European Review for Medical and Pharmacological Sciences 2019; 23: 10631-10637

MiR-325-3p promotes locomotor function recovery in rats with spinal cord injury *via* inhibiting the expression of neutrophil elastase

J.-D. SUN[1,2], Y.-H. ZENG[3], Y. ZHANG[1], X.-X. YANG[1], W.-J. ZENG[1], L.-S. ZHAO[1], C.-G. LIANG[4]

[1]Department of Spinal Cord Injury Rehabilitation, Gansu Province Hospital Rehabilitation Center, Lanzhou, China
[2]Department of Bone and Joint Rehabilitation, Gansu Province Hospital Rehabilitation Center, Lanzhou, China
[3]Department of Internal Medicine, Gansu Province Hospital Rehabilitation Center, Lanzhou, China
[4]Department of Pain Rehabilitation, Gansu Province Hospital Rehabilitation Center, Lanzhou, China

Abstract. – OBJECTIVE: This study aims to investigate the potential function of miR-325-3p in vascular integrity and inflammatory response following spinal cord injury (SCI).

MATERIALS AND METHODS: The protein levels of ANG-1, ANG-2, and caspase-3 in HUVECs incubated with 0, 100, 200, 400, and 800 ng/ml NE for 24 h were determined. The regulatory effect of overexpressed miR-325-3p on the protein levels of ANG-1 and ANG-2 was determined by Western blot. The SCI model in SD rats was established by spinal injury at T10. Subsequently, the relative levels of miR-325-3p, ANG-1, and ANG-2 were determined in SCI rats and controls. Furthermore, SCI rats were administrated with miR-325-3p mimics or negative control and the relative levels of miR-325-3p, ANG-1, and ANG-2 were examined as well. At day 14, the protein levels of iNOS and GFAP in SCI rats and those overexpressing miR-325-3p were detected. BBB (Basso, Beattie, and Bresnahan) locomotor rating scale was applied for evaluating the locomotor function recovery at day 1, 3, 7, 14, 21, and 28 following SCI.

RESULTS: NE treatment in HUVECs downregulated ANG-1 and upregulated ANG-2 and caspase-3 in a dose-dependent manner. The overexpression of miR-325-3p upregulated NE-induced decreased the level of ANG-1 and downregulated NE-induced increased level of ANG-2. After the establishment of the SCI model in rats, the miR-325-3p level gradually decreased in SCI rats relative to controls in a time-dependent manner. ANG-1 level in SCI rats decreased to the lowest on the first day following SCI, and gradually increased at day 3, 5, and 7. ANG-2 level was firstly upregulated and achieved the peak on day 3, and then decreased at day 5 and 7. Moreover, SCI rats overexpressing miR-325-3p showed a higher level of ANG-1 and lower level of ANG-2 than those of SCI rats. Overexpression of miR-325-3p downregulated the protein levels of iNOS and GFAP in SCI rats. BBB scale showed elevated locomotor function recovery in SCI rats overexpressing miR-325-3p compared with SCI rats.

CONCLUSIONS: MiR-325-3p protects the integrity of the vascular wall, reduces infiltration of inflammation, and improves locomotor function recovery at post-SCI.

Key Words:
MiR-325-3p, Neutrophil elastase, Vasopermeability, Spinal cord injury.

Introduction

Spinal cord injury (SCI) is one of the serious diseases of the central nervous system. The pathological process of SCI is very complicated, involving many biological changes at the cellular level that could lead to the disintegration of neural structure and the absence of function[1,2]. In vascular structure, endothelial cell injury caused vascular wall defect and increased permeability, eventually resulting in the occurrence of spinal cord ischemia and the expanded infiltration of inflammation[3,4]. This is also one of the most important reasons for the secondary injury following SCI.

Neutrophil elastase (NE) is a proteolytic enzyme secreted by neutrophils. It can destroy

Corresponding Author: Chunguang Liang, BM; e-mail: 461560723@qq.com

the connective components between endothelial cells and damage the endothelial cells[5,6]. Secreted leukocytes and inflammatory factors infiltrate to the blood vessels and adjacent organs, further expanding the injury[7,8]. As a result, normal spinal cord tissues suffer from inflammation and apoptosis[9].

