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Original Article

MicroRNA-124-3p and Brain-derived Neurotrophic Factor in Rat Spinal Cord Injury: Inverse Expression Pattern

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Abstract

Background and objectives: Spinal cord injury (SCI) significantly impacts the central nervous system, with limited effective treatments available. Brain-derived neurotrophic factor (BDNF) plays a crucial role in neuronal growth, survival, and regeneration after SCI. MicroRNAs, particularly miR-124-3p, have been implicated in SCI pathophysiology. However, the relationship between miR-124-3p and BDNF in the context of SCI remains unclear. This study aimed to investigate the correlation between miR-124-3p expression and BDNF levels in a rat model of spinal cord injury and to assess how the timing of injury affects this relationship.

Methods: This study included 72 male Wistar rats divided into three groups: intact (n = 8), sham (n = 32), and SCI (n = 32). SCI diagnosis was confirmed through behavioral-motor function analysis using the Basso, Beattie & Brenham score and histological examination with crystal violet staining. The expression levels of miR-124-3p and BDNF were assessed using real-time polymerase chain reaction in all groups at four time points (one hour, one day, three days, and seven days post-injury).

Results: In the SCI group, a marked reduction in miR-124-3p expression was observed relative to both the sham and intact groups. Conversely, there was a substantial elevation in BDNF expression within the SCI group in comparison to the sham and intact groups. The findings underscore a negative association between miR-124-3p expression and BDNF messenger RNA levels.

Conclusions: The downregulation of miR-124-3p and concurrent upregulation of BDNF suggest a potential regulatory role of miR-124-3p in modulating BDNF expression during SCI. These findings provide new insights into the molecular mechanisms underlying SCI and suggest that miR-124-3p and BDNF could serve as potential therapeutic targets. Further research is needed to explore the translational potential of these findings for developing novel diagnostic and therapeutic strategies for SCI.

Introduction

Spinal cord injury (SCI) is a widespread and impactful condition of the central nervous system. Despite global research efforts, effective treatments have not been found. SCI can cause changes in mobility, muscle strength, and organ functions, either temporarily or enduringly.[1,2] There are two classifications for SCI: primary and secondary injuries.[3] The incidence of new cases is consistently increasing, affecting millions of individuals worldwide.[4] People with SCI are at a higher risk of early death and depression, and the costs of their care greatly impact their quality of life.[5,6] Research emphasizes the importance of identifying biomarkers with diagnostic and prognostic significance for SCI.[7]

Understanding the molecular mechanisms involved in SCI is crucial for developing effective treatments. One key player in these mechanisms is the brain-derived neurotrophic factor (BDNF), which is acknowledged for its significant role in the functioning of the central nervous system (CNS).[8] This factor is essential in aiding the processes of neuronal growth, survival, and differentiation, as well as supporting neuronal regeneration, synaptic plastic-

Keywords: MicroRNA; miR-124-3p; Spinal cord injury; SCI; Brain-derived neurotrophic factor; BDNF.

