Research Article

Regulatory effect of neuroglobin in the recovery of spinal cord injury

Ji-Lin Dai^{1*}, Yun Lin^{2*}, Yong-Jian Yuan¹, Shi-Tong Xing¹, Yi Xu¹,
Qiang-Hua Zhang¹, Ji-Kang Min¹

¹Department of Orthopaedic, Huzhou First People's Hospital of Zhejiang Province, Huzhou City 313000, China,

²Department of Editor, Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, Chengdu 610072, China

Objective: The present study was aimed to investigate the therapeutic potential of neuroglobin in the recovery of spinal cord injury.

Methods: The male albino Wistar strain rats were used as an experimental model, and adeno associated virus (AAV) was administered in the T12 section of spinal cord ten days prior to the injury. Basso Beattie Bresnahan (BBB) locomotor rating scale was used to determine the recovery of the hind limb during four weeks post-operation. Malondialdehyde (MDA), catalase and superoxide dismutase (SOD) were determined in the spinal cord tissues. Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay was carried out to determine the presence of apoptotic cells. Immunofluorescence analysis was carried out to determine the neuroglobin expression. Western blot analysis was carried out to determine the protein expressions of caspase-3, cytochrome c, bax and bcl-2 in the spinal cord tissues.

Results: Experimental results showed that rats were recovered from the spinal cord injury due to increased neuroglobin expression. Lipid peroxidation was reduced, whereas catalase and SOD activity were increased in the spinal cord tissues. Apoptosis and lesions were significantly reduced in the spinal cord tissues. Caspase-3, cytochrome c and bax levels were significantly reduced, whereas bcl-2 expression was reduced in the spinal cord tissues.

Conclusion: Taking all these data together, it is suggested that the increased neuroglobin expression could improve the locomotor function.

Keywords: Neuroglobin, Caspase-3, MDA, Spinal cord injury, Cytochrome c

Introduction

The neuroglobin is a globin protein involves in the oxygen homeostasis of a cellular system in animals. It is a hemoprotein found in the cerebrospinal fluid (CSF), retinal cells, central nervous system (CNS), peripheral nervous system (PNS) and endocrine tissues.¹ Furthermore, it is found in jellyfish, acoel and in the astrocytes of rodent brain under the diseased condition, and seal brain under physiological condition.²⁻⁴ Neuroglobin bind with oxygen with more affinity compared to hemoglobin and protects the brain by providing oxygen under ischemic and hypoxic conditions. Neuroglobin has been reported as a neuroprotective

agent and resistant to cerebral ischemia in transgenic mice,⁵ and reduces oxidative stress and improves mitochondrial function under hypoxic condition.⁶ Neuroglobin has been overexpressed to protect brain following mechanical injury,⁷ and it has been used to treat reactive oxygen species (ROS) related disorders.⁶ Brittain¹ have reported that the neuroglobin is actively participating in the mitochondrial release of cytochrome c and, thereby apoptosis pathway.

A spinal cord injury (SCI) is a damage of spinal cord with altered function, and it can be either as complete or incomplete injury based on the level of damage. SCI can lead to the severe catastrophic cellular and physiological dysfunction,⁸ with symptoms of numbness, pain, paralysis, and incontinence. Complication can occur either as short term or long term and influence infections, muscle atrophy, blood pressure and

Correspondence to: Ji-Kang Min, Department of Orthopaedic, Huzhou First People's Hospital of Zhejiang Province, No 158 Square posterior Road, Huzhou City 313000, China. Tel 0086-572-2039275, Email: jikangmin108@hotmail.com

*These two authors contribute equally to this work.

respiratory disease. The primary and secondary mechanism of injury has been associated with SCI.⁹ Blight¹ have reported that the primary SCI is irreversible, whereas secondary SCI alters several cellular and metabolic conditions such as free radical production, vascular changes, lipid peroxidation, apoptosis and mitochondrial dysfunction.

Therefore, the present study was aimed to investigate the functional role of neuroglobin expression in the recovery of SCI.

Materials and Methods

Materials

Animals

Healthy male albino Wistar strain rats were purchased from the animal house (Shanghai, China) weighing from 160–180 g, was selected for the present study. Animals were kept in polypropylene cages at temperature of $25 \pm 0.5^\circ\text{C}$ with relative humidity of $60 \pm 5\%$ and a photoperiod of 12 hours/day.

Treatment

Adeno-associated virus (AAV) vector was synthesized according to the method of Wen-Bin *et al.*¹⁰ Neuroglobin expression was determined at 0, 7, 14 and 28 days following AAV administration. SCI was induced at T12 position by the chloral hydrate (600 mg/kg) administration. AAV (5 ml) was administered using gauge needle. Rats were grouped into 5 groups of six rats each. The experimental groups were designated as follows; Group I–V.

Group I: Normal saline

Group II: AAV (5 ml, 0 days)

Group III: AAV (5 ml, 7 days)

Group IV: AAV (5 ml, 14 days)

Group V: AAV (5 ml, 28 days)

Rats were sacrificed at the end of our treatments. The blood and tissues were collected for the analysis.

