Beneficial effects of local profound hypothermia and the possible mechanism after experimental spinal cord injury in rats

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Objective: The primary focus of this study was to investigate the effects of local profound hypothermia and to explore the possible mechanism in adult rats with spinal cord injury.

Study Design and Methods: Spinal cord injury models were established by placing aneurysm clips on T10. An epidural perfusion device was applied to maintain a steady temperature (18 °C) for 120 min with gradual rewarming to 37 °C Total hypothermic duration lasted up to about 170 min. The expression of axon regeneration inhibitors was tested by Western blot and real-time PCR. Luxol Fast Blue (LFB) stain and Bielschowsky silver stain were used to observe spinal cord morphology. Motor function of the hind limbs (BBB score) was monitored for 21 days.

Results: The expressions of RhoA, ROCK-II, NG2, Neurocan, Brevican, and Nogo-A were downregulated by regional hypothermia (RH) after spinal cord injury. Subsequent observation showed that rats that had received RH had an alleviated demyelinating condition and a greater number of nerve fibers. Furthermore, the RH group achieved higher BBB scores than the spinal cord injury (SCI) group.

Conclusions: Recovery of hind limb function in rats can be promoted by local profound hypothermia; this may be caused by the suppression of axon regeneration inhibitors.

Keywords: Profound hypothermia, Spinal cord injury, RhoA, ROCK

Introduction

Spinal cord injury (SCI) is a catastrophic neurological event that can seriously affect quality of life; however, there are no treatments with relatively superior curative effects. After years of exploration several therapeutic methods, such as stem cell transplantation[1] and hyperbaric oxygen therapy,[2] have been used to treat patients suffering from SCI. Of all the methods, hypothermia has been used to protect the central nervous system (CNS) after cardiac arrest hypoxic ischemic encephalopathy,[3] aortic aneurysm surgery,[4–6] and spinal cord injury, in many experimental and clinical studies.[7–9] However, some controversial issues need to be resolved, such as the temperature range, detrimental consequences, the method of introduction, and the duration of hypothermia.

The pathophysiology of SCI is complex and involves both primary and secondary mechanisms. During the period after SCI a host of biochemical changes occur, some of which are immune activation, apoptosis, intracellular Ca^2+ overload, impaired spinal cord blood flow, excess release of excitatory amino acids, and altered gene expression.[10–14] In addition to the changes mentioned above, the failure of axon regeneration is another important detrimental consequence of SCI.[15] Chondroitin sulfate proteoglycans (CSPGs) are a family of inhibitory extracellular matrix molecules highly expressed at CNS injury sites, where they can restrict anatomical plasticity by inhibiting sprouting and reorganization.[16–18] In animal SCI models the expression levels of some well-characterized CSPGs, such as NG2 and neurocan, increase rapidly within 24 h, and then reach a peak around 8–14 days after injury. The levels may remain high for at least several weeks.[19,20] Apart from the CSPGs, Nogo-A also plays an important role in inhibiting axon regeneration by causing growth cone collapse.[21,22] Unlike CSPGs, Nogo transcripts are downregulated in the central parts of lesion sites but upregulated at the borders.[23,24] Together with CSPGs, Nogo-A actives RhoA (a substance belonging to the Rho subfamily of GTPases

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within the Ras superfamily) and can regulate organization of the actin cytoskeleton, gene expression, and cell proliferation. Through regulating growth cone formation and elongating neuritis, both RhoA and its main downstream protein Rho kinase II (ROCK-II) play a significant role in the regenerative process. The regeneration of axons is restrained by the upregulation and activation of the RhoA-ROCK pathway after spinal cord injury.[25,26] In rat SCI models, RhoA mRNA as well as protein expression are enhanced significantly 7 d after injury.[27] Recently, a large number of studies have reported the beneficial effects of Rhoa-ROCK inhibitors (C3-exoenzmye, fasudil, Y-27632, ibuprofen, siRhoA, and p21),[28–32] Nogo-A antibody,[33] and the catabolic enzyme of CSPGs (chondroitinase ABC)[34] on the regeneration of inhibited axons, plasticity of uninjured pathways, and neuroprotection of injured projection neurons.