Angiopoietin (ANG) is a kind of angiogenic factor secreted by vascular endothelial cells, which not only promotes angiogenesis and maturation, but also participates in the regulation of vascular stability[10,11]. ANG-1 is widely expressed in normal blood vessels, which is beneficial to maintain the stability of blood vessels and the integrity of the vessel wall, and prevent the leakage of plasma[12,13]. ANG-2 is highly expressed after vascular injury and antagonizes the effect of ANG-1[14]. Therefore, the upregulation of ANG-1 and downregulation of ANG-2 following endothelial cell injury contribute to protect the integrity of the vascular wall[15]. In addition, the extensive infiltration of inflammatory factors through the blood vessels activates many glial cells, which aggravates the area and density of glial scar tissue[16]. Neurons are extensively affected following SCI, accompanied by regeneration of axons and remodeling of neural structures[17]. Therefore, it is important to maintain microvascular homeostasis and enhance vascular wall integrity after SCI[18].

MicroRNA-325-3p (miR-325-3p) exerts an important role in ischemic-hypoxic brain injury[19]. MiR-325-3p may be a potential target on improving the stable state of spinal microvasculature under the condition of ischemia and hypoxia after SCI. However, the effects of vascular regulation and inflammatory divergence and glial scar remain to be investigated. In the present study, we identified the role of miR-325-3p in the vascular integrity and inflammatory response after SCI and proposed the protective role of miR-325-3p in the SCI rats.

Materials and Methods

Cell Culture and Transfection

Human umbilical vein endothelial cells (HUVECs) provided by YRGene (Changsha, China) were cultured in the endothelial growth medium. HUVECs were incubated with NE at different doses (0, 100, 200, 400, and 800 ng/ml) for 24 h. Transfection of miR-325-3p mimics or negative control was performed using Lipofectamine 2000.

Establishment of the SCI Model in Rats

A total of 90 male Sprague-Dawley (SD) rats aged 6-8 weeks and weighted at 180-220 g were selected. They were housed in a standard chamber with 22-25°C temperature, 50-55% humidity and 12 h/12 h light/dark cycle. They were given free access to food and water.

The rats received anesthesia *via* peritoneal injection of 0.8 mL of chloral hydrate (10%). The rats were fixed on the surgical table, and the anadesma and muscle were bluntly dissected to expose the T_{10} vertebral plate. T_{10} laminate was removed, and an impact (10 g × 5 cm) by a striker on T_{10} spinal cord was performed. The bleeding point of the spinal cord and delayed neurological symptoms proved that the SCI procedures were successful. The rats in the control group were subjected to T_{10} laminate removal without impact injury. MiR-325-3p mimics or negative control was epidurally injected into SCI rats. We disinfected the incision in rats by iodophor and placed on the heat pad to maintain body temperature. This study was approved by the Animal Ethics Committee of Gansu Province Hospital Rehabilitation Center Animal Center.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted using TRIzol reagent, chloroform, and isopropanol (Invitrogen, Carlsbad, CA, USA). The extracted RNAs were quantified using a NanoDrop spectrophotometer. QRT-PCR was carried out using an SYBR Green Master Mix at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 30 s. GAPDH was used as an internal reference. Relative data was calculated using the $2^{-\Delta\Delta CT}$ method. The primer sequences for the interested genes and the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as follows: miR-325-3p, forward: 5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCACAACC-3'; ANG-1, forward: 5'-TTTAGATTGGAAGGGCCACA-3'; Reverse: 5'-ATGCGCCCTTATGCTAACAG-3'; ANG-2, forward: 5'-CCCACTTCTGAGCTTCACATC-3'; Reverse: 5'-CATAGGAGGAAACCTGTTCACC-3'; GAPDH, forward: 5'-CAACTCCCTCAAGATTGTCAGCAA-3'; Reverse: 5'-GGCATGGACTGTGGTCATGA-3'.

