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Original Article

Changes in nitric oxide synthase expression in young and adult rats after spinal cord injury

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Objective: To examine the clinical meaning of the changes in nitric oxide synthase (NOS) expression and activity after spinal cord injury (SCI) according to the age of the experiment animal.

Material and method: Ten 5- and 16-week-old Sprague–Dawley rats were laminectomized at T10 and SCI induced at this level using a New York impactor. Outcome measures to assess SCI utilized the Basso–Beatti–Bresnahan scale to quantitate hind limb motor dysfunction as a functional outcome measure. NOS isoforms (nNOS, neuronal NOS; iNOS, inducible NOS; and eNOS, endothelial NOS) were also immunolocalized in sections of control and *spinal cord injury* in the two sample groups using specific monoclonal antibodies. Student's *t*-test evaluated the difference between the young and adult rats, and $P<0.05$ was considered as significant value.

Result: As the expression of nNOS on the spinal gray matter of the adult rat decreased, eNOS activity increased. Different from the adult rat, expression of the nNOS in the young rat was maintained until 1 day after SCI, and compared with the adult rat; eNOS activity was increased in the vessels from the damaged gray matter area after 7 days of SCI. iNOS expression was maintained until the 7th day of SCI on the adult rat, but iNOS expression after 7 days of SCI on young rat decreased. The young rat showed relatively less motor disability on the hind limb when compared with the adult rat, and had a rapid recovery.

Conclusion: Neural protective eNOS activity increased after SCI in the young rat, and neural destructive iNOS expression was more remarkable in the adult rat.
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Introduction

The process of inflammation after spinal cord injury (SCI) is initiated by glial cells and T cells that activate macrophages to mount an immune response. Macrophages and neutrophils cause lesion enlargement and tissue destruction. Many cytokines including tumor necrosis factor and interleukins 1, 6, and 10 are released during the secondary immune reaction that is mediated by macrophages and microglia. These cytokines induce the activation of other substances such as chemokines and nitric oxide (NO) and also induce the secondary immune reaction.

NO is a neurotransmitter that is not stored in synaptic vesicles and is synthesized from L-arginine by NO synthase (NOS) only when required. NO can have neurotoxic effects and has been implicated in several central nervous system (CNS) diseases. The genetic and amino acid sequences of three isoforms of NOS were discovered recently. The three NOS isoforms are neuronal NOS (nNOS; also known as brain NOS) that is located within nerve cells, endothelial NOS (eNOS) that is located within vessel endothelium, and inducible NOS (iNOS) that is found in macrophages, astroglia cells, and microglial cells.[1–5]

When the spinal cord is injured, the tissue that surrounds the primary lesion is transformed into a secondary lesion. Reactive oxygen species (ROS) are important mediators of SCI.[6] One such ROS, namely NO, has major cytotoxic effects within the secondary lesion.[7] For example, peroxynitrite is a superoxide derivative of NO that has been shown to destroy proteins, lipids, and DNA.[8,9] The neurotoxic effects of NO vary according to the concentration of NO, redox conditions, cell type, and the tissue milieu. However, NO can also produce neuroprotective effects,[10] and under normal conditions or when the spinal cord is

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under pressure, NO has been shown to maintain the vascular tension of small arterioles.[11,12] NOS expression increases after SCI, although the different NOS isoforms exhibit increased expression at different times. For example, expression of nNOS and eNOS increases dramatically 4–8 h after SCI, whereas iNOS expression is markedly elevated 72 h after SCI.

The inflammation response to CNS injury varies according to age. For example, young rats with SCI exhibit few spinal cord lesions and limited motor dysfunction, whereas SCI-induced lesions and motor dysfunction are much more severe in adult rats. In the present study, we compare changes in NOS expression after SCI between young and old rats.

Materials and methods

Animals
We used 5- (young) and 16-week-old (adult) male Sprague–Dawley rats (10 of each type). The average weight of the young and adult rats was between 115 and 324 g, respectively. Animals were anesthetized with halothane before a laminectomy was performed at the level of the 10th thoracic vertebra. Care was taken to avoid damaging the dura mater. After laminectomy, SCI was induced using a New York impactor (10 g/20 mm). The muscle and skin were then sutured using 3-0 silk sutures. Following surgery, we placed 100 mm Petri dishes containing mash (food pellets soaked in water and four 1-inch pieces of apple) into the cages. To prevent urinary tract infections and to maintain bladder function, artificial urination was performed twice a day and administered subcutaneous Cefazolin injections for 5 days at 25 mg per kg per day.

