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ORIGINAL ARTICLE

Myelotomy reduces spinal cord edema and inhibits aquaporin-4 and aquaporin-9 expression in rats with spinal cord injury

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Objective: Spinal cord edema contributes to the pathophysiological mechanisms underlying spinal cord injury (SCI) and is associated with functional recovery after SCI. Early myelotomy may be a promising surgical intervention for reducing SCI-induced edema. However, it remains unclear whether myelotomy can reduce SCI-induced edema. In addition, aquaporin-4 (AQP4) and aquaporin-9 (AQP9) have important roles in the regulation of water homeostasis. Here, we aimed to determine the effects of myelotomy on AQP4 and AQP9 expression and spinal cord edema in a rat model of moderate SCI.

Methods: Rats were randomly assigned to three groups: the sham control group ($n=22$) receiving laminectomy alone; the contusion group ($n=44$) receiving laminectomy plus contusion; and the myelotomy group ($n=44$) receiving laminectomy plus contusion followed by myelotomy at 24 h. Functional recovery was estimated by the open-field and inclined plane tests. Spinal cord edema was determined by measuring the water content. The expression of AQP4 and AQP9 was determined by western blot.

Results: Compared with the contusion group, myelotomy significantly improved the Basso, Beattie and Bresnahan scores in the open-field test and resulted in a higher mean angle value in the incline plane test. Myelotomy significantly reduced SCI-induced edema at 4 and 6 days after SCI, which was accompanied by downregulation of AQP4 and AQP9 expression.

Conclusion: Myelotomy improves locomotor function, reduces edema in rats with SCI and is associated with decreased expression of AQP4 and AQP9.

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INTRODUCTION

Although surgical, physical, biological and pharmacological treatment methods have been adopted for the treatment of spinal cord injury (SCI), currently there is no effective therapy to improve the neurological outcome of SCI patients.[1] Decompression of the extradural elements is an important therapeutic strategy for the treatment of SCI and is believed to have substantial benefit in terms of neurological recovery.[2] However, decompression cannot fully remove the constraint from the dura and pia maters. Although durotomy or pia incision has been used to release the constraint from the meninges, hemorrhagic and necrotic tissues cannot be removed using these surgical procedures.[3] Myelotomy not only removes hemorrhagic and necrotic tissues by opening the dura and pia maters and swollen spinal cord but also limits the development of secondary injury induced by hematoma accumulation.[4] With the application of computer-assisted microsurgical techniques, myelotomy can be performed with precise localization of the lesion and avoidance of non-lesioned tissue.

Since Allen first showed that myelotomy improved functional outcomes in dogs with SCI in 1911,[5] myelotomy has been demonstrated to be effective for the treatment of SCI in one clinical and one animal study. In the clinical study, myelotomy was shown to relieve cord swelling and improve functional recovery of the extremities in patients with acute cervical cord injury.[4] In a rat model of acute spinal cord contusion injury, myelotomy effectively reduced tissue injury and improved functional recovery.[6]

The SCI pathophysiology involves irreversible primary injury induced mainly by mechanical compression and potentially preventable secondary injury involving a cascade of vascular, cellular and biological events such as hemorrhage, edema, inflammation, oxidative stress and cell death. Spinal cord edema is important for the development of secondary injury after SCI. Both clinical and animal studies have shown that water accumulates in the parenchyma of the spinal cord in the acute phase of SCI, and the severity of edema is closely associated with poor functional recovery.[7,8] To date, no significant progress in the treatment of SCI has been made, partly because the pathogenesis of spinal cord edema after SCI remains poorly understood.

As Agre *et al.*[9] first identified aquaporins, a family of water channel proteins that serve as selective pores through which water crosses the plasma membrane, aquaporins have been extensively studied in the field of edema. Of the 13 members of the aquaporin family, aquaporin-4 (AQP4) and aquaporin-9 (AQP9) have been found to

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be closely associated with edema in the central nervous system.[10–12] Both AQP4 and AQP9 are located in the plasma membrane. AQP4 is widely expressed in glial and ependymal cells of the spinal cord and has an important role in the regulation of spinal cord edema. In addition, AQP4 is likely to contribute to the function of the blood brain barrier and may act as an extracellular osmoreceptor. AQP9 is expressed in astrocytes, catecholaminergic neurons and endothelial cells of sub-pial blood vessels. AQP9 promotes the flow of water, glycerol, monocarboxylates and urea and has a role in water homeostasis and energy metabolism in the central nervous system. However, it remains unknown whether myelotomy affects the expression of AQP4 and AQP9 and spinal cord edema in rats with SCI.