Western Blotting

The cells were extracted from spinal cord tissues in cell lysis buffer containing protease

inhibitor and phosphatase inhibitors at 4°C. The supernatant was centrifuged at 4°C, 14,000 rpm for 15 min. Protein sample quantified by Bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) was loaded on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). After transferring to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), they were blocked in 5% skim milk for 1 h. The membranes were incubated with primary antibodies (ANG-1, 1:5000; ANG-2, 1:1000; Caspase-3, 1:1000; iNOS, 1:500; GFAP, 1:1000; GAPDH, 1:10000) at 4°C overnight and secondary antibodies at room temperature for 1 h. The blots were visualized with enhanced chemiluminescence (ECL) detection system (Thermo Fisher Scientific, Waltham, MA, USA).

Determination of Hindlimb Locomotor Function

The hindlimb locomotor function of rats was evaluated *via* the open-field Basso, Beattie, and Bresnahan (BBB) locomotor test at day 1, 7, 14, 21, and 28 days following SCI. The locomotor score ranged from 0-21 points. This experiment was independently conducted by two researchers who were blinded to treatment group.

Statistical Analysis

The data were analyzed with Statistical Product and Service Solutions (SPSS 16.0) software (SPSS Inc., Chicago, IL, USA). All data were expressed as mean±SEM ($\bar{x} \pm s$). The intergroup difference was analyzed by the *t*-test. $p<0.05$ was considered statistically significant.

Results

NE Treatment Regulated Levels of ANG-1 and ANG-2, and Induced Apoptosis of HUVECs

HUVECs were incubated with NE at different doses (0, 100, 200, 400, and 800 ng/ml) for 24 h. The protein level of ANG-1 gradually decreased, whereas ANG-2 level increased in a dose-dependent manner (Figures 1A-C). The protein level of caspase-3 was upregulated with the increased dose of NE, indicating the induced apoptosis of HUVECs due to NE treatment (Figures 1A, D).