Measurement of motor dysfunction
Motor dysfunction in the hind limbs was measured using the Basso–Beatti–Bresnahan scale.[13,14]

Tissue preparation
Rats were killed at day 1 or 7 after SCI. Tissue was fixed by perfusing the animal with 4% paraformaldehyde. Fixed tissue at the site of the SCI was excised and immersed in the same fixative for 24 h. Thereafter, the tissue was preserved in 30% sucrose. Frozen sections of fixed tissue were prepared at a thickness of 30 μm and collected into 0.1 M phosphate-buffered saline (PBS) in a 24-well plate.

Immunohistochemistry for nNOS and iNOS
Sections were preincubated with a 1% solution of hydrogen peroxide and methanol for 30 min to neutralize endogenous peroxidases. Sections were later incubated with 3% normal goat serum (NGS), 1% bovine serum albumin (BSA), and 0.3% Triton X-100 in 0.1 M PBS before the addition of a primary antibody raised against nNOS (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or iNOS (1:500; Calbiochem, San Diego, CA, USA) diluted in a solution of 1% NGS and 1% BSA in 0.1 M PBS. Sections were incubated with the primary antibody for 18 h at 4°C. Thereafter, a secondary antibody (1:200 biotinylated anti-rabbit IgG; Vector Laboratories, Burlingame, CA, USA) diluted in the same solution used to dilute the primary antibody was added to the sections, which were incubated for 2 h more at room temperature. Thereafter, the ABC reagents of the Vectastain kit (Vector Laboratories) diluted to 1:100 in 0.1 M PBS were added to the sections, which were incubated for 2 h at room temperature. Finally, a solution of 0.1 M PBS with 0.05% 3,3-diaminobenzidine tetrahydrochloride and 0.02% hydrogen peroxide was added to produce a color reaction. Sections were then washed in distilled water before being mounted on slides using standard procedures.

Immunohistochemistry for nicotine adenine dinucleotide phosphate hydrogen-diaphorase
Some of the sections that had been stained for nNOS or iNOS (see above) were used to double stain nicotine adenine dinucleotide phosphate hydrogen-diaphorase (NADPH-d) for eNOS. The sections were washed with 0.1 M PBS before being incubated in 0.1 M PBS with 1 mM NADPH, 0.1 mM nitroblue terazolium chloride, and 0.3% Triton X-100 for 1 h at 37°C. Thereafter, the tissue was washed with saline and was then stained using the methods described above.

Quantitative analysis
The immunoreactive cells were counted by two investigators using five slides selected randomly from each rat. The number of cells counted by each investigator was averaged and expressed per unit area. If the immunostaining was weak (ie generalized background staining together with a weak acidophilic reaction), the slide was considered to be immunonegative. A positive contrast study was used as a quality control measure for slides that were pseudopositive or pseudonegative. Differences in the number of cells counted in spinal cord tissue from young and adult rats were compared using Student's *t*-test. Differences were considered to be statistically significant when P was <0.05.

Results

Changes in nNOS and NADPH-d expression in adult rats
In normal (uninjured) spinal cord from adult rats, nNOS-immunoreactive cells were localized to the areas that surrounded the central canal and to the lateral gray matter (Figure 1a). One day after SCI, there was hemorrhaging around the central canal and no nNOS-immunoreactive cells were observed (Figure 1b). Similarly, there was severe hemorrhaging and tissue injury and an absence of nNOS-immunoreactive cells 7 days after SCI (Figure 1c).