We have previously found that myelotomy improves functional recovery in rats with acute SCI and that the optimal time window of myelotomy is 24 h after SCI.[13] In the present study, we aimed to investigate the effects of myelotomy performed 24 h after SCI on the expression of AQP4 and AQP9 and spinal cord edema in a rat model of moderate SCI.

MATERIALS AND METHODS

Animals and experimental procedures

All experimental protocols were approved by the Institutional Animal Care and Use Committee of Capital Medical University. A total of 110 female Sprague Dawley rats (10 weeks old) were used in this study. The animals were obtained from the Animal Care Center of Academy of Military Medical Sciences. Animals were housed at constant temperature (20–28 °C) with 50–60% humidity and a 12 h light/dark cycle. They were habituated to the housing conditions for at least a week before the surgical procedure. Animals were fed with standard rat chow and water *ad libitum*. The bladders were emptied manually every 8 h until recovery of reflex bladder emptying or euthanization. The 110 rats were randomly assigned to three groups using a computer-generated randomization schedule: the sham control group ($n=22$); the contusion group ($n=44$); and the myelotomy group ($n=44$). Animals in the sham group were treated with laminectomy alone without contusion. Animals in the contusion group received laminectomy plus contusion. Animals in the myelotomy group were treated with myelotomy after laminectomy and contusion. Researchers who performed surgeries were blinded to the experimental groups. In the sham group, 12 rats were used for evaluation of motor function, 5 rats for determination of spinal cord water content and 5 rats for western blot analyses. In the contusion and myelotomy groups, 12 rats were used for evaluation of motor function, 15 rats for determination of spinal cord water content at 2, 4 and 6 days after SCI ($n=5$ for each time point) and 15 rats for western blot analyses at 2, 4 and 6 days after SCI ($n=5$ for each time point).

Spinal cord injury

All animals were anesthetized by intraperitoneal injection of 10% chloral hydrate (400 mg kg^{-1}), and were placed in a stereotaxic frame. The surgical site was shaved and sterilized. A 4-cm-long longitudinal incision was made to expose the T8–T10 spinal column. After stripping the paraspinal muscles, laminectomy was performed at the T10 level to expose the spinal cord. Contusion injury was induced using a New York University weight-drop device.[14] A 10-g weight was dropped from a height of 25 mm onto the exposed dura, resulting in a moderate injury in the T10 spinal cord. The surface of the spinal cord exhibited signs of congestion. The wound was then sutured layer by layer. Immediately after SCI, those rats that shook their bodies, quickly retracted their lower limbs and wagged their tails were included in the study as per previously published criteria.[15] Of the 44 rats in the contusion group and 44 rats in the myelotomy group that received laminectomy plus contusion, 2 rats in each group were excluded from the study because of lack of the above-mentioned signs immediately after SCI.

Microsurgical myelotomy

Twenty-four hours after SCI, rats were anesthetized again. Microsurgical myelotomy was performed under a Zeiss operating microscope (Oberkochen, Germay) as we previously described.[13] The original incision was disinfected and opened layer by layer. The spinal cord tissues with dark purple appearance were exposed. A 1-ml disposable syringe needle (27 G) was used to puncture a small hole on the dura mater, slightly away from the middle line to avoid the dorsal vessels. The dura mater and arachnoid were cut open with a microscissor (Micro Scissor Straight 22.5 cm, Daddy D Pro Surgical Company, Miami, FL, USA). A 3.5-mm-long incision in the spinal cord was made with a blunt microprobe (its tip size is 0.2 mm in diameter, modified from KRAYENBUHL NERVE HOOK, WI-GS-3382, Wrangler Instruments, Sialkot, Pakistan) along the posterolateral region at a depth of approximately half-way through the spinal cord (1.0–1.5 mm). The incision was then gently washed twice with warm saline to remove necrotic tissues and blood clots. A piece of gelform was then placed on the surface of the dura mater. The muscles and skin were sutured.

Evaluation of motor function

Locomotor function was evaluated according to the open-field (Basso, Beattie and Bresnahan, BBB) and inclined plane tests. The 21-point open-field BBB Scale was used to assess hindlimb locomotion.[16] The Open-field BBB test was carried out once a week until the end of the experiments. The BBB score ranged from 0 (no hindlimb movement) to 21 (normal movement). The BBB scores were evaluated by two observers who were blinded to the animal groups, and the final score was given by consensus.

