neuronal dysfunction (48), the nerve injury-induced neuropathic pain model (49), the nitric oxide-induced neurotoxicity model (50) and NGF inducing the differentiation of PC12 cells by functioning through the tropomyosin receptor kinase A receptor (51).

In the present study, the Ad-Nrf2 gene was successfully transferred into PC12 cells and the spinal cord (by local injection). Furthermore, the protein levels of nuclear Nrf2 and its regulated gene products, HO-1 and NQO1, were significantly increased in PC12 cells compared with the control, $P<0.01$), and the number of positive cells for Nrf2, HO-1 and NQO1 were promoted in the neurons/glial cells of the spinal cord grey matter. The function of the hind limb in SCI rats was significantly improved following Ad-Nrf2 gene transfer compared with the SCI group ($P<0.05$). According to these results, it was postulated that the gene transfer of Nrf2 was able to alleviate SCI and promote the functional recovery of the injured spinal cord. In view of the fact that neurons are more susceptible than glial cells to oxidative stress in Ad-Nrf2 group (23,52), it may be considered that the Nrf2-ARE pathway may serve a protective function in the pathological process of SCI.

Nrf2 mediates a group of cytoprotective enzymes. It is believed to be the key regulator in CNS diseases by inducing the expression of a group of antioxidant and detoxification enzymes (26,53). In the present study, it was revealed that SCI-induction significantly increased the number of positive cells for Nrf2, HO-1 and NQO1 in grey matter neurons compared with the control ($P<0.01$), suggesting that the Nrf2-ARE pathway was activated. Such phenomena have additionally been observed in TBI (54).

Increasingly, evidence suggests that a group of cytoprotective enzymes mediated by the Nrf2-ARE pathway serve a pivotal role in antioxidant, anti-inflammation and detoxification functions, including HO-1 and NQO1 (55,56). HO-1 produces biliverdin and reduces bilirubin to reduce ROS production. The expression of ferritin (HO-1 dependent) may prevent the conversion of H_2O_2 to hydroxyl radicals by the Fenton reaction (57). NQO1 catalyzes the double-electron reduction and detoxification of quinones and their derivatives, therefore protecting cells from the harmful effects of quinones and their associated compounds (58). In SCI, it is considered important to reduce the neurons apoptosis or necrosis and maintain the associations between neurons and axons. However, neurons have a low antioxidant capacity and are highly sensitive to oxidative stress (59). It is reported that Nrf2-mediated neuroprotection is conferred primarily by glia (23). SCI induces an increase in oxidative stress and simultaneously causes glial dysfunction (60). It is therefore important to activate the remaining functional glia and neuronal self-protection. The present study hypothesized that the gene-transfer of Nrf2 may promote the Nrf2-ARE pathway to reduce neuron necrosis or apoptosis, particularly by self-protection. The results revealed that Ad-Nrf2 increased nuclear Nrf2, HO-1 and NQO1 expression in PC12 cells and neurons in the contusion site of SCI, and hind limb functional recovery was also observed. Certain phase II enzyme inducers (activating Nrf2), even fibroblast growth factor-1, have demonstrated neuroprotective effects on motor neuron survival in traumatic SCI (61-63). Genetic ablation of the transcription repressor Bach1, a transcriptional repressor of the HO-1 gene, may substantially increase HO-1 expression and cytoprotection against SCI (64). The present study did not successfully isolate the spinal cord neurons of adult rats. PC12 is a tumor cell, which is still different from the neurons themselves. This is the main limitation of the present experiment.

Therefore in conclusion, Nrf2 gene transfer may be a direct method of protecting neurons/glial cells in SCI. Although local injection may injure nerve cells, this method works and will still benefit clinical treatment. Nrf2-adenovirus-mediated *in vivo* gene transfer may promote functional recovery following spinal cord contusion.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

FCZ was responsible for the study design, literature research, experiments, manuscript preparation, editing and review. DMJ was responsible for the conception of the study and the guarantor of integrity of the entire study. MHZ was responsible for the definition of intellectual content and acquisition of data. BZ was responsible for data acquisition. CH was responsible for data analysis. JY was responsible for statistical analysis. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experimental procedures were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23, revised 1996), and the number of animals used and their suffering were minimized. Ethical approval was provided by the Yongchuan Hospital of Chongqing Medical University (Chongqing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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