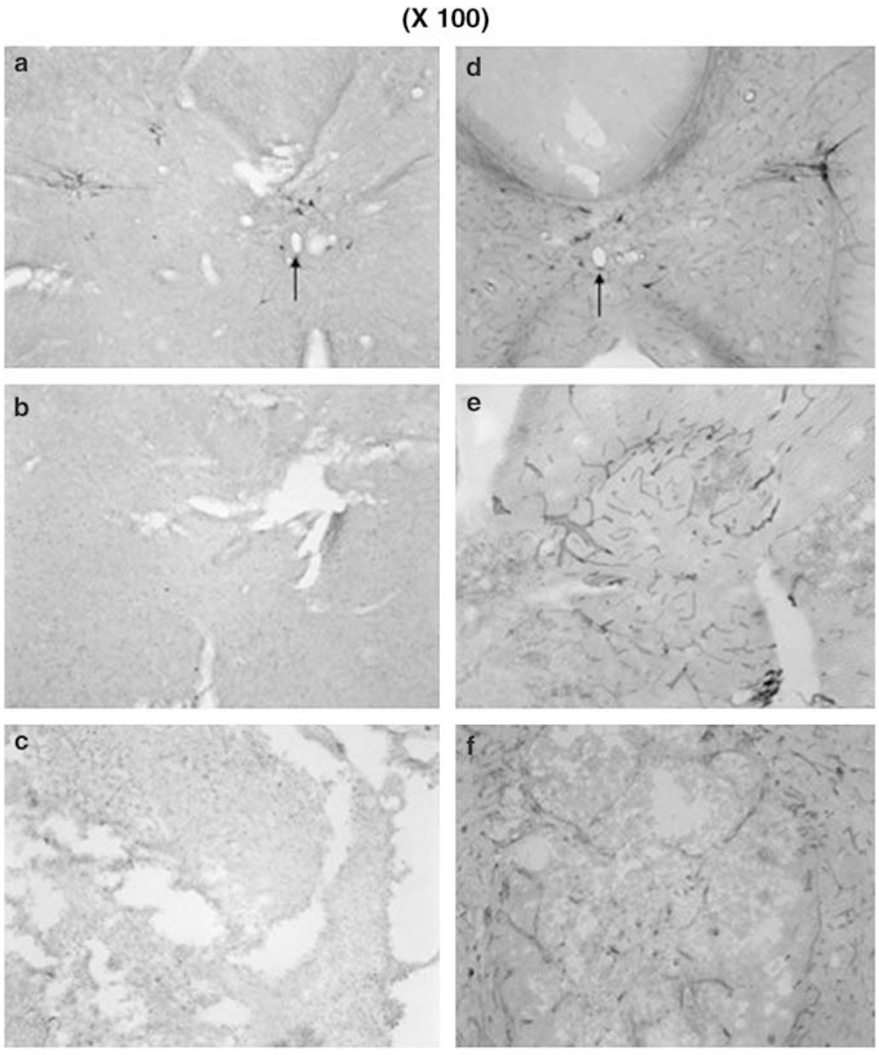

Figure 1 Immunoreactivity for nNOS (**a**–**c**) and NADPH-d (**d**–**f**) in spinal cord tissue from adult rats (× 100 magnification). (**a**) A few nNOS-immunoreactive cells were present in the area that surrounds the central canal (arrow) and within the lateral gray column in control rats. No nNOS-immunoreactive cells were detected 1 (**b**) and 7 days (**c**) after SCI. (**d**) Strongly NADHP-d-immunoreactive cells and weakly NADHP-d-immunoreactive endothelia were present in the gray matter of the spinal cords of control rats. Arrows indicate the central canal. The intensity of NADHP-d immunoreactivity was elevated within intact endothelia in gray matter 1 day after SCI (**e**) and had decreased in injured gray matter 7 days after SCI (**f**)

NADPH-d-immunoreactive cells were localized to the central canal and lateral gray matter in a pattern that resembled the localization of nNOS-immunoreactive cells, and there was also weak NADPH-d immunostaining within the blood vessels that surrounded gray matter (Figure 1d). Although no NADPH-d-immunoreactive cells expressed nNOS 1 day after SCI, there were numerous NADPH-d-immunoreactive blood vessels within the gray and white matter (Figure 1e). Seven days after SCI, numerous NADPH-d-immunoreactive blood vessels were observed within the less severely injured gray matter, whereas a few NADPH-d-immunoreactive blood vessels were seen within the relatively more severely injured gray matter. These observations indicated that nNOS expression decreased, whereas eNOS expression increased in gray matter after injury to the spinal cord of adult rats.