In the current study we explored the neuroprotective effects of local profound hypothermia and the possible mechanism of action after experimental spinal cord injury in rats.

Methods

Animals

Seventy-nine adult male Sprague–Dawley rats weighing 240–260 g were used in this study. Experimental protocols were approved by the Animal Care and Use Committee of Nanjing University and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. All rats were housed in standard conditions and given *ad libitum* access to food and water.

Spinal cord injury

Firstly, the rats were anesthetized with 10% chloral hydrate (0.35 mL/100 g) through intraperitoneal injection. Half the quantity (0.17 mL/100 g) was then injected into the rats every hour during the entire experiment to achieve continuous anesthesia. Rats were placed in the prone position on a warming pad. They were shaved, aseptically prepared, and a midline longitudinal incision was created to expose the region of interest (T8–T12). Subsequently, a laminectomy was performed at the T10 level using an operating microscope (M500-N, LEICA, Heerbrugg, Switzerland) to expose the surface of the spinal dura mater. Finally, a 10 g aneurysm clip (Kent Scientific, Torrington, CT, USA) was used to induce a 2-min compressive spinal cord injury.

Application of local profound hypothermia

An epidural hypothermia device was applied at the injured site according to our previous study.[35] Hypothermia was initiated by infusing 4 °C saline into the epidural area through the inflow catheter, which was connected to an infusion pump. A catheter on the other side ensured sufficient outflow. By adjusting the speed of perfusion, the temperature was allowed to stabilize at target and the temperature of the surface of the spinal cord was kept at 18 °C for about 120 min. The hypothermia device was then removed to allow gradual whole body rewarming. Because of the low speed of temperature variation, the total hypothermia time was longer than 160 min (Figure 1).

Western blot analysis

The 20 rats for the Western blot analysis were divided into four groups (SCI 2 d $n = 5$, SCI 8 days, $n = 5$, SCI + hypothermia 2 days, $n = 5$, SCI + hypothermia 8 days, $n = 5$). Protein concentrations were determined by the Bradford method. Equal amounts of protein per lane were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene-difluoride (PVDF) membranes. The membranes were blocked for 2 hours in blocking buffer [Tris-buffered saline/0.05% Tween 20 (TBST) containing 5% skim milk] and then incubated overnight at 4 °C with primary antibodies against RhoA (1:1200; Abcam, Cambridge, MA, USA), ROCK-II (1:1000;

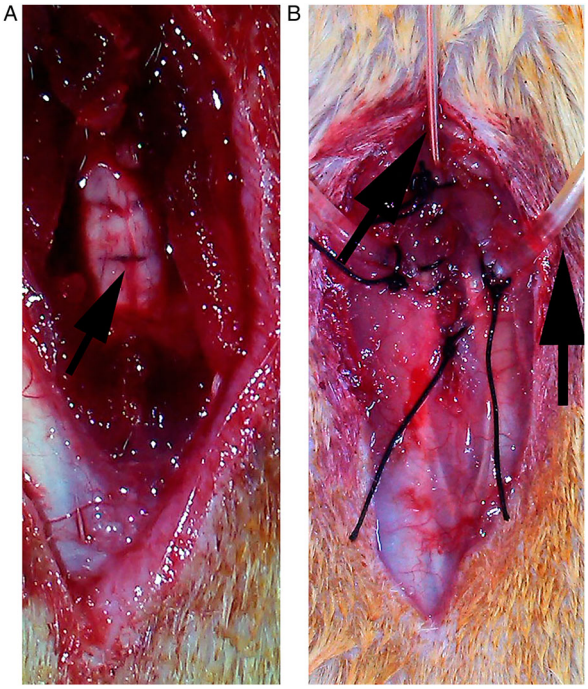

Figure 1 Rats with spinal cord injury (A). Rats with the hypothermic device (B).

Abcam, USA) and β-actin (1:5000; Bioworld Technology, St. Louis Park, MN, USA) in blocking buffer. After being washed with TBST (3×10 min), the membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated IgG (1:5000; Bioworld Technology, USA) for 2 hours at room temperature. The protein bands were visualized by enhanced chemiluminescence (ECL) Western blot detection reagents (Millipore, Billerica, MA, USA). Band density was quantified using Un-Scan-It 6.1 software (Silk Scientific Inc., Orem, UT, USA).