For the inclined plane test, rats were placed on an adjustable inclined plane as previously described.[17] The maximum angle on the inclined plane at which each animal maintained a stable position for at least 5 sec was evaluated by two observers who were blinded to the animal groups, and the average angle was recorded.

Determination of spinal cord water content

The spinal cord water content was measured as previously reported.[10] Rats were anesthetized after laminectomy in the sham control group and at 2, 4 and 6 days after SCI in the contusion and myelotomy groups. Spinal cords were removed, and 1.5-cm-sized spinal tissues were obtained at the edge of the injury site. The tissues were weighed on an electronic analytical balance to determine the wet weight and then dried in an oven at 95 °C for 48 h to obtain the dry weight. Spinal cord water content was calculated by the formula (wet weight–dry weight)/wet weight $\times 100$. Spinal cord water content was determined by researchers blinded to the experimental groups.

Western blot

Rats were decapitated after laminectomy in the sham control group and at 2, 4 and 6 days after SCI in the contusion and myelotomy groups. The spinal cord from the rats in each group was removed and stored at − 80 °C. Western blot experiments were performed by researchers blinded to the experimental groups. The spinal tissues were homogenized on ice in lysis buffer supplemented with a cocktail of phosphatase and proteinase inhibitors (Sigma-Aldrich, St Louis, MO, USA). Lysates were centrifuged at 12 000 *g* for 30 min at 4 °C. Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes by electroblotting. Membranes were incubated with primary antibodies against AQP4 (sc-20812, rabbit anti-ratAQP4, dilution 1:500, Santa Cruz Biotechnology, Inc., USA) and AQP9 (sc-28623, rabbit anti-rat AQP9, dilution 1:500) at 4 °C overnight. β-actin was used as a loading control. Membranes were then incubated with horseradish peroxidase-linked goat anti-rabbit secondary antibodies (dilution 1:5000) at room temperature for 2 h. Bands were visualized using a chemiluminescence detection system and were analyzed using Quantity One 4.6.2.

Statistical analysis

Statistical analyses were performed using SPSS 11.5. All data are presented as mean and s.d. Repeated-measures analysis of variance followed by the Tukey test was used to compare differences in the locomotor function, spinal cord water content, and expression of AQP4 and AQP9 among different

time points for each group. Probability values <0.05 were considered statistically significant.

RESULTS

Myelotomy improves locomotor functions of rats with SCI

Hindlimb motor performance was evaluated using two behavioral tests, including the open-field BBB test and the inclined plane test. Before SCI, BBB scores in all groups were 21. Rats in the sham control group continued to score 21 during the observed period. In the contusion and myelotomy groups, all rats scored 0 on the first day after SCI. Rats in the myelotomy group showed better performance than those in the contusion group from 7 days after SCI and reached significant difference from 14 days after SCI until the end of the study ($P<0.05$, Figure 1). At 42 days after injury, the average BBB score was 15 points in the myelotomy groups, which was 4 points more than that (11 points) in the contusion groups.

For the inclined plane test, the mean angle in all groups was ~65 degrees before SCI. Rats in the sham control group continued to score 65 during the observed period. For the contusion and myelotomy groups, all rats scored ~22 on the 7th day after SCI. After myelotomy, the average angle in the myelotomy groups was significantly higher than that in the contusion groups from 14 days after SCI until the end of the study ($P<0.05$, Figure 2). At 42 days after injury, the average angle in the myelotomy group was 49 degrees, which was 10 degrees more than that (39 degrees) in the contusion group. Both the open-field BBB test and the inclined plane test suggested that myelotomy remarkably improves hindlimb motor performance in rats with SCI.

Myelotomy reduces spinal cord edema in rats with SCI

Compared with the sham control group, the spinal cord water content was significantly increased at 2, 4 and 6 days after SCI in the contusion and myelotomy groups. There was no significant difference in the water content at 2 days after SCI between the contusion and myelotomy groups. The water content decreased with time in both contusion and myelotomy groups. The water content at 4 and 6 days after SCI was significantly lower in the myelotomy group than in the contusion group ($P<0.05$, Figure 3).