Changes in nNOS and NADPH-d expression in young rats

In normal (uninjured) spinal cord from young rats, nNOS-immunoreactive cells were localized to the central canal and lateral gray matter (Figure 2a). Although hemorrhaging and tissue injury was evident around the central canal 1 day after SCI, the number of nNOS-immunoreactive cells around the central canal and within the lateral gray matter was indistinguishable from the uninjured controls (Figures 2b and 3). Seven days after SCI, the white matter of the spinal cord

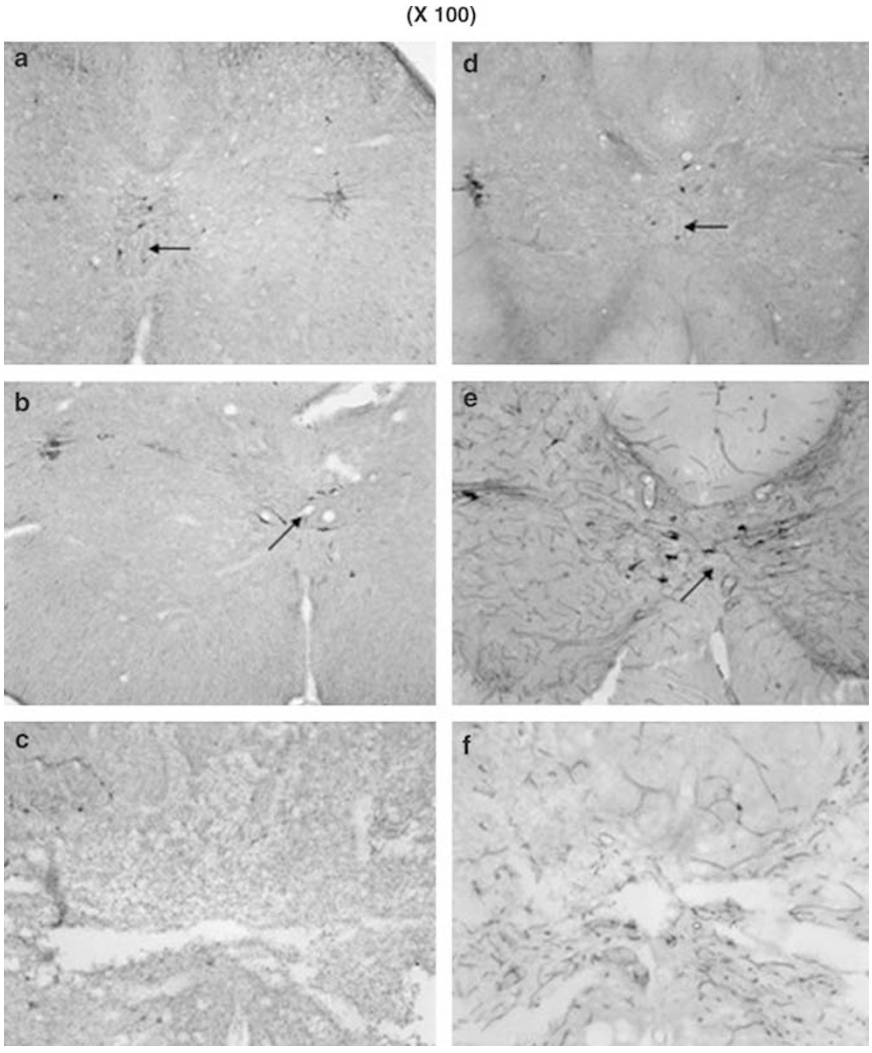

Figure 2 Immunoreactivity for nNOS (**a**–**c**) and NADPH-d (**d**–**f**) in spinal cords of young rats (× 100 magnification). Arrows indicate the central canal. A few nNOS-immunoreactive cells were present around the central canal and within the lateral gray column in control rats (**a**) and 1 day after SCI (**b**). (**c**) No nNOS-immunoreactive cells were present 7 days after SCI. (**d**) Strongly NADHP-d-immunoreactive cells and weakly NADHP-d-immunoreactive endothelia were present in the gray matter of the spinal cords of control rats. (**e**) The intensity of NADHP-d immunoreactivity was markedly higher in the endothelia in the gray matter 1 day after SCI. (**f**) NADHP-d immunoreactivity was maintained in the injured gray matter 7 days after SCI

exhibited severe damage without any sign of nNOS-immunoreactive cells (Figure 2c).