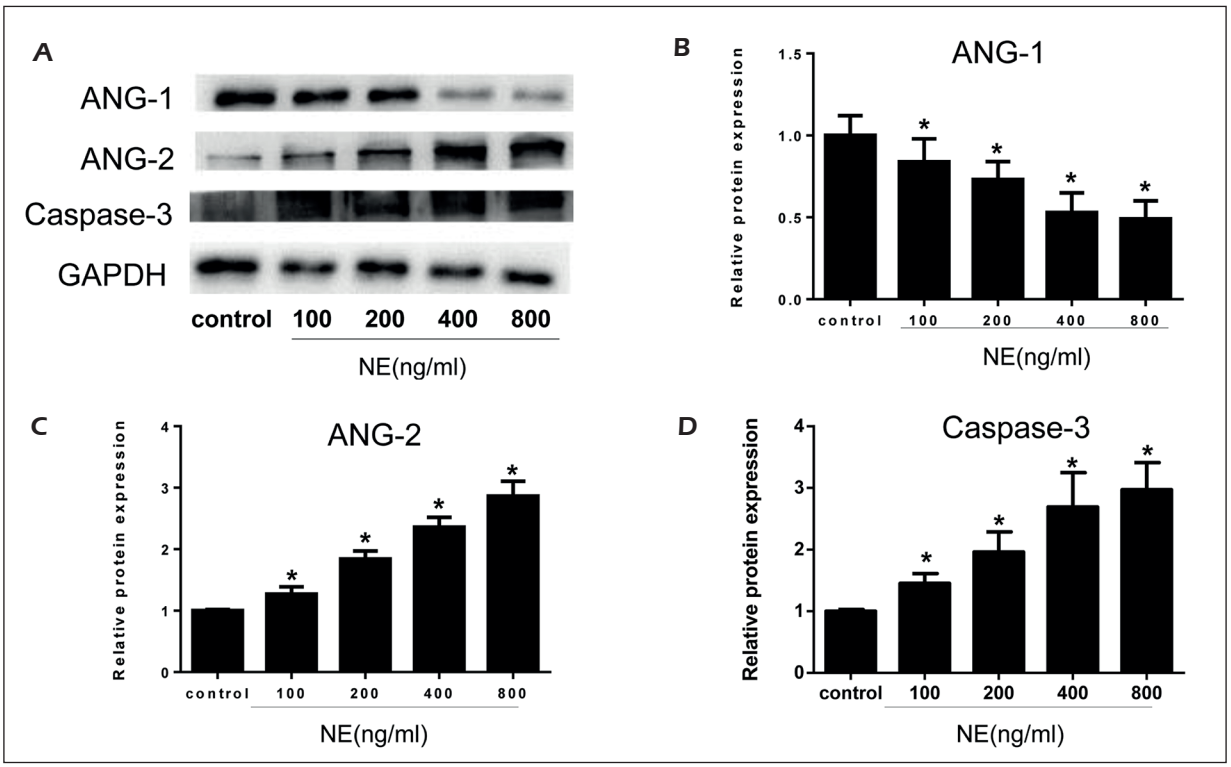

Figure 1. NE treatment regulated the levels of ANG-1 and ANG-2, and induced apoptosis of HUVECs. HUVECs were incubated with NE at different doses (0, 100, 200, 400, and 800 ng/ml) for 24 h. **A,** Western blot analyses of ANG-1, ANG-2, and caspase-3 in HUVECs. **(B)** Quantification of grey bands of ANG-1, **(C)** ANG-2 and **(D)** caspase-3.

Overexpression of MiR-325-3p Protected NE Damage to HUVECs

HUVECs transfected with miR-325-3p mimics or negative control were subjected to 800 ng/ml NE treatment for 24 h. The Western blot analyses revealed that the overexpression of miR-325-3p upregulated the NE-induced decreased level of ANG-1 and downregulated the NE-induced increased level of ANG-2 (Figures 2A, B). It is suggested that overexpressed miR-325-3p alleviated NE damage to HUVECs.

Overexpression of MiR-325-3p Alleviated SCI-Induced Dysregulated Vascular Integrity

At 1, 3, 5, and 7 days at post-SCI, the relative level of miR-325-3p, ANG-1, and ANG-2 in SCI rats and controls were determined by qRT-PCR. MiR-325-3p gradually decreased with the prolongation of SCI (Figure 3A). Besides, the mRNA level of ANG-1 reduced to the lowest at on first day post-SCI, and gradually increased within the first week. The mRNA level of ANG-2, on the contrary, first increased to the peak at day 3, and subsequently decreased at day 5 and 7 (Figure 3B). To elucidate the *in vivo* function of miR-325-3p in SCI, the SCI rats were administrated with miR-325-3p mimics or negative control. MiR-325-3p level was markedly upregulated within the first week of SCI after administration of miR-325-3p mimics in SCI rats (Figure 3C). Higher abundance of ANG-1 and lower abundance of ANG-2 were observed in SCI rats overexpressing miR-325-3p relative to SCI rats administrated with negative control (Figure 3D). The above data suggested that the overexpression of miR-325-3p alleviated the dysregulated vascular integrity at post-SCI.

MiR-325-3p Reduced Microglia Activation and Formation of Glial Scar

Microglia and astrocytes were activated at day 14 at post-SCI. However, the protein levels of iNOS and GFAP were downregulated in SCI rats overexpressing miR-325-3p (Figures 4A, B). It is believed that miR-325-3p could ameliorate microglia activation and glial scar hyperplasia following SCI.

Overexpression of MiR-325-3p Improved the Locomotor Function Recovery Following SCI

Locomotor function of SCI rats for four weeks was tested. BBB locomotor test was conducted on day 1, 3, 7, 14, 21, and 28 following SCI. The SCI rats presented a remarkably lower grade during the four weeks relative to controls. Notably, the overexpression of miR-325-3p in SCI rats greatly elevated BBB, indicating an improved locomotor function recovery after SCI (Figure 5).

Discussion

Vascular damage causes secondary injury at post-SCI, especially tissue ischemia and inflammatory infiltration[20]. Early stabilization of blood vessels and enhancement of wall integrity can effectively control the attenuation of secondary injury[21]. The inhibition of the activation of the