Real-time quantitative polymerase chain reaction (PCR)

Thirty-five rats were divided into three groups [sham $n = 5$, SCI $n = 15$ (2, 8, and 14 days, $n = 5$), SCI + hypothermia $n = 15$ (2, 8, and 14 days, $n = 5$)] Total RNA was extracted from spinal cord samples (in the test of Nogo-A, we used the samples around contusion injury sites) with RNAiso Plus (TaKaRa Bio, Japan). The concentration and purity of total RNA were determined by a spectrophotometer (OD260/280 1.8–2.0) and 1% agarose gel electrophoresis. To avoid RNA degradation, some of the RNA was immediately reverse transcribed to cDNA with the PrimeScript RT reagent kit (TaKaRa Bio, Kusatsu, Japan), and the surplus RNA was kept at −80 °C. The primers were designed according to PubMed GenBank and synthesized by Invitrogen Life Technologies (Shanghai, China). The primer sequences are given in Table 1. The quantitative real-time PCR analysis was performed by using the Mx3000P System (Stratagene, CA, USA) with real-time SYBR Green PCR technology. The PCR amplification program consisted of an initial denaturation step of 95 °C for 30 seconds, followed by 40 cycles of 95 °C for 5 seconds, and a 30 seconds annealing and elongation step at 60 °C. All samples were analyzed in triplicate. β-Actin was used as an endogenous reference "housekeeping" gene. Relative change in mRNA expression was determined by the equation:

$$\text{fold change} = 2 - (\Delta\Delta\ \text{CT}).$$

Table 1 Primer sequences of RhoA, ROCK-II, NG2, Neurocan , Brevican and Nogo-A

Primer Sequences (5′–3′)	Name
GTAAGACATGCTTGCTCATA	Rhoa forward
CTCCGTCTTTGGTCTTTGCT	Rhoa reverse
CATACACCACATGTCGCTCG	ROCK-II for
AGCCCAGACAAACCTCTCCA	ROCK-II rev
GAG ACC CTT TTT GCT CTT CCT G	Nogo-A for
AAT GAT GGG CAA AGC TGT GCT G	Nogo-A rev
CTG TGT ACC GCT TCG CCA AC	Neurocan for
TGG GAC CCC CTG GAG TAG AA	Neurocan rev
CAG GAG GAC CTG TGG GTG TG	Brevican for
CAG GGG CTG GGG ATA CAG TC	Brevican rev
TTG CTC CAG CTC CAC TCA GG	NG-2(cspg 4) for
CAG GCC CAC TTC ATC ACC AG	NG-2(cspg 4) rev
CCCATCTATGAGGGTTACGC	actin for
TTTAATGTCACGCACGATTTC	actin rev

Tissue processing and histochemistry

Following animal perfusion with 4% paraformaldehyde, the spinal cords containing the lesion area were dissected out. Spinal cord blocks (sham $n = 4$, SCI $n = 4$, SCI + hypothermia $n = 4$) were transversely cut (8 μm) at the indicated levels rostral or caudal to the lesion. Myelination of the spinal cord was evaluated via Luxol Fast Blue (LFB) staining. Briefly, spinal cord sections were dehydrated in a gradient of ethanol, and stained in 0.1% solvent blue 38 (Sigma-Aldrich Co., LLC, St. Louis, MO, USA) in acidified 95% ethanol overnight at 60 °C. After rinsing with 95% ethanol and distilled water, sections were then differentiated with 0.05% Li2CO3 and 35% ethanol several times until the contrast between gray matter and white matter was clearly detected. The pictures were analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Silver Spring, MD, USA).

Other spinal cord blocks (sham $n = 4$, SCI $n = 4$, SCI + hypothermia $n = 4$) were cut longitudinally (8 μm). Nerve fibers were observed via modified Bielschowsky's silver staining. Spinal cord sections were dehydrated in a gradient of ethanol, rinsed in distilled water three times, and stained in 3% silver nitrate solution at 37 °C for 30 min away from light. After being rinsed three times, sections were reduced for 5 min with 10% formaldehyde until they turned a light brown color and then rinsed a further three times. Ammonium silver alcohol solution was then added to sections (200 μl/section) for 5 min. Finally, sections were reduced again in 8% formaldehyde until the color became dark brown. All sections were visualized using an Olympus IX71 inverted microscope system.