The pattern of expression of NADPH-d-immunoreactive cells in normal spinal cord was similar to that of nNOS immunoreactivity around the central canal and lateral gray matter, whereas NADPH-d immunoreactivity around the blood vessels within gray matter was relatively weak (Figure 2d). NADPH-d immunoreactivity was intense around the central canal 1 day after SCI as well as within blood vessels (Figure 2e). Seven days after SCI, although the damage to the gray matter was severe, there were many NADPH-d-immunoreactive blood vessels (Figure 2f). These observations indicated that in young rats, nNOS expression was unchanged 1 day after SCI, whereas eNOS expression in blood vessels within injured gray matter had increased 7 days after SCI.

Changes in iNOS expression in young and adult rats

There were no iNOS-immunoreactive cells in spinal cord tissue from normal adult (Figure 4a) and young (Figure 4d) rats. In adult rats, iNOS immunoreactivity was markedly more intense 1 day (Figure 4b) and 7 days (Figure 4c) after SCI. In contrast, iNOS immunoreactivity 1 day after SCI in young rats was significantly weaker than that observed in the adult rats (Figures 4e and 5) and had declined 7 days after SCI (Figures 4f and 5).

Assessment of hind limb motor dysfunction after SCI

In the young and adult rats, there was motor dysfunction of the hind limbs 1 day after SCI. This motor dysfunction was reduced by day 7 in both types of

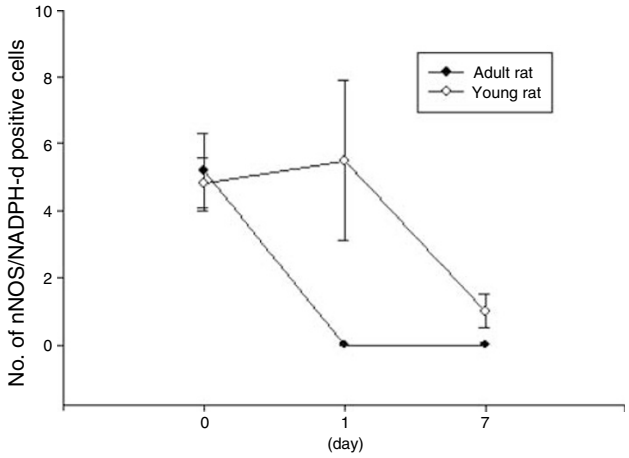

Figure 3 The number of nNOS- and NADPH-d-immunoreactive cells around the central canal in young and adult rats after SCI. The error bars indicate the SE

animal, but the young rats exhibited significantly better recovery of motion of the hind limbs compared with the adult rats (Figure 6).

Discussion

SCI is defined as an insult to the spinal cord that partially or completely interrupts the motor, sensory, and reflex functions of the cord. Our basic understanding of the pathophysiology of SCI is based on studies of the evolution of the lesion in experimental model of SCI. These studies have provided evidence to support the division of the injury into primary and secondary changes. The lack of regenerative properties of the spinal cord is probably attributable to a combination of factors, such as the inhibitory character of glial scars, white matter in CNS, and lack of trophic support. In addition, it is well established that a series of secondary degenerative processes takes place, which lead to the further loss of tissue. This secondary reaction involves a variety of neurochemical changes that initiate an excitotoxic cascade leading to changes in the physiological state of spinal neurons. This excitotoxic cascade includes a series of cytoplasmic events that direct changes in gene expression. To restore the function of injured nerves, axons must regrow and migrate to the site of injury, and adequate amounts of oxygen and nutrients must be supplied. In addition, various cytokines that are related to the immune reaction can promote the regeneration of neuronal function. For example, inhibition of apoptosis by inflammation-responsive substances such as NO may mitigate damage to neurons and promote regeneration following nerve injury even in the face of the necrosis that occurs immediately after injury. Therefore, studies related to the actions of pro-inflammatory substances have become increasingly important in the development of clinically relevant therapies for CNS injuries.

