The neuroprotective effect of N-acetylcysteine in spinal cord-injured rats


Department of Physiology, Medical University of Silesia, Katowice, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

Abstract

Background. Spinal cord injury (SCI) is an important cause of impairment of sensory and motor nerve function. It has been shown that free-radical species play an important role in the pathogenesis of acute tissue trauma after SCI. There are no proven pharmacological therapies that provide neuroprotection and stimulate axonal growth after trauma.

Objectives. The aim of this study was to investigate the neuroprotective effect of N-acetylcysteine (NAC) on the regeneration of spinal cord injuries in rats.

Material and methods. A total of 20 male Wistar C rats were subjected to SCI and divided into control and experimental groups. In the control group (n = 10) trepanation and SCI by means of a pressure impactor was performed without any therapy. In the study group (n = 10), 1 dose of NAC was applied intraperitoneally (150 mg/kg b.w.) immediately after SCI, and another one after 24 h. The functional outcome on the Basso-Beattie-Bresnahan (BBB) scale and sciatic functional index (SFI) and morphological features of regeneration were analyzed during a 12-week follow-up. The spinal cords and brains were collected 12 weeks after SCI for histopathological and immunohistochemical analyses.

Results. The rats treated with NAC presented some improvement in locomotor activity and spinal cord morphology when compared to the control group. Namely, the hind paw angle of rotation was significantly lower in the NAC group than in the control group. No differences were observed between the control and study groups in terms of interlimb coordination. The area of the main lesion was only slightly decreased in the NAC group as compared to the control group. The length of lesions in the injured spinal cord in the NAC group was diminished in comparison to the control group. The number of FG-positive cells was higher in the NAC group than in the control group.

Conclusions. The study showed that the neuroprotective activity of NAC had limited positive influence on the regeneration of the isolated SCI in rats.

Key words: neuroprotection, N-acetylcysteine, spinal cord injury, neuroregeneration
Spinal cord injury (SCI) is an important cause of impairment globally, with an incidence between 236 and 1,009 cases per million people. The usual causes of spinal cord injury are motor crashes, sport-related accidents, and incidents associated with community violence and the workplace. Injury of the spinal cord causes sensory and motor dysfunction distally to the place of trauma.

There are 2 main mechanisms that lead to spinal cord injury. The first is related to mechanical damage to the spinal cord structure. The other is secondary injury, which plays a dominant role in a cascade of biochemical events at the cellular level. It has been shown that free-radical species play an important role in the pathogenesis of acute tissue trauma after spinal cord impact injury.

Many authors have suggested that this oxidative stress is associated with such processes as edema, hypoperfusion, conduction disturbances, impairment of metabolism, and Wallerian degeneration of neurons. It has been shown that neutralization of reactive oxygen species (ROS) and nitrogen in the first 3–4 h after the onset of trauma or ischemia reduces oxidative stress in neurons and shows a neuroprotective effect.

Currently, there are no proven pharmacological therapies for spinal cord injury that provide neuroprotection and stimulate axonal growth after trauma.

N-acetylcysteine (NAC) is a thiol compound possessing antioxidant properties and the precursor of glutathione. It acts by scavenging reactive oxygen species and inhibits the activity of cyclooxygenase-2 and membrane lipid peroxidation induced by inflammation. Some studies have indicated that NAC might have neuroprotective effect following brain ischemia or traumatic brain injury in rats.

The aim of this study was to investigate the neuroprotective effect of NAC therapy on the regeneration of isolated spinal cord injuries in rats.

### Material and methods

All the procedures were performed in accordance with EU animal protection laws and were approved by the Local Animal Research Ethics Committee. Twenty adult male Wistar C rats (approximate body weight: 300 g) were randomly divided into 2 groups: 1) the control group (n = 10) – animals subjected to trepanation of the vertebra and a single injury blast of the spinal cord (explained in detail below) without any repair therapy; and 2) the NAC group (n = 10) – animals subjected to trepanation and injury in the same way as the animals in the control group and then administered intraperitoneal doses of 2.55% N-acetylcysteine solution, both immediately and 24 h after the injury, at a dose of 150 mg/kg b.w.