Myelotomy inhibits SCI-induced upregulation of AQP4 and AQP9

Compared with the sham control group, the expression of AQP4 and AQP9 was significantly increased at 2, 4 and 6 days after SCI in the contusion and myelotomy groups. The expression of AQP4 and AQP9 was not significantly different at 2 days after SCI between the contusion and myelotomy groups. The expression of AQP4 and AQP9 was significantly lower at 4 and 6 days after SCI in the myelotomy group compared with the contusion group ($P<0.05$, Figure 4).

DISCUSSION

In the present study, we found that myelotomy at 24 h after SCI improved locomotor functions of rats with SCI as measured by the BBB and inclined plane tests. The findings are consistent with ours and other previous reports showing that myelotomy is an effective therapeutic measure for rats with SCI.[5,7,13] In addition, we found that

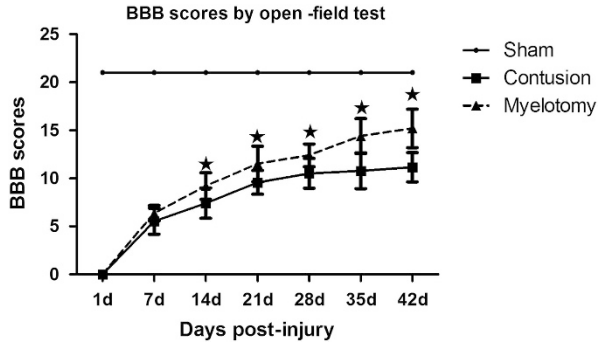

Figure 1 The time course of locomotor function measured by BBB locomotor rating scores in rats from the sham control group (sham), contusion group (contusion) and myelotomy group (myelotomy). $^{*}P<0.05$ vs contusion group. $n=12$.

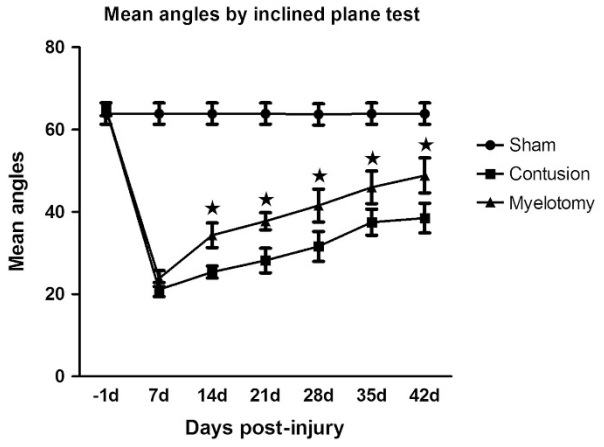

Figure 2 The time course of mean angle value measured by the inclined plane test in rats from the sham control group (sham), contusion group (contusion) and myelotomy group (myelotomy). $^{*}P<0.05$ vs contusion group. $n=12$.

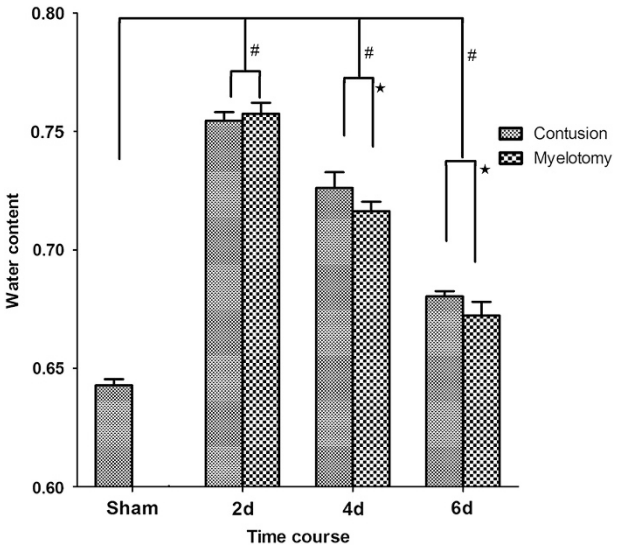

Figure 3 Spinal cord water content after operation in rats from the sham control group (sham) and at 2, 4 and 6 days after SCI in the contusion group (contusion) and myelotomy group (myelotomy). $^{\#}P<0.05$ vs the sham group. $^{*}P<0.05$ vs the contusion group. $n=5$ per time point.