After injury to the spinal cord, a deficiency in the supply of nutrients results in progressive tissue destruction. The ability of CNS axons to grow declines with age, and the degree of petechia, regression of nerve cells, the degree of vacuolization, and the degree of sedimentation of inflammatory cells differ between young and adult rats.

At present, it is not possible to control the secondary changes that occur after SCI. Recent studies have focused on the suppression of apoptosis by altering the function of cytotoxic substances such as NO, NOS, and mediators of these substances.[15] At high concentrations, NO is toxic to cells; therefore, the concentration of NO in the CNS is strictly regulated. The suppression of iNOS expression occurs at the same physiological concentration of NO synthesized by NOS, and experiments have revealed that nNOS expression decreases in rats with SCI. Although injury-induced changes in eNOS expression are more variable, eNOS is not normally expressed in injured spinal cord tissue. However, although expression of the various isoforms of NOS change after injury to the spinal cord, the relationship between these changes and the age of the animal has not been elucidated.

NO and peroxynitrite inhibit enzymes that depend on metal cofactors or amino acids that can be oxidized. Hence, NO should modulate the activity of various cytokines and mediators of inflammation; therefore we studied the changes in NOS expression in the present study. We found that whereas nNOS expression in the gray matter of spinal cord tissue from adult rats decreased after injury, eNOS expression increased. In young rats with SCI, the elevated expression of nNOS was maintained until 1 day after the injury, whereas the expression of eNOS in the gray matter 7 days after the injury was greater than the level of expression observed in the adult rats. The elevated expression of iNOS was maintained until day 7 in the adult rats, but decreased in young rats. Hind limb motor dysfunction in young rats was less severe than that in the adult rats, and the rate of recovery of motor function was also faster in the young rats.

Others have reported that nNOS and eNOS expression 4 and 80 h after SCI was elevated by 138 and 96%, respectively, whereas iNOS expression was 103% of the controls 72 h after injury.[16] The increase in NOS expression is a calcium-dependent mechanism that is related to glutamate release and other factors that cause cytotoxicity within 4–8 h of injury,[17] whereas the increase in iNOS expression that follows injury is thought to be the result of inflammation.[18] During the early stages of SCI, NO and peroxynitrite play an important role in injury and regeneration of spinal cord tissue.[17] However, the neurotoxic and neuroprotective effects of NO depend on the time and site of release.[19,20] In the brain, NO has a neuroprotective effect and maintains blood vessel tone by increasing cerebral perfusion and decreasing platelet aggregation and interactions between leukocytes and endothelial cells.[21–24] How injury affects perfusion of the spinal cord has not yet