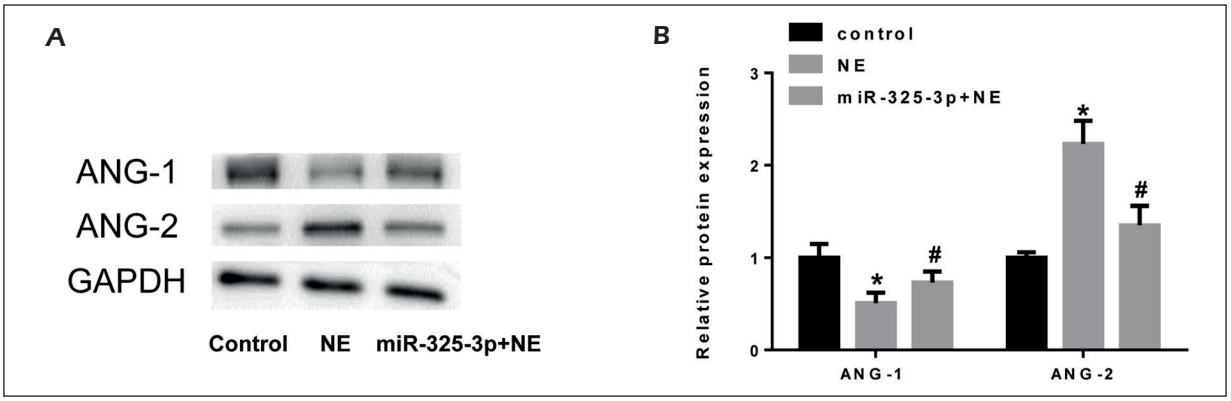

Figure 2. Overexpression of miR-325-3p protected NE damage to HUVECs. HUVECs with no treatment, NE induction or NE induction + transfection with miR-325-3p mimics. **A,** Western blot analyses of ANG-1 and ANG-2 in HUVECs. **B,** Quantification of grey bands of ANG-1 and ANG-2.