Behavioral testing

BBB scoring

Animals were evaluated using the BBB open field locomotor test[36] immediately after the introduction of injury, and on postoperative days 1, 3, 5, 7, 14, and 21. BBB scores reflect a 21-point open field locomotor scale, where 0 indicates no locomotion and 21 normal motor functions. Rats' hind limb movements, trunk position and stability, stepping, coordination, paw placement, toe clearance, and tail position were analyzed

during the evaluation period. Two blinded observers evaluated the scores individually, and the mean value of the two observers' scores was used.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics, version 19 (IBM Corp., Armonk, NY, USA). The data are expressed as mean ± S.E.M. Paired *t*-tests, 1-way ANOVAs with Tamhane's T2 and repeated measures ANOVAs were used in data analysis. Values of ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$ were considered statistically significant.

Results

Temperature management and duration

The temperature level reached 18 °C within 10 min after the hypothermic device was applied. The regional temperature was maintained at the required level within a small fluctuation range (within 0.5 °C) for 120 min, and the rewarming period lasted 38.21 ± 2.35 min. Using a warming pad, rectal temperature was maintained at 37.5 ± 0.3 °C during the experiment.

Expression of RhoA and ROCK-II proteins

Western blot testing demonstrated that RhoA and ROCK-II proteins were expressed at relatively low levels at 2 days after SCI but were elevated at 8 days in both the SCI group and the SCI + hypothermia group. RhoA and ROCK-II protein expression was significantly higher in the SCI group than in the SCI + hypothermia group at both 2 days (the relative expression of RhoA $P < 0.001$, ROCK-II $P < 0.05$) and 8 days (RhoA $P < 0.01$, ROCK-II $P < 0.01$; Figure 2).

Expressions of RhoA, ROCK-II, NG2, Neurocan, Brevican, and Nogo-A mRNAs

Except for Brevican, other mRNA expression began to increase as early as 2 d after SCI and peaked at 8 days. Similarly, the expression levels of mRNA were higher in the SCI group than in the SCI + hypothermia group at 2 days, 8 days, and 14 days. Brevican mRNA expression was downregulated at 2 days and upregulated at 8 days after SCI, even though the expression level was still higher in the SCI group than in the SCI + hypothermia group at 2 days, 8 days, and 14 days (Figure 3).

LFB and modified Bielschowsky's silver staining

Six representative images of LFB staining are shown in Figure 4. The LFB staining results indicated that axonal myelination at the SCI site increased significantly at D 21 in the SCI + hypothermia group compared with that in the SCI group. The integrated optical density (IOD) of the SCI + hypothermia group (442.67 ± 50.36) was much higher than that of the SCI group (picture B 186.35 ± 35.69, $P < 0.01$). More characteristic vacuolation was observed in the SCI group than in the SCI + hypothermia group.

Representative images of modified Bielschowsky's silver staining are shown in Figure 5. Compared with sections in the SCI group, there were more nerve fibers observed at the lesion site in SCI rats at D 21 in the SCI + hypothermia group.

Locomotor function recovery

The preoperative motor function of all rats was normal (BBB Scale = 21). At 1 day after SCI, all animals in the SCI and SCI + hypothermia groups were completely paraplegic. In the following days, a slow recovery of hind limb locomotion, characterized by slight