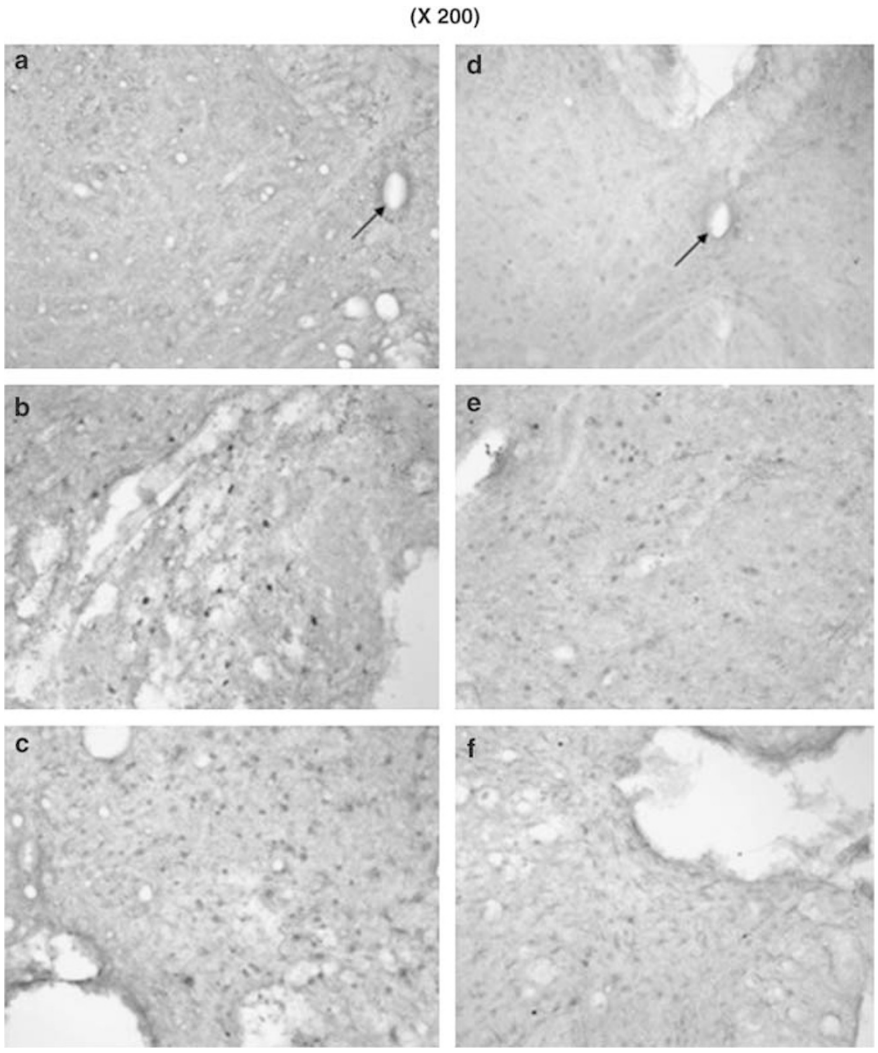

Figure 4 Immunoreactivity for iNOS in spinal cords of young and adult rats after SCI ($\times$ 200 magnification). Arrows indicate the central canal. There were no iNOS-immunoreactive cells in the spinal cords of control adult (**a**) and young (**d**) rats. In adult rats, the number of iNOS-immunoreactive cells in the gray matter had increased 1 day after SCI (**b**) and remained elevated 7 days after SCI (**c**). In young rats, the number of iNOS-immunoreactive cells in the gray matter had increased 1 day after SCI (**e**), but decreased 7 days after SCI (**f**)

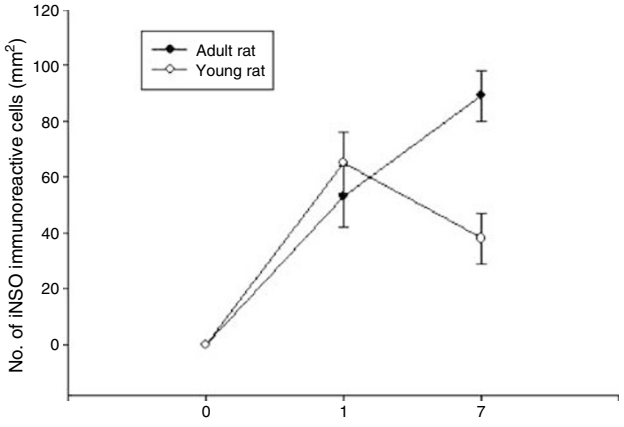

Figure 5 Immunoreactivity for iNOS in spinal cord tissue after SCI. The error bars indicate the SE

been determined. To summarize the information available at present, NO has important physiological functions such as the autoregulation of blood vessel tone, platelet function, and inflammation. Several reports have suggested that although NO is a nontoxic mediator of cerebral blood vessel dilatation in the normal brain, high concentrations of NO in the limbs may be neurotoxic. NO seems to play a pivotal role in the pathology of ischemic injury in neurons. Treatment with L-NMMA (a NOS inhibitor) promotes the recovery of spinal cord reflexes after injury. Nitroprusside, a NO donor, was reported to slow the rate at which the polysynaptic reflex potential recovers after spinal cord ischemia. Another interesting finding was that the recovery of rats with injuries to the spinal cord improved by treating these animals immediately with angiostatin, a suppressor of blood vessel proliferation.