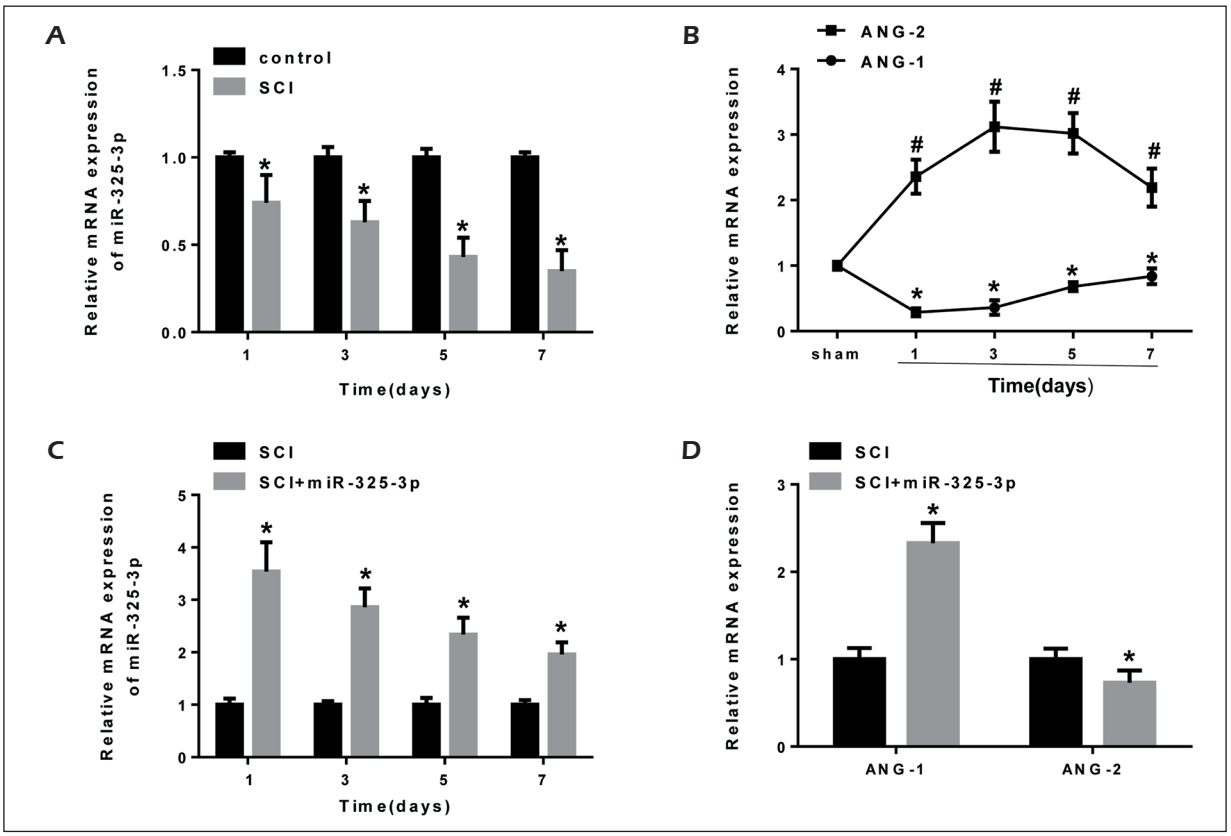

Figure 3. Overexpression of miR-325-3p alleviated SCI-induced dysregulated vascular integrity. **A,** Relative level of miR-325-3p in the control rats and SCI rats at day 1, 3, 5, and 7 following SCI. **B,** Relative levels of ANG-1 and ANG-2 in control rats and SCI rats at day 1, 3, 5, and 7 following SCI. **C,** Relative level of miR-325-3p in SCI rats and SCI rats overexpressing miR-325-3p at day 1, 3, 5, and 7 following SCI. **D,** Relative levels of ANG-1 and ANG-2 in SCI rats and SCI rats overexpressing miR-325-3p at day 1, 3, 5, and 7 following SCI.

inflammatory glial cells and the formation of glial scar tissue are beneficial to neuronal damage recovery and axon regeneration[22]. Non-violent external microenvironment and loose scar tissue can make the nerve fibers better extended to form a new loop[23].

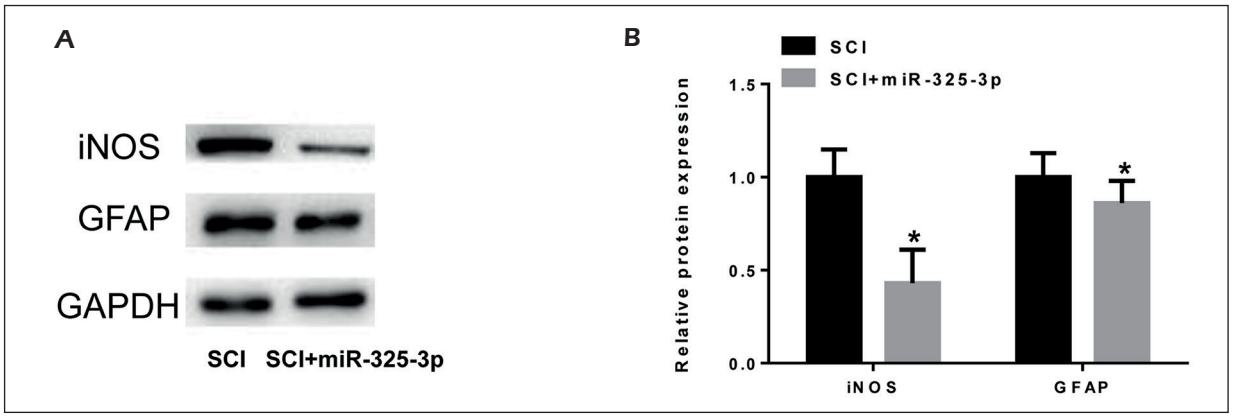

Figure 4. MiR-325-3p reduced microglia activation and formation of glial scar. **A,** Western blot analyses of iNOS and GFAP in SCI rats and SCI rats overexpressing miR-325-3p at day 14 following SCI. **B,** Quantification of grey bands of iNOS and GFAP.

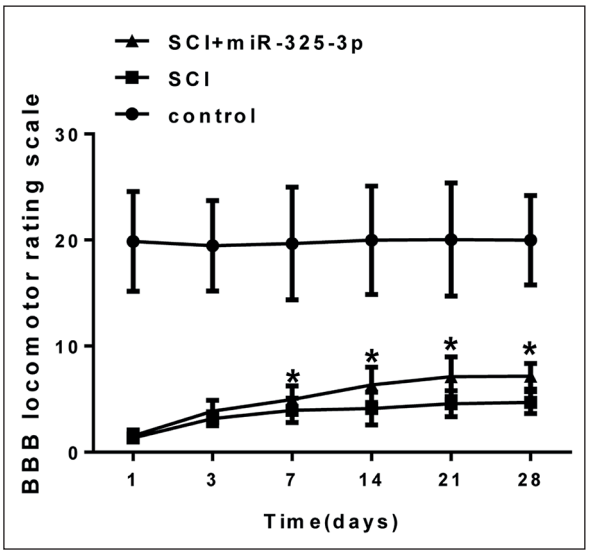

Figure 5. Overexpression of miR-325-3p improved the locomotor function recovery following SCI. BBB locomotor rating scale in control rats, SCI rats, and SCI rats overexpressing miR-325-3p at day 1, 3, 7, 14, 21, and 28 following SCI.