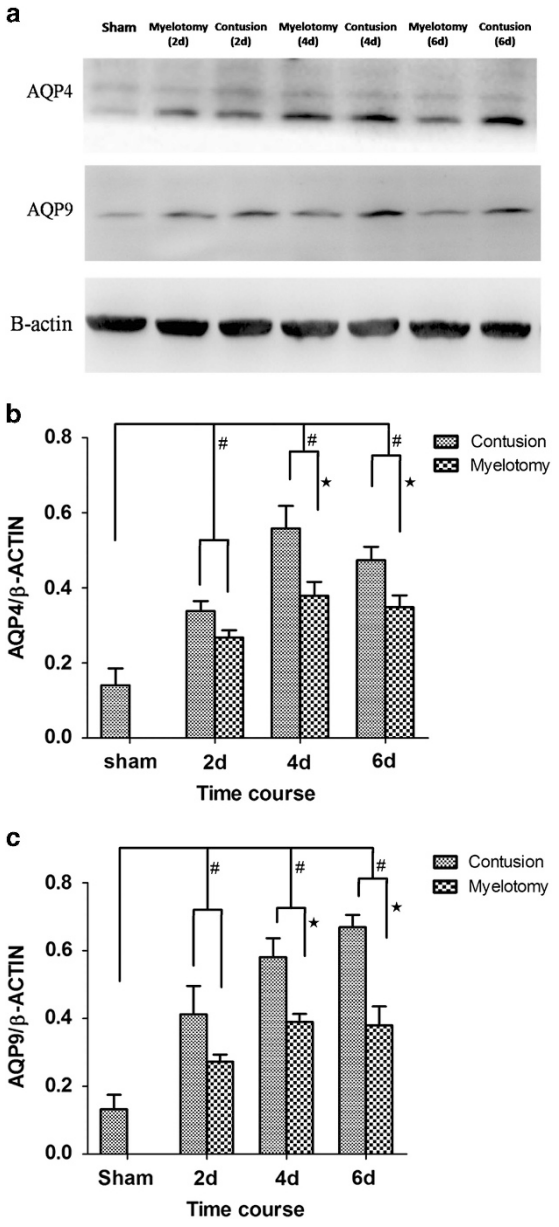

Figure 4 The expression of AQP4 and AQP9 after operation in rats from the sham control group (sham) and at 2, 4 and 6 days after SCI in the contusion group (contusion) and myelotomy group (myelotomy). (**a**) Representative western blot showing the expression of AQP4 and AQP9 after operation in the sham group and at 2, 4 and 6 days after SCI in the contusion group (contusion) and myelotomy group (myelotomy). β-actin was used as the loading control. Quantification of AQP4 (**b**) and AQP9 (**c**) expression normalized to β-actin. $^{\#}P<0.05$ vs the sham group, $^{*}P<0.05$ vs the contusion group. $n=5$ per time point.

myelotomy reduced SCI-induced edema in rats with SCI, which was accompanied by downregulation of AQP4 and AQP9 expression. Our study suggests that myelotomy may reduce edema in rats with SCI through the inhibition of AQP4 and AQP9 expression, thus improving locomotor functions.

Clinical and animal studies have demonstrated that edema is closely associated with the neurological outcome of SCI.[18] Consistent with previous reports, we found that myelotomy reduced SCI-induced edema, which was accompanied by better locomotor performance. Because edema develops from the center to the periphery of the injury site, and the center of the injury site usually contains hemorrhagic and necrotic tissues,[19] we selected the edge and not the center of the injury site to measure the water content. We found that the expression of AQP4 and AQP9 in the spinal cord increased after SCI. In agreement with our findings, several studies have shown that SCI induces upregulation of the expression of AQP4.[10] Furthermore, we found that the water content at 4 and 6 days after SCI was significantly reduced in the myelotomy group compared with the contusion group, which was accompanied by a significant decrease in the expression of AQP4 and AQP9. Similarly, Sun *et al.*[20] reported that the time course of AQP4 and AQP9 expression is consistent with that of edema formation in a rat model of intracerebral hemorrhage. These findings suggest that AQP4 and AQP9 may have an important role in the regulation of edema formation.