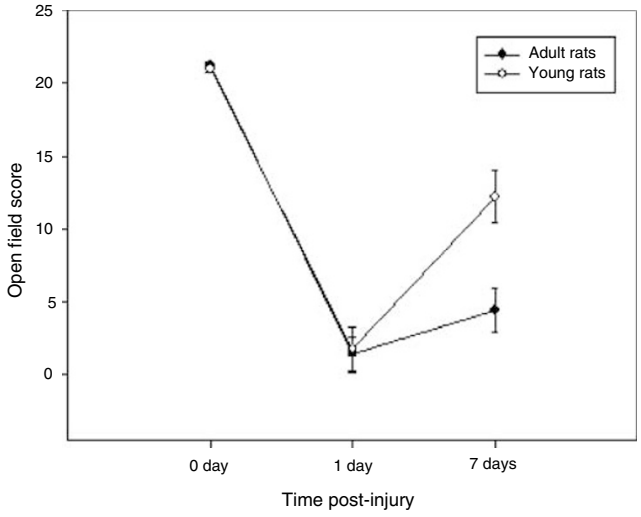

Figure 6 Open field score for the hind limbs after SCI in young and adult rats. An open field score of 0 reflected an absence of observable hind limb movement; an open field score of 21 reflected normal hind limb movement. The error bars indicate the SE

This may be due to altered regulation of the inflammatory reaction rather than a direct effect on blood vessels.

In the present study, nNOS-immunoreactive cells were absent 1 day after SCI. We assume that nNOS expression would have been elevated 4–8 h after SCI, but that damage to the gray matter within which nNOS-forming cells reside would have reduced nNOS expression. NADPH-d expression within blood vessels in the gray matter increased after SCI. This may have a neuroprotective effect and may promote spinal cord regeneration. In the young rats, numerous nNOS-immunoreactive cells were localized in the area that surrounds the central canal, which exhibited relatively little tissue damage. These nNOS-forming cells were still observed 1 day after injury. In addition, in the young rats, eNOS expression around the injured gray matter 7 days after SCI was relatively greater than that observed in the adult rats. These findings suggest that young rats are less prone to spinal cord damage and are better able to regenerate neuronal function after SCI.

During SCI, iNOS is formed by neurons, astroglia, microglia, and macrophages. The increase in iNOS expression in the CNS mediates the process of inflammation that causes tissue damage.[21–23] In the present study, the increase in iNOS expression after SCI was more prominent in adult rats, which was consistent with the role of this substance in the induction of SCI and severe motor dysfunction. In our study, fixation with 4% paraformaldehyde was carried out for 24 h. When fixation with 4% paraformaldehyde is relatively brief (6 h), some NOS immunoreactivity is still evident.[25,26] Therefore, the absence of NADPH-d immunoreactivity for eNOS in our study was likely attributable to the relatively long fixation time.

When compared with the adult rats, there was relatively less motor dysfunction in the young rats, and the young rats also recovered motor function faster than the adult rats. The motor dysfunction that results from SCI is due to tissue damage and involves changes in the activity of nNOS, iNOS, and eNOS. But we need to further study about the relationship between NO and various cytokines and the control of NO and NOS after acute SCI.

The results from these studies may lead to alternative therapeutic targets of apoptosis to SCI, providing the basis for developing agonist and antagonist systems for apoptosis and also encouraging better strategies for treatment of acute SCI. Whereas these experiment results after SCI have not yet resulted in complete difference between young and adults rats and effective control of regeneration of spinal cord, they show considerable points the way toward future experimentation.

Conclusion

In adult rats with SCI, nNOS expression in the spinal cord decreased whereas eNOS expression increased. In young rats with the same injury, the decrease in nNOS expression was less pronounced than that in the adult rats, whereas eNOS expression increased. Whereas iNOS expression in adult rats remained elevated up to 7 days after SCI, iNOS expression decreased in young rats. Motor dysfunction was less prominent in young rats with SCI compared with adult rats, and young rats also exhibited a higher recovery rate.

To summarize, we found that eNOS had a neuroprotective effect in the young rats after SCI, whereas neurotoxic iNOS expression was more prominent in adult rats after SCI.

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