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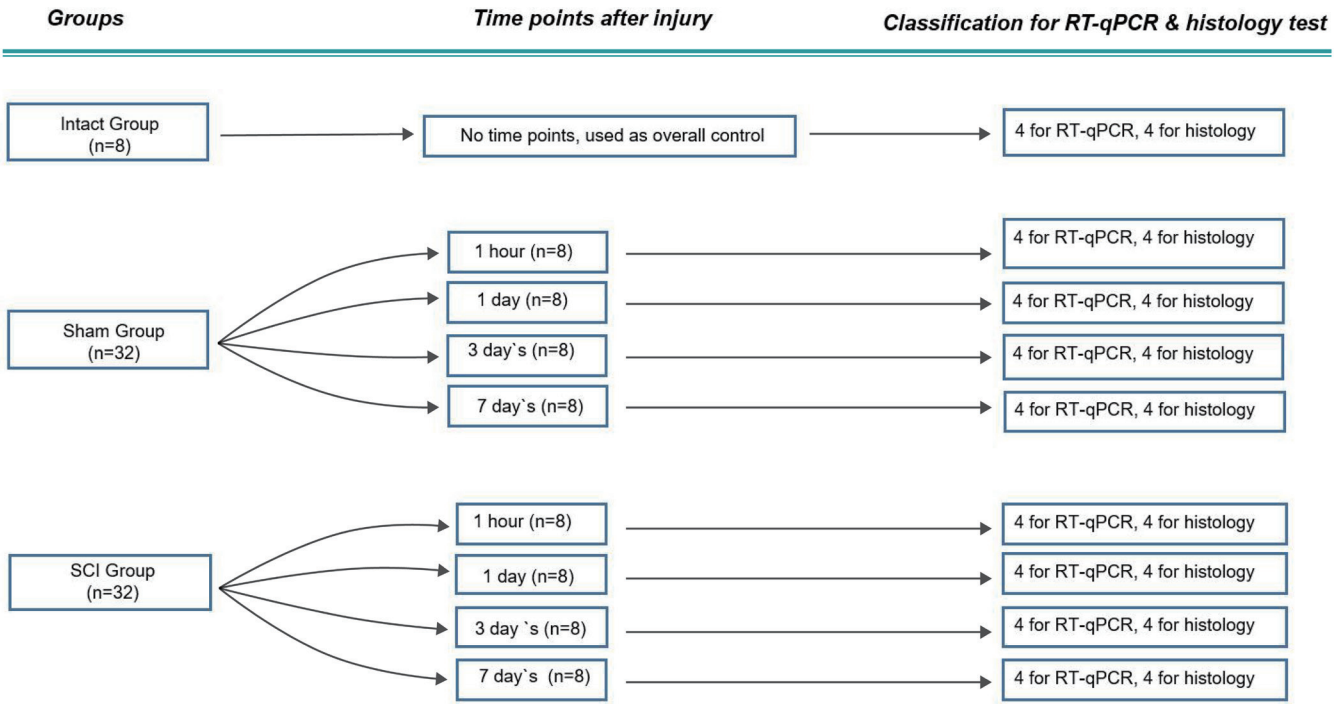

Fig. 1. Flowchart of the procedure and sample size. RT-qPCR, real time-qPCR; SCI, spinal cord injury.

ity, and the restoration of function after SCI.[9] BDNF's significance in the nervous system suggests it could provide neuroprotective benefits and encourage axon regeneration in cases of SCI.[10]

The regulation of gene expression, including that of BDNF, plays a crucial role in the response to SCI. MicroRNAs (miRNAs) are important regulators in this process. These short, non-coding RNA molecules, typically composed of 19 to 24 nucleotides, have been identified in the mammalian brain and spinal cord.[11,12] Following SCI, there is an abnormal regulation of different miRNAs in the spinal cord, which may lead to the development of the injury's pathology.[13] One of the key miRNAs involved in the healing mechanisms of SCI is miR-124-3p.[14] miR-124-3p is mainly expressed in neurons and neural tissues in a variety of organisms, including nematodes and primates. Its main role is to promote the formation of new neurons during embryonic development.[15] In SCI, there is a notable reduction in miR-124-3p expression, leading to a disruption in the regulation of neural stem cells and a decrease in the spinal cord's protective mechanisms.[16] Recent studies have suggested a potential regulatory relationship between miR-124-3p and BDNF in the context of SCI, but this relationship remains to be fully elucidated.

Hence, this current study aimed to investigate the correlation between miR-124-3p expression and BDNF levels in a rat model of spinal cord injury and to assess how the timing of injury affects this relationship. By elucidating this temporal relationship, we aimed to contribute to the understanding of SCI pathophysiology and potentially identify new targets for therapeutic interventions.

Materials and methods

Animals and grouping

In this study, 72 male Wistar rats, aged 12–13 weeks and weighing 220–230 g, were divided into three groups: sham (n = 32), SCI (n = 32), and control (n = 8). Each group was assessed at four different time points (one hour, one day, three days, and seven days). For real time-qPCR (RT-qPCR) analysis and histological assessments, four rats from each group were used (Fig. 1). The sample size was determined using GPOWER software to ensure a minimum of 72 animals with a statistical power of 0.85 (1-B). The rats were housed in clean facilities with controlled temperature (22–26°C) and humidity (40–60%). All research procedures and animal experiments adhered to the guidelines outlined in the Manual for the Proper Care and Utilization of Laboratory Animals. Ethical approval was obtained from the Review Board and Ethics Committee of Arak University of Medical Sciences (IR.ARAKMU.REC.1401.019).

Creation of the SCI model

The rats were divided into three groups: (1) intact group (n = 8), which underwent no manipulation; (2) sham group (n = 32), which underwent surgical procedures without spinal cord injury; and (3) SCI group (n = 32), in which SCI was induced by applying controlled pressure to the T9-T10 region. This site was selected based on previous research due to its clear anatomical visibility and impact on rat movement.[17] The rats were anesthetized with ketamine (70 mg/kg) and xylazine (10 mg/kg), their heads were immobilized, and the surgical site was prepared. A hole was carefully drilled in the T9-T10 region of the vertebral column without damaging the spinal cord. A weighted lead cylinder was then used to apply controlled pressure, inducing SCI. After the procedure, the incision was sutured and sterilized, and the rats were placed in suitable cages for post-operative care and observation.