Behavioral test

The behavioral test was performed according to Basso *et al.*¹¹ The behavioral test was targeted to assess the recovery of motor function after SCI. Six rats were used for treatment, and the behavioral test was carried out pre- and post-injury.

Preparation of spinal cord tissues

Rats were sacrificed at the end of treatments. Rats were perfused with phosphate buffered saline (PBS) and formaldehyde. Spinal cord tissue (2–3 cm) were surgically removed and immersed in the fixative solution. The tissue sections (5 μm) were prepared using microtome

(Leica Biosystems RM2245 Semi-Automated Rotary Microtome).

Determination of lipid peroxidation

Lipid peroxidation (LPO) was determined by the kit method (ab118970, Abcam). This was based on the spectrophotometric method of Muthuraman *et al.*¹² MDA was measured by determining the thiobarbituric acid reactive species (TBARS). The absorbance of the resultant product was measured at 534 nm (Agilent Technologies, Cary 100 UV-Vis spectrophotometer).

Determination of SOD and catalase enzyme activities

SOD and catalase enzyme activities were determined by using the kit method (ab65354, ab83464, Abcam), which was based on the method of Muthuraman *et al.*¹²

TUNEL assay

Tunel assay was used to detect apoptosis, which was carried out by using the commercially available kit (Cayman, USA). Spinal cord tissues were washed and permeabilized with Triton-X 100, and then washed with PBS. Spinal cord tissue sections were immersed in Tunel assay reaction mixture and incubated at room temperature for 1 hour. Cells were stained with Hoechst 33342 (14533, Sigma-Aldrich) for 20 minutes and images were obtained (IX83 Inverted Microscope, Olympus, Japan).¹³

Immunofluorescence

Spinal cord tissues were incubated with caspase-3 antibody (Anti-human caspase-3/Casp-3 antibody, Santa Cruz Biotechnology, USA) with a dilution of 1:500 for overnight at 4°C . Then, the sections were incubated with goat anti-rabbit IgG H&L (FITC) (ab6717, abcam) for 2 hours at room temperature. The images were taken with the use of a fluorescence microscope (IX83 Inverted Microscope, Olympus, Japan).¹⁴

Western blot analysis

Cell lysates were washed with PBS and lysed in lysis buffer. The equal amounts of protein lysates were investigated on Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Polyvinylidene difluoride (PVDF) membrane was used for transferring protein, and non-specific binding has been blocked with use of a Tris-buffered saline-Tween buffer (TBST) that contained 5% nonfat dry milk. Then, samples were incubated with primary antibodies of neuroglobin (ab37258), bcl-2 (ab59348), bax (ab32503) and cytochrome c (ab13575) for 10–12 hours at 4°C . Then, samples were incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG for 1 hour.

Figure 1 Up-regulation of neuroglobin expression in the spinal cord tissues following AAV treatment. The letter “A” represents the protein bands of neuroglobin at different time periods and “B” represents the densitometry analysis of “A”. * $P < 0.05$ and ** $P < 0.01$.

The protein bands were analyzed using the enhanced chemiluminescent method.¹⁵

Statistical analysis

All the results are expressed as mean $\pm$ standard error of the mean (SEM). The data between control and treated

group were evaluated using Student’s t-test. A $P < 0.05$ was considered statistically significant.

Result

Neuroglobin expression

The neuroglobin expression was determined in control and AAV treated spinal cord tissues at specified times. Neuroglobin expression was significantly increased at 14 and 28th day compared to control group. Neuroglobin was increased 0.45 and 0.73 folds at 14 and 28th day respectively (Figure 1, $P < 0.05$).

Effect of neuroglobin on neuronal survival

Tunel assay was used to determine the viable and non-viable neurons using specific stain. Neuron expression was found low in the control group. However, AAV treated groups showed reduced apoptotic cell index compared to control. The number of positive apoptotic cells was reduced in the treated group especially on the 28th day (Figure 2).

Effect of neuroglobin on motor disturbances

The impact of neuroglobin expression on motor disturbances was noted in control and treated rats. The BBB score was slightly decreased in experimental control rats. However, it was recovered after 7 days. The BBB score was 7, 9, 10.1 and 13 at 0, 7, 14 and 28th days

Figure 2 Effect of neuroglobin on neuronal survival. Tunel assay was used to determine the neuron expression. Representative images were from six independent experiments. Scale bar is 50 μ m.

Figure 3 Effect of overexpressed neuroglobin on the locomotor disturbances. Values are expressed mean $\pm$ SEM, * $P < 0.05$ and ** $P < 0.01$.

respectively. At 28th day, the overexpression of neuroglobin significantly improved locomotor disturbances (Figure 3, $P < 0.05$).