### Spinal cord injury technique

Focal spinal cord injury was performed using the authors’ original apparatus – a pressure impactor producing a precisely controlled air blast. After intraperitoneal anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg), the animals were placed on a heated plate and immobilized by means of head holding bars and spine clamps at the level of Th-9 and Th-11, making Th-10 stable and easily accessible for further steps. The skin was then incised over the spinal cord, and the vertebral surface was exposed dorso-laterally from Th-9 to Th-11. Under control of a stereomicroscope (Nikon, Tokyo, Japan), a 2 mm diameter opening was drilled in the Th-10 vertebral arch on the right side. To avoid overheating and thermal lesion, the trepan was cooled down with chilled phosphate-buffered saline (PBS). Then, the impactor tip was placed close to the drilled opening and adjusted by means of a micromanipulator. The penetration depth of the tip was set up to establish contact with the dura mater but without exerting any pressure on it. After setting the impact parameters (150 kPa pressure; 0.1 s duration), the air blast system was activated. The “shot” was observed under the stereomicroscope and recorded by the attached camera (Nikon, Tokyo, Japan). After the procedure was complete, the hole was secured with wax, the muscles were sutured in layers, the skin was closed, and the wound was covered with a sterile bandage.

To avoid dehydration, all the animals were subcutaneously injected with 2 mL of sterile saline. Because the autonomic function of the urinary bladder was impaired due to the spinal shock, it was emptied manually twice a day until the recurrence of bladder function. To prevent pain, 400 mg of paracetamol (in a 125 mg/5 mL suspension) was dissolved in 100 mL of drinking water, providing an average drug dosage of 200 mg/kg b.w. per day.

### Assessment of locomotor function

All the animals were observed and analyzed regarding development or regression of neurological deficits for 12 weeks following the SCI procedure. Behavioral observations included gait analysis (the footprint test) and open field tests using the Basso-Beattie-Bresnahan (BBB) locomotor rating scale.

### Footprint tests

Footprint tests were carried out in the 1st, 4th, 7th and 12th week postoperatively. The animals were tested on a 100 cm long and 7 cm wide runway with side walls and a transparent floor. The walk of a rat along the runway was recorded with a digital camera and automatically analyzed frame by frame (Catwalk XT 8.1, Noldus Information Technology BV, Wageningen, Netherlands). Each animal was tested 3 times consecutively. Framed foot images were analyzed with respect to the foot rotation angle (the angle made by 2 lines connecting the 3rd toe and the stride line at the center of the paw) of the right hind paw (ipsilateral to the injury site), and interlimb coordination (the smallest distance between the middle point of the hind paw and the forepaw on the same side).
BBB open field test

Open field tests were carried out in the classical manner on a plexiglass surface. During the test, the motor function of the joints was analyzed to assess the stepping ability of the animal. Additionally, the general coordination and stability of the body were evaluated. The value range was 0–22, where 0 was a complete lack of motor capability and 22 indicated normal locomotion. The results obtained were presented as average values from both hind extremities.

Retrograde neuroanatomical tracing

Retrograde neuroanatomical tracing was used to determine the extent to which supraspinal axons regenerate to reach spinal cord segments caudal to the injury. The application of Fluoro-Gold (FG) (Fluorochrome Inc., Englewood, USA) was performed using methods originally described by Coumans et al.17

Only 4 animals in each group were randomly selected for this procedure. The remaining animals from each group were used for functional testing, due to restrictions of the Local Animal Research Ethics Committee limiting the number of animals used in all the experiments. For retrograde tracing, 1 week before the end of the experiment, anesthesia was administered as described above, then the spinal cord was exposed by laminectomy below the injury site and 2 microcrystals of Fluoro-Gold were placed bilaterally inside the spinal cord 10 mm caudally from the injury site. To ensure that there was no unintended diffusion of FG into the spinal cord rostral to the injury, the injection sites and lesions were examined in all the animals. None of the animals had any spread of the tracer.

To quantify the number of FG-positive neurons present in the brain stem (red nucleus) and primary motor cortex, on the last day of experiment, the animals were rapidly perfused with a bolus of cold PBS, and whole brains were carefully dissected, dehydrated in 15% sucrose, embedded in Tissue-Tek matrix (Sakura Finetechical Co., Tokyo, Japan), frozen, and then cut coronally into 10 µm sections. Every 6th slide was analyzed, giving 18–22 sections per animal. The sections were viewed under UV excitation (at a 365 nm wavelength) in a fluorescent microscope using the appropriate filter (Labophot 2, Nikon, Tokyo, Japan) and photographed. Microphotographs were taken near the 4 corners and the center of the areas where the most cells were present. The number of cells from each section was then summed for each rat for both the brain stem and the motor cortex; the number of cells per mm2 was calculated and finally averaged for the whole group.