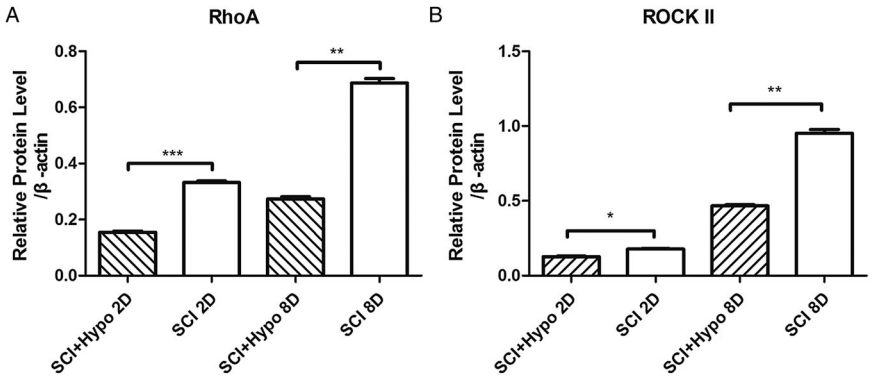

Figure 2 Time course of RhoA and ROCK-II protein expression after SCI. Expression levels of proteins were normalized to those of β-actin. The expression levels of RhoA and ROCK-II proteins were significantly decreased at 2 d and 8 d in the SCI + hypothermia group compared with those in the SCI group (the relative expression of RhoA at 2 d: SCI + hypothermia 0.155 ± 0.007 times; SCI 0.332 ± 0.012 times, $P < 0.001$, ROCK-II: SCI + hypothermia 0.128 ± 0.005 times; SCI 0.178 ± 0.006 times, $P < 0.05$. RhoA at 8 d: SCI + hypothermia 0.273 ± 0.014 times; SCI 0.087 ± 0.03 times, $P < 0.01$, ROCK-II: SCI + hypothermia 0.467 ± 0.01 times; SCI 0.9521 ± 0.04 times, $P < 0.01$). (A) A typical Western blot result. (B) Quantification of data.