Neuroglobin on oxidative stress

MDA content, catalase and SOD activity were determined. MDA content was increased in the control rats, whereas AAV treatment significantly reduced MDA content 9, 16.8, 34.9 and 52.2% at 0, 7, 14 and

28th days respectively (Figure 4, $P < 0.05$). Catalase and SOD activity were drastically reduced in the control rats. Catalase enzyme activity was increased 30, 63, 109.5 and 155% at 0, 7, 14 and 28th days of AAV treatment respectively. SOD enzyme activity was increased 100, 158.3, 163.6 and 320% at 0, 7, 14 and 28th days of AAV treatment respectively (Figure 4, $P < 0.05$).

Cytochrome c, bax, and bcl-2 expression

Protein expression of cytochrome c, bax and bcl-2 were determined by using Western blot analysis. Cytochrome c expression was increased in the cytoplasm, and reduced in the mitochondrial region of SCI. Cytochrome c expression increased 0.34 and 1.12 folds at 14 and 28th days of AAV treatment respectively. The overexpression of neuroglobin normalized the altered cytochrome c expression in the cytosol and mitochondria. To confirm further, the bax and bcl-2 expression were carried out to understand the effect of neuroglobin expression on apoptosis. The bax expression was increased, whereas bcl-2 expression was reduced in the control groups. The bcl-2 expression increased 0.45 and 0.97 folds at 14 and 28th days of AAV treatment respectively. The bax expression reduced 0.21 and 0.44

Figure 4 Effect of neuroglobin overexpression on the MDA content (A), catalase (B) and SOD (C) activity after SCI. Values are expressed mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Figure 5 Protein expression of cytochrome c, bax and bcl-2 in the spinal cord tissues following AAV treatment. The letter “A” represents the protein bands of cytochrome c, bax and bcl-2 at different time periods and “B” represents the densitometry analysis of “A”. * $P < 0.05$ and ** $P < 0.01$.

folds at 14 and 28th days of AAV treatment respectively (Figure 5, $P < 0.05$).

Caspase-3 expression

Caspase-3 expression was determined in the SCI tissue by using immunofluorescence. Caspase-3 expression was increased in the control group. However, caspase-3 expression was reduced in the AAV treated rats. Overexpression of neuroglobin could inhibit the apoptosis by reducing caspase-3 expression (Figure 6).

Discussion

In the present study, we have evaluated the effect of neuroglobin overexpression on the recovery of SCI.

Neuroglobin expression was determined in the intracellular region of spinal cord tissues following AAV treatment. Overexpression of neuroglobin significantly promoted SCI recovery. The number of neuron positive cells was significantly increased in the lesion region of spinal cord tissues following AAV treatment compare to controls. Overexpression of neuroglobin reduced caspase-3 and bax expression, whereas bcl-2 expression was increased in the injured spinal cord tissues. In addition, the reduced cytochrome c release from mitochondria to cytosol was observed due to overexpression of neuroglobin.

Gene therapies are considered as a valid treatment for SCI. Zhao *et al.*¹⁶ have reported that the gene therapies

Figure 6 Effect of overexpressed neuroglobin on caspase-3 expression in the spinal cord tissues. Representative images were from six independent experiments. Scale bar is 50 μ m.

has been successful for the treatment of SCI by using specific genes with a viral vector. Snyder *et al.*¹⁷ have reported that the AAV vectors are ideal for gene therapies for SCI, and it can transduce mitotic cells. AAV has been selected for the gene delivery based on these salient characters and neuroglobin was tremendously expressed in the AAV injected site. The brain has been protected from hypoxia and traumatic brain injury by the overexpression of neuroglobin.¹⁸

We have showed the therapeutic potential of overexpressed neuroglobin on SCI recovery following AAV treatment. Elmore¹⁹ has reported that the apoptosis is programmed cell death, which plays a vital role in the growth and development of normal and tumor cells. Yin *et al.*²⁰ have reported that the apoptosis is a crucial mechanism in the SCI. Apoptosis was reduced in the injured spinal cord tissues following overexpression of neuroglobin. Lipid peroxidation was induced by the increased intracellular ROS level. Xu *et al.*²¹ have reported that the increased lipid peroxidation, and ROS level could significantly contribute to the neuronal cell death in the injured spinal cord tissues.

Liu *et al.*⁶ have reported that the MDA content and catalase and SOD activity majorly participates in the oxidative damage. Kalayci *et al.*²² have reported that the increased MDA content and reduced catalase and SOD activity in SCI. Nayak *et al.*²³ have reported that the reduced intracellular ROS following the overexpression of neuroglobin. Reduced MDA content and increased catalase and SOD activity was observed in this study. Kim *et al.*²⁴ have reported that the consistent mitochondrial damage and ROS level. Xu *et al.*²¹ have reported that the increased production of ROS could significantly contribute to the neuronal cell death in the injured spinal cord tissues.

Conclusion

Overexpression of neuroglobin significantly improved the spinal cord injury following the delivery of AAV. The release of cytochrome c from mitochondria to cytosol was significantly reduced. Overexpression of neuroglobin significantly reduced the apoptosis in injured spinal cord tissues. Oxidative makers such as MDA, ROS, catalase, and SOD were renormalized following the overexpression of neuroglobin. The possible mechanism of SCI recovery could be based on the functional maintenance of mitochondria in the injured spinal cord tissues.

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