Histopathologic examination

After 12 weeks, the animals were re-anesthetized and perfused transcardially with 100 mL PBS (pH = 7.4) followed by 100 mL of 4% paraformaldehyde solution in the same buffer. Fragments of spinal cord (~2 cm) incorporating the injured area were dissected and dehydrated in 20% sucrose in PBS for 24 h at 4°C. The spinal cords were then embedded in Tissue-Tek matrix (Sakura Finetechical Co., Tokyo, Japan), frozen, and cut sagitally or transversally into 10 µm sections mounted on Super-Frost Plus slides (Thermo Fisher Scientific, Waltham, USA). The slides were subjected to routine hematoxylin-eosin staining, 1% toluidine staining or immunohistochemical labeling. The anti-rat antibodies used were rabbit GFAP (for astrocytes) and mouse GAP-43 (both from Chemicon Europe Ltd., Southampton, UK). The sections were incubated with the primary antibody overnight at 4°C, and then, after triple rinsing in PBS, they were treated with secondary goat anti-rabbit or anti-mouse IgG antibodies conjugated with Alexa 488 and/or Alexa 568 (Molecular Probes, Eugene, USA). Sections co-stained in VectaShield with DAPI (Vector Laboratories, Burlingame, USA) were examined under a confocal laser scanning microscope (FluoView, Olympus Corporation, Tokyo, Japan). The images were digitally stored and then analyzed. Twelve weeks after surgery the total average lengths and sizes of lesions (cavity areas) were measured.

Statistical analysis

The ANOVA and nonparametric Kruskal-Wallis ANOVA were used to analyze normally distributed data and non-continuous data, respectively. Differences between the group means in footprint test results, histological and BBB scores at each time point after the injury were identified using the Student’s t-test. The level of statistical significance was set at p ≤ 0.05. All data was expressed as mean ±SD.

Results

Functional tests

All the functional tests were performed by the same person, properly trained and experienced in conducting this type of research. Immediately after SCI most of the animals were monoplegic. The hind paw angle of rotation increased in all the animals in the 1st week after the injury, reaching 19.6 ±1.2° in the control group at the end of the 12-week observation, indicating significant deterioration of locomotor function following the injury. In the NAC group, this parameter was significantly lower by the 12th week (15.1 ±1.2°; p < 0.05). The differences appeared in the 7th week of observation (Fig. 1).

Interlimb coordination in the animals treated with NAC did not improve, reaching a value of 2.17 ±0.26 cm, which was comparable with the results in the control group (2.13 ±0.27 cm) (Fig. 2).
The analysis of the BBB tests revealed comparable dynamics of recovery of function in the NAC group and in the control group. BBB scores 1 week after SCI were lower than 10 in all the animals, and after 12 weeks they had not significantly improved in either the control group (10.3 ±0.6) or the NAC group (11.9 ±0.7; p > 0.05) (Fig. 3).

The area of the main lesion (cavity area) was slightly decreased in the NAC group (0.81 ±0.29 mm²) as compared to the control group (1.03 ±0.21 mm²) (p > 0.05) (Fig. 4). These values, however, were not significantly different.

**Morphology**

The length of the spinal cord lesions differed between the 2 groups. In the control group it was 6.11 ±2.1 mm, which was significantly higher than in the NAC group (4.07 ±0.56 mm; p < 0.05) (Fig. 5).

**Neuronal tracing**

FG-positive cells in the brain stem and primary motor cortex, proving the survival of long-tract neurons, were more numerous in the NAC group (96.7 ±43.1) than in the control group (17.3 ±2.6), and this difference was significant (p < 0.05) (Fig. 6).

**Discussion**

Spinal cord injury leads to a loss of motor and sensory function distally to the site of the trauma. The most important goal in limiting spinal cord injury should be reducing edema and free radical generation in damaged neurons and controlling inflammation in order to decrease secondary injury to the spinal cord parenchyma after the initial insult, promoting neural growth and demyelination repair to reduce conduction deficits.2 In the present study, NAC was used because of its ability to reduce oxidative stress by interfering with free radicals or up-regulating antioxidant systems.18,19

Some beneficial effects of NAC application in SCI in an animal model were observed in the present study. The angle of hind paw rotation was significantly lower in the NAC group than in the control group. However, interlimb coordination did not improve in the animals treated with NAC in comparison to the control group. Karimi-Abdolrezaee et al. revealed that after trauma, the normal rotation angle (about 8°) increased to 30°, while interlimb coordination was about 1.5 cm in healthy rats and increased to 3 cm or more after injury.20 Similarly, the area of the main lesion and the length of lesion in the injured spinal cords were only slightly decreased in the NAC group as compared to the control group.