Neutrophil elastase is a destructive proteolytic enzyme, which is secreted by neutrophil to damage the structure of the vascular wall and stimulate the inflammatory cells chemotaxis into injured tissue[24]. At post-SCI, neutrophil infiltration and vascular destruction are induced, and the inflammatory mediators activate a wide range of glial cells and promote the proliferation and aggregation of glial scar[25].

This study demonstrated that miR-325-3p can consolidate vascular stability and protect the wall from vascular damage following SCI. By early protection of blood vessels, inflammation and scar formation are reduced, and neural function reconstruction is promoted. In this study, we proposed a new approach to interfere with the recovery of the nerve function at post-SCI. By the interaction between miRNA and NE, the stability of vascular structure was maintained, and the recovery of motor function was promoted by improving the microenvironment of the spinal cord in the early stage. However, this study failed to elucidate the specific cellular pathway in the protective effect of miR-325-3p, which remained to be discovered by further experimental studies.

Conclusions

MiR-325-3p protects the integrity of the vascular wall, reduces infiltration of inflammation, and improves locomotor function recovery at post-SCI, which may be served as a potential therapeutic target for SCI.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Mitchell CS, Lee RH. Pathology dynamics predict spinal cord injury therapeutic success. J Neurotrauma 2008; 25: 1483-1497.
2) Wu C, Chen J, Liu Y, Zhang J, Ding W, Wang S, Bao G, Xu G, Sun Y, Wang L, Chen L, Gu H, Cui B, Cui Z. Upregulation of PSMB4 is associated with the necroptosis after spinal cord injury. Neurochem Res 2016; 41: 3103-3112.
3) Fan L, Wang K, Shi Z, Die J, Wang C, Dang X. Tetramethylpyrazine protects spinal cord and reduces inflammation in a rat model of spinal cord ischemia-reperfusion injury. J Vasc Surg 2011; 54: 192-200.
4) Huang Y, Xie K, Li J, Xu N, Gong G, Wang G, Yu Y, Dong H, Xiong L. Beneficial effects of hydrogen gas against spinal cord ischemia-reperfusion injury in rabbits. Brain Res 2011; 1378: 125-136.
5) Nadel JA. Role of neutrophil elastase in hypersecretion during COPD exacerbations, and proposed therapies. Chest 2000; 117: 386S-389S.
6) Zheng JQ, Zhang GR, Li J, Bi HW. Neutrophil elastase inhibitor suppresses oxidative stress in obese asthmatic rats by activating Keap1/Nrf2 signaling pathway. Eur Rev Med Pharmacol Sci 2019; 23: 361-369.
7) Henriksen PA. The potential of neutrophil elastase inhibitors as anti-inflammatory therapies. Curr Opin Hematol 2014; 21: 23-28.
8) Mittendorf EA, Alatrash G, Qiao N, Wu Y, Sukhumalchandra P, St JL, Philips AV, Xiao H, Zhang M, Ruisaard K, Clise-Dwyer K, Lu S, Molldrem JJ. Breast cancer cell uptake of the inflammatory mediator neutrophil elastase triggers an anticancer adaptive immune response. Cancer Res 2012; 72: 3153-3162.
9) Tonai T, Shiba K, Taketani Y, Ohmoto Y, Murata K, Muraguchi M, Ohsaki H, Takeda E, Nishisho T. A neutrophil elastase inhibitor (ONO-5046) reduces neurologic damage after spinal cord injury in rats. J Neurochem 2001; 78: 1064-1072.
10) Ryu JK, Cho CH, Shin HY, Song SU, Oh SM, Lee M, Piao S, Han JY, Kim IH, Koh GY, Suh JK. Combined angiopoietin-1 and vascular endothelial growth factor gene transfer restores cavernous angiogenesis and erectile function in a rat model of hypercholesterolemia. Mol Ther 2006; 13: 705-715.