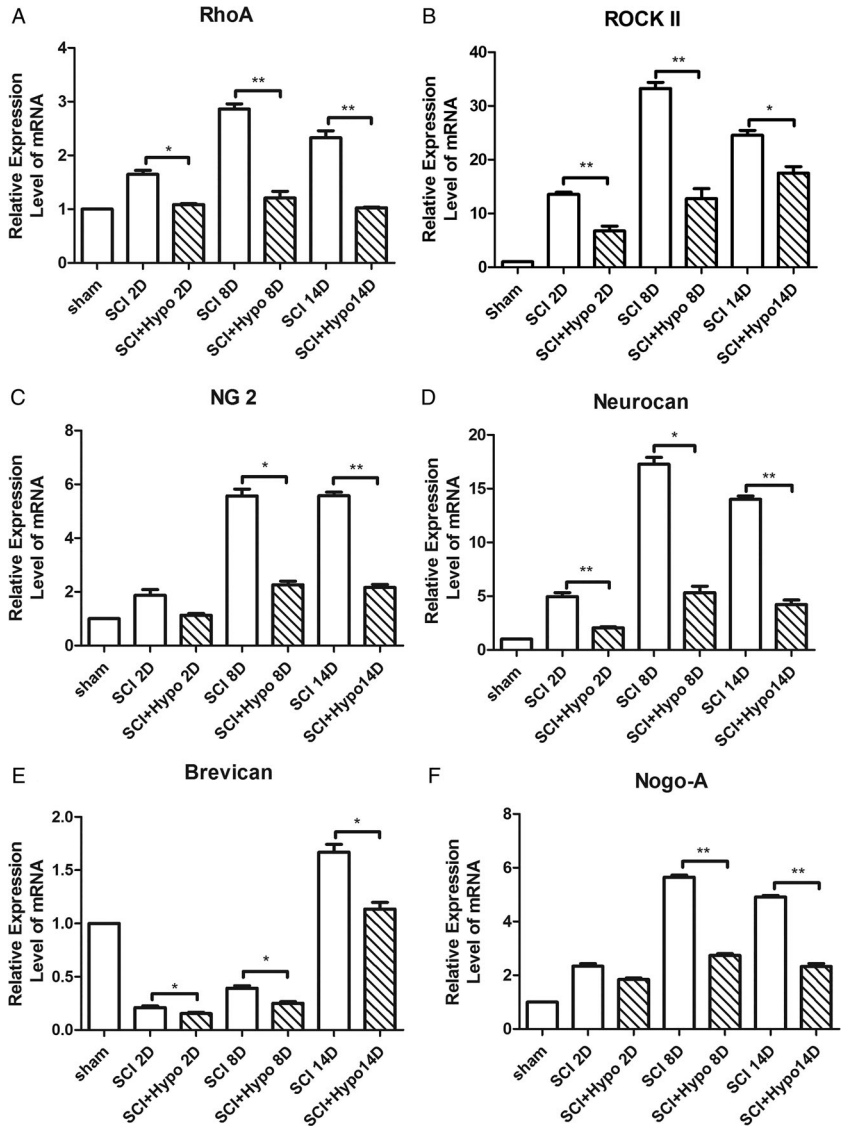

Figure 3 Time course of RhoA (A), ROCK-II (B), NG2(C), Neurocan (D), Brevican (E), and Nogo-A (F) mRNAs expressions after SCI. The data were normalized to β-actin and were expressed as fold increase over the sham group. The expression levels of mRNAs significantly decreased at 8 days and 14 days in the SCI + hypothermia group compared with those in the SCI group.

movement in one or two joints, was observed in both groups. The RH group achieved a higher BBB score than the SCI group at D 7 (SCI + hypothermia 9.15 ± 1.02; SCI 6.33 ± 0.58, P = 0.016), D 14 (SCI + hypothermia 13.25 ± 1.26; SCI 9.75 ± 0.96, P = 0.004) and D 21 (SCI + hypothermia 15.25 ± 1.32; SCI 12.25 ± 0.86, P = 0.018; Figure 6).

Discussion

Axon regeneration is much more problematic in the central nervous system than in the peripheral nervous system. CNS injury, especially of the spinal cord, often causes serious damage to patients and can lead to life-long disabilities. When the neuroprotective effects of hypothermia were identified in the 1940s, it was proven to reduce oxygen consumption and metabolic rate, steady the blood flow, and inhibit the release of excitatory neurotransmitters, apoptosis, and inflammation.[10–14] As a result, hypothermia has been widely used in the treatment of cardiac arrest hypoxic ischemic encephalopathy, aortic aneurysm surgery, strokes, and spinal cord injury.[3–9]

The beneficial effect of regional hypothermia and its possible mechanism

The effect of hypothermia on axons has rarely been studied, unlike its effect on metabolism, hemodynamics, apoptosis, and inflammation.[10–14] In the present study, the LFB staining results showed that axonal myelination at the lesion site in SCI rats was significantly increased at

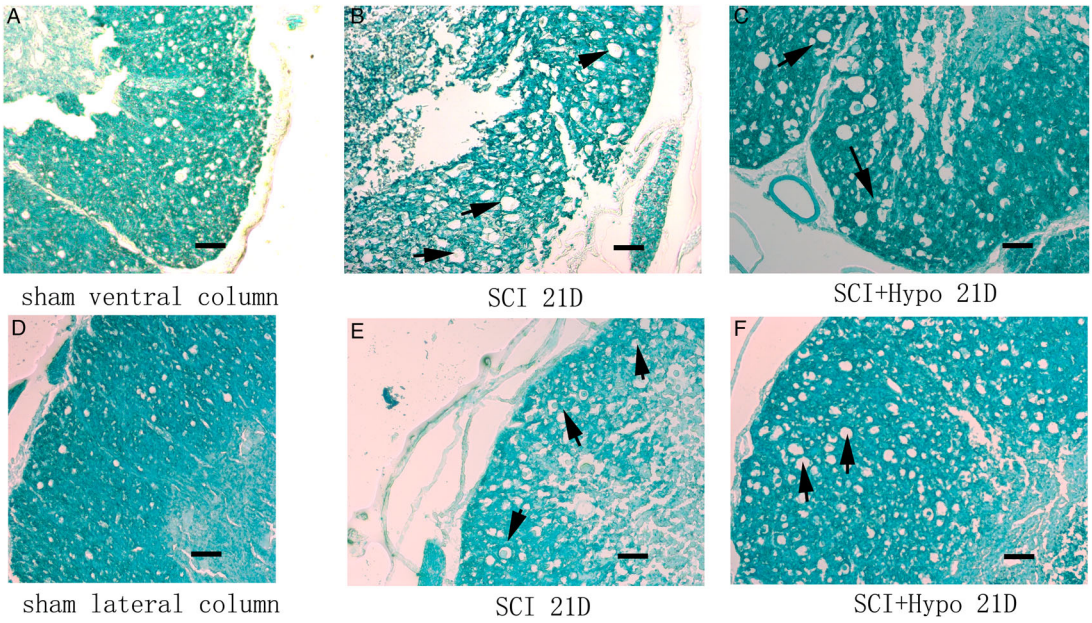

Figure 4 Representative LFB staining of spinal cord (A-C ventral column, D-E: lateral column) on D 21 from the sham, SCI and SCI + hypothermia groups. All black arrows indicate characteristic vacuolation. Scale bar = 200 μm.