There are only a few articles about the protective role of NAC on injured spinal cord neurons in vivo. Hanci et al. investigated the effectiveness of methylprednisolone, NAC and a combination of both compounds in spinal cord injury in rats. In that study, injuries were performed extradurally with aneurysm clips at the T4–T5 level. After injury, methylprednisolone was applied intraperitoneally in the 1st group (30 mg/kg and maintenance doses of 5.4 mg/kg); in the 2nd group NAC (150 mg/kg)
was administered; and in the 3rd group both compounds were administered in the doses given above. The authors showed that administration of methylprednisolone, NAC and a combination of both compounds prevented secondary trauma in experimental spinal cord injury in the rats.21

Karalija et al. demonstrated the neuroprotective efficacy of NAC and acetyl-L-carnitine in cases of spinal motor-neuron degeneration. They showed that both substances restored the density of dendritic branches and axons in the ventral horn of hemisectioned rat spinal cords.22 However, Kaynar et al. reported that a single dose of NAC administered intraperitoneally was ineffective after experimental spinal cord injury with an aneurysm clip in rats.23

Naziroglu et al. examined the effects of NAC and selenium on apoptosis and oxidative stress in the hippocampus of rats following brain injury. NAC and selenium were administered intraperitoneally in 32 rats. The authors observed that both substances had a protective effect against oxidative stress and apoptosis in the hippocampus, and that the effect of NAC was greater than that of selenium.14

Some authors have shown that N-acetylcysteine has favorable effects on brain ischemia and ischemia/reperfusion injury. Khan et al. investigated the neuroprotective potential of NAC administered intraperitoneally (150 mg/kg) in rats with temporary focal cerebral ischemia immediately and 6 h after the reperfusion. They observed a significant reduction in the infarct area and volume, and also an improvement in neurologic scores. Their results showed that NAC protected against free oxygen radical injury, apoptosis, and inflammation.24 However, Thomale et al. found that after brain contusion, NAC was not effective at reducing the area of injury and did not influence post-traumatic brain edema formation.25

Cakir et al. investigated the effect of NAC on spinal cord ischemia-reperfusion injury in rabbits. Ischemia was induced by clamping the aorta both below the left renal artery and above the aortic bifurcation. They observed that administering 50 mg/kg of NAC resulted in significant reduction of motor dysfunction, and that a combination of hypothermia and NAC led to complete recovery of motor function in the animals. The authors stated that NAC and hypothermia following ischemia and reperfusion of the spinal cord protected against spinal cord injury.26

Hicdonmez et al. assessed the effects of a single dose of NAC on tissue malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase and catalase activity in rats subjected to experimental closed head trauma. They reported favorable effects of NAC treatment on the oxidative brain tissue injury induced by the trauma.27 However, in a study on rats with moderate left focal cortical contusion trauma, Thomale et al. found that 163 mg/kg of NAC applied intraperitoneally 2–4 h after the brain injury was ineffective against post-traumatic perfusion, brain edema or contusion volume.28

Cuzzocrea et al. investigated the effect of NAC on brain ischemic injury in gerbils. Ischemia-reperfusion injury was induced in Mongolian gerbils by a single bilateral occlusion of the common carotid arteries, and NAC (20 mg/kg) was given intraperitoneally 30 min before and 1 h, 2 h, and 6 h after reperfusion. The authors observed a reduction
in brain edema after the injury, and an increase in MDA and myeloperoxidase (MPO) levels in the hippocampus. They showed that NAC administration increased survival and reduced the hyperactivity of neurons associated with post-traumatic neurodegeneration, and also caused a reduction in neuronal loss in the treated animals.

In the present study, the neuroprotective activity of NAC had limited positive influence on the regeneration of isolated spinal cord injuries in rats. This may be due to the relatively low permeability of NAC through the blood-brain barrier. The ability of NAC to cross the blood-brain barrier is questionable and could be dependent on the dose and schedule of administration.

Further confirmation of the neuroprotective efficacy of NAC in spinal cord injury is needed. It is important to establish the optimal dose of this compound, especially in comparison to other substances with antioxidant properties previously studied in animal models.

References