AQP4, which is abundantly expressed in astrocytes, has an important role in the regulation of water and salt balance. AQP4 allows bidirectional water flow and has different roles in different types of edema. AQP4 deficiency reduces edema and improves neurological outcome in models of cytotoxic (cellular) edema,[21] whereas AQP4 deletion has the opposite effect and worsens outcome in models of vasogenic (fluid leak) edema.[22] The mechanisms underlying regulation of water balance by AQP4 in the spinal cord remain unclear. AQP4 may be upregulated in response to cell swelling after SCI, and the upregulation of AQP4 in astrocytes may protect the neurons from damage arising from dramatic changes in osmotic pressure following SCI. However, AQP4 may mediate water entry into the injury site, which would lead to increased intramedullary pressure and spinal cord swelling, thus aggravating SCI. Therefore, inhibition or downregulation of AQP4 may represent a novel therapeutic strategy for reducing spinal cord edema after SCI.[10] In agreement with this idea, we found that myelotomy reduced spinal cord edema, improved motor function and was associated with downregulated AQP4 in rats with SCI.

However, some studies using different models of SCI or traumatic brain injury show contrasting results with respect to the role of AQP4. For example, Kimura *et al.*[23] have reported that AQP4 has a protective role after contusion SCI in mice. The differences between the study by Kimura *et al.* and ours are likely to be due to the use of different animal species, injury models and severity of injury. For instance, in the present study, we induced moderate SCI with a 25×10 gcm force using an New York University weight-drop device. In contrast, Kimura *et al.* generated a mouse model of SCI with a 60 kdyn force using an infinite Horizons impactor that the authors speculate might predominantly lead to vasogenic edema. Although the extent of cytotoxic versus vasogenic edema in spinal cord swelling is not well defined in traumatic spinal injury, both vasogenic and cytotoxic edema are believed to be major contributors to the pathological outcome. Further studies are required to elucidate whether the extent of cytotoxic versus vasogenic edema in different animal models of traumatic spinal injury contributes to the conflicting findings among different studies.

AQP4 has an important role in water balance, astroglial cell migration and neural signal transduction and is associated with several neurological diseases such as neuromyelitisoptica and epilepsy.[24] Autoantibodies (NMO-IgG) against AQP4 that are associated with neuromyelitisoptica disrupt the blood brain barrier and activate astrocytes, leading to inflammation and neuronal toxicity in the central nervous system. However, it remains unclear whether NMO-

IG antibodies directly cause alteration of AQP4 function in NMO patients.

The expression of AQP9 in the spinal cord has not been extensively studied. AQP9 is permeable to water as well as to monocarboxylates, glycerol, purines and urea. AQP9 has been found to be expressed in the liver, epididymis, testis, spleen, brain and spinal cord.[11,25] In the brain, it is mainly expressed in astrocytes, endothelial cells and neurons,[25] and in the spinal cord it is extensively expressed in astrocytes in the white matter.[11] In the present study, we found that the expression pattern of AQP9 was similar to that of AQP4 in rats with SCI, suggesting that, similar to AQP4, AQP9 may mediate regulation of spinal cord edema in response to SCI.

There are some limitations to this study. First, we used a model that induces only contusion and does not incorporate compression. However, patients with SCI commonly experience contusion, followed by transient compression, which causes more damage than the contusion-only model used in this study.[26,27] A future study with the contusion plus compression model will be performed to confirm the effect of myelotomy in the treatment of SCI. Second, although we found that myelotomy significantly improved BBB scores in the open-field test and resulted in a higher mean angle value in the incline plane test, the sample size is relatively small ($n = 12$ per group). Larger sample sizes may need to be used in future experiments to examine the effect of myelotomy and strengthen the validity of our results. Third, we did not examine the molecular mechanisms underlying the protective effect of myelotomy. Future studies should be performed to examine the effect of myelotomy on histopathological and ultrastructural changes in the spinal cord as well as the expression of inflammatory/oxidative stress markers.

In summary, the present study, for the first time, investigated the effects of myelotomy on the expression of AQP4 and AQP9 in rats with moderate SCI. We found that myelotomy reduced spinal cord edema, improved motor function and was associated with downregulated AQP4 and AQP9 in rats with SCI. Our study suggests that myelotomy has the potential to become a valuable treatment strategy for SCI. In the clinic, patients classified as American Spinal Injury Association-A have poor neurological recovery, and early myelotomy might have potential benefit for these patients, especially those with obvious spinal cord edema. However, much more knowledge is needed before justifying a clinical study involving incisions into the parenchyma of the acutely injured human spinal cord. Future animal studies are needed to better elucidate the physiological mechanisms, the potential risks and the types of injury most likely to benefit from myelotomy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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