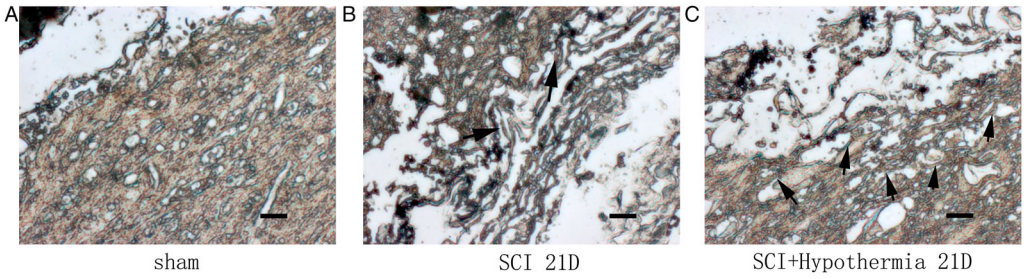

Figure 5 Bielschowsky's silver staining of the spinal cord. The spinal cord tissues on D 21 from the Sham group (A), the SCI + hypothermia group (B), and the SCI group (C). All black arrows indicate the nerve fibers. Scale bar = 200 μm.

21 days in the SCI + hypothermia group compared with the SCI group. The modified Bielschowsky's silver staining results also showed that there were more nerve fibers observed at the lesion site in SCI rats at D 21 in the SCI + hypothermia group. All of these findings may explain the reason why the BBB scores were markedly increased by the regional/local profound hypothermia at D 7, D 14 D, and D 21 after SCI.

In our study, the mRNA levels of NG2, Neurocan, Brevican, and Nogo-A were downregulated significantly in the SCI + hypothermia group. NG2, Neurocan, and Brevican [three important types of CSPG mainly expressed at CNS injury sites] were rapidly upregulated at the lesion site by reactive astrocytes in the glial scar tissues. Spinal cord trauma usually induces overexpression of CSPGs in the lesion penumbra, with higher levels in the epicenter of scar tissue.[37] In addition to the physical barrier of scar tissue including reactive astrocytes, meningeal cells, fibroblasts, and microglia, the increased levels of CSPGs form a potent chemical barrier for axon regeneration by preventing

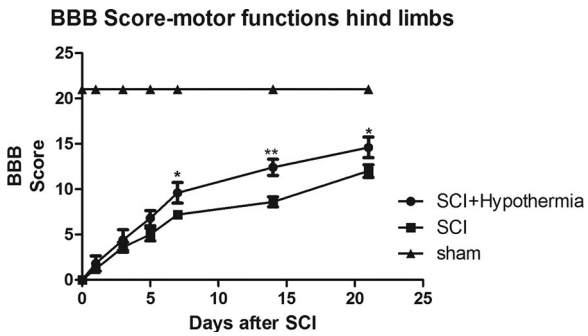

Figure 6 The BBB scores of rats after SCI in the Sham, SCI and SCI + hypothermia groups. The BBB scores of rats in the SCI + hypothermia group were higher than those of the SCI group at D 7 (SCI + hypothermia 9.15 ± 1.02; SCI 6.33 ± 0.58, P = 0.016), D 14 (SCI + hypothermia 13.25 ± 1.26; SCI 9.75 ± 0.96, P = 0.004) and D 21 SCI + hypothermia 15.25 ± 1.32; SCI 12.25 ± 0.86, P = 0.018).