11) Hattori K, Dias S, Heissig B, Hackett NR, Lyden D, Tateno M, Hicklin DJ, Zhu Z, Witte L, Crystal RG, Moore MA, Rafii S. Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. J Exp Med 2001; 193: 1005-1014.

12) Thurston G. Complementary actions of VEGF and angiopoietin-1 on blood vessel growth and leakage. J Anat 2002; 200: 575-580.

13) Nambu H, Nambu R, Oshima Y, Hackett SF, Okoye G, Wiegand S, Yancopoulos G, Zack DJ, Campochiaro PA. Angiopoietin 1 inhibits ocular neovascularization and breakdown of the blood-retinal barrier. Gene Ther 2004; 11: 865-873 .

14) Xu J, Lan D, Li T, Yang G, Liu L. Angiopoietins regulate vascular reactivity after haemorrhagic shock in rats through the Tie2-nitric oxide pathway. Cardiovasc Res 2012; 96: 308-319.

15) Zhang M, Zhou SH, Zhao SP, Liu QM, Li XP, Shen XQ. Irbesartan attenuates Ang II-induced BMP-2 expression in human umbilical vein endothelial cells. Vasc Med 2008; 13: 239-245.

16) Wang SM, Hsu JC, Ko CY, Chiu NE, Kan WM, Lai MD, Wang JM. Astrocytic CCAAT/enhancer-binding protein delta contributes to glial scar formation and impairs functional recovery after spinal cord injury. Mol Neurobiol 2016; 53: 5912-5927.

17) Navarro X, Vivó M, Valero-Cabré A. Neural plasticity after peripheral nerve injury and regeneration. Prog Neurobiol 2007; 82: 163-201.

18) Ohri SS, Hetman M, Whittemore SR. Restoring endoplasmic reticulum homeostasis improves functional recovery after spinal cord injury. Neurobiol Dis 2013; 58: 29-37.

19) Yang Y, Sun B, Huang J, Xu L, Pan J, Fang C, Li M, Li G, Tao Y, Yang X, Wu Y, Miao P, Wang Y, Li H, Ren J, Zhan M, Fang Y, Feng X, Ding X. Up-regulation of miR-325-3p suppresses pineal aralkylamine N-acetyltransferase (Aanat) after neonatal hypoxia-ischemia brain injury in rats. Brain Res 2017; 1668: 28-35.

20) Zhu Y, Xu Q, Sha WP, Zhao KP, Wang LM. MiR-219-5p promotes spinal cord injury recovery by inhibiting NEUROD2-regulated inflammation and oxidative stress. Eur Rev Med Pharmacol Sci 2019; 23: 37-43.

21) Abraham S, Kogata N, Fässler R, Adams RH. Integrin beta1 subunit controls mural cell adhesion, spreading, and blood vessel wall stability. Circ Res 2008; 102: 562-570.

22) Lewis GP, Chapin EA, Luna G, Linberg KA, Fisher SK. The fate of Muller's glia following experimental retinal detachment: nuclear migration, cell division, and subretinal glial scar formation. Mol Vis 2010; 16: 1361-1372.

23) Aoki Y, Akeda K, An H, Muehleman C, Takahashi K, Moriya H, Masuda K. Nerve fiber ingrowth into scar tissue formed following nucleus pulposus extrusion in the rabbit anular-puncture disc degeneration model: effects of depth of puncture. Spine (Phila Pa 1976) 2006; 31: E774-E780.

24) Speer CP, Pabst MJ, Hedegaard HB, Rest RF, Johnston RB Jr. Enhanced release of oxygen metabolites by monocyte-derived macrophages exposed to proteolytic enzymes: activity of neutrophil elastase and cathepsin G. J Immunol 1984; 133: 2151-2156.

25) Genovese T, Mazzon E, Esposito E, Di Paola R, Murthy K, Neville L, Bramanti P, Cuzzocrea S. Effects of a metalloporphyrinic peroxynitrite decomposition catalyst, ww-85, in a mouse model of spinal cord injury. Free Radic Res 2009; 43: 631-645.