elongation.[16,19,20] Nogo-A was discovered as a myelin-associated inhibitor of axon growth and its mRNA was upregulated around the lesion. Nogo-A protein was strongly expressed in injured dorsal column fibers and their sprouts that entered the lesion site. Binding to its receptors, Nogo-A could obviously inhibit axon regeneration.[21–24] Thus, surmounting strong suppression of CSPG inhibitors and Nogo-A is a major target for therapeutic intervention following CNS injuries, including SCI.[33,34] Our study found that hypothermia can downregulate the gene expressions of NG2, Neurocan, Brevican, and Nogo-A. This may be one of the important mechanisms for explaining why more axons remained in the SCI + hypothermia group. Hypothermia could inhibit the activation of astrocytes and microglia.[38,39] The regeneration inhibitors were expressed in this sense. Therefore, we assumed that the effect of local profound hypothermia on expressions of NG2, Neurocan, Brevican, and Nogo-A may be caused by the inhibition of astrocytes and microglia.

The intracellular effects of most axon growth-suppressing proteins, including CSPGs and Nogo-A, are mediated by the activation of the small GTP-binding protein RhoA, a signaling molecule that regulates neuronal morphogenesis via interaction with a number of other molecules, including serine/threonine kinases, tyrosine kinases, lipid kinases, lipases, oxidases, and scaffold proteins. In particular, the activated (GTP-bound) form of Rho can bind and directly activate ROCK. This activation leads to phosphorylation of several target proteins, including myosin light chain, and mediating cytoskeletal rearrangements, and disassembly in neurons and collapse of growth cones.[25,26,40] In the current study, the protein and mRNA expression levels of RhoA and ROCK-II were downregulated in the SCI + hypothermia group; we believe that regional profound hypothermia could suppress this pathway, including RhoA and ROCK-II. As a result, more axons and improved behavioral recovery were observed after SCI. Since hypothermia could downregulate the expression of CSPGs and Nogo-A, which could in turn active RhoA by binding to their respective receptors, activation of the RhoA and ROCK-II pathway may be inhibited by hypothermia.

Local profound hypothermia

The efficacy of hypothermia is associated with three important factors: the methods of hypothermia induction, the duration, and the temperature range. According to existing small clinical trials, systematic hypothermia both increases the incidence of cardiac arrhythmias and pneumonia and causes serious consequences such as chills, immunosuppression, electrolyte disorders, and mild coagulopathy.[41–43] RH can largely avoid the complications mentioned above and achieve a lower temperature range than systematic hypothermia. While there are numerous advantages of RH,[5,9] its use is restricted because of the technical complexity of laminectomy procedures[44] and the relatively shorter duration of hypothermia.

In this study we used a mixture of ice and saline for perfusion, as the temperature could approach the ideal level more quickly (10 min) and remain more stable during the perfusion than with pure cold saline. Although the duration of hypothermia in our study (120 min) was shorter than those of other studies (duration could be as long as three or more days),[45,46] a neuroprotective effect was found. This may be because we used profound hypothermia with a lower temperature level of 18C instead of mild hypothermia, since profound hypothermia has been proved to be more efficient in reducing oxygen consumption and metabolic rate, and inhibiting inflammation. As a result, we observed a significant recovery of locomotor function despite the shorter duration of hypothermia.

In some clinical trials, ice blankets, medicine, and endovascular devices have been used to induce hypothermia.[41–43] These methods could maintain the level of temperature for a long time, but failed to achieve the lowest temperature the patients could tolerate. A recent clinical study[9] found that the use of regional/local profound hypothermia during fixation surgery could improve the prognosis of patients with severe SCI. The majority of patients may need emergency surgery because of the severity of SCI, and this may make intraoperative regional/local profound hypothermia a potential adjunct in treating SCI in the future.

Conclusion

The present experimental study demonstrated the beneficial effects of regional profound hypothermia on the recovery of locomotor function. Moreover, our results strongly suggest that regional profound hypothermia could improve locomotor function by inhibiting the pathways of CSPGs, Nogo-A, RhoA, and ROCK-II. We hope these results will be an inspiration for further studies and will promote the potential clinical utilization of regional profound hypothermia in the future.

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Disclaimer statements

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Conflicts of interest There are no conflicts-of-interest.

Ethics approval This study was performed on rats, the paper has received approval from Research Ethical Commitee of Jinling hospital.

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