Histological examination

The spinal cord specimens were preserved at −80°C to maintain molecular integrity for future gene expression analyses. Four rats from each experimental group were randomly selected at each des-

Table 1. Primer sequences for reverse transcription and RT-qPCR analysis

Primer name	Sequence
Stem-miR-124-3p	5′-GTC GTATGGAGAGCAGGGTCCGAGGTATTCGCACTCGATACGAGGCATTC-3′
Rno-miR-124-3P	F: 5′- GCACGCGGTGAATGCC-3′
Stem-Snord-47 (U47)	5′GTCGTATGCAGAGCAGGGTCCGAGGTATTCGCACTGCATACGACAACCTCA-3′
Rno-Snord-47 (U47)	F: 5′-ATCACTGTAAAACCGTTCCA-3′
Common reverse	R: 5′-AGAGCAGGGTCCGAGGT-3′
Rno-BDNF	F: 5′-CAGGAGCGTGACAACAATG-3′
	R: 5′-TCACCTGGTGGAACTCAG-3′
Rno-GAPDH	F: 5′-CGGTGTGAACGGATTTGG-3′
	R: 5′-CTGGCTCCTGGAAGATGG-3′

BDNF, brain-derived neurotrophic factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-qPCR, real time-qPCR.

ignated time point for histopathological assessment. After achieving deep anesthesia, the spinal cord was surgically excised and immersed in 10% formalin overnight for fixation. The fixed spinal cord specimens were embedded in paraffin, and 5-µm-thick coronal sections were prepared for further analysis. After alcohol-based dehydration, the sections were stained with crystal violet (Nissl staining) and mounted onto glass slides. The slides were examined under a light microscope (×40 objective lens; BX51, Japan), and images were captured with a digital camera (Olympus DP 11, Japan). Ultimately, the region of injury and cellular alterations were identified based on their distinct morphological characteristics.

The assessment focused on identifying key histopathological features of SCI, including: Tissue architecture (Evaluation of overall spinal cord structural integrity), Cellular morphology (Assessment of neuronal cell body size, shape, and staining intensity), Nissl substance (Examination of the presence and distribution of Nissl body presence within neurons, an indicator of neuronal health), and Cavitation (Detection of fluid-filled cavities within the spinal cord tissue). The region of injury and cellular alterations were identified based on their distinct morphological characteristics, allowing for differentiation of the injured areas from the normal tissue in the sham and control groups. The extent of tissue damage was semi-quantitatively analyzed by measuring the cavitation area and inflammatory cell density using ImageJ software.

Behavioral examination

Hind limb motor function was assessed using the Basso, Beattie & Bresnahan (BBB) motor rating score.[18] To ensure unbiased evaluation, rats showing behavioral impairments post-surgery were randomly selected for histological and RT-qPCR analyses. Motor function was assessed in a 1m × 1m circular Plexiglas activity box, where movement was analyzed over 5-m observation periods. The evaluation included: limb movement, paw placement, stride, coordination, toe spacing, and tail position. Two experienced researchers, blinded to experimental conditions, conducted the scoring process.

RNA extraction

RNA was extracted from spinal cord tissue using the RNX-Plus kit (SinaClon, Iran). The procedure involved homogenizing 50 mg of spinal cord tissue in 0.5 mL of RNX-Plus solution on ice. This mixture was incubated at room temperature for 5 m before adding chloroform, followed by vigorous shaking and another incubation. The samples were then centrifuged at 12,000 g for 30 m at 4°C, and the upper phase was transferred to new tubes and mixed with isopropanol. After a 10-m incubation, the samples were centrifuged again, and the resulting pellet was washed with 70% ethanol. Finally, the RNA was dried, reconstituted in DEPC (diethylpyrocarbonate)-treated water, incubated at 55°C, quantified using a NanoDrop spectrophotometer, and stored at −80°C.

Complementary DNA (cDNA) synthesis and RT-qPCR

cDNA was synthesized from total RNA using stem-loop primers for miRNA and random hexamer primers for BDNF messenger RNA (mRNA). The reaction mixture (1 µg RNA, 1 µM primers) was incubated at 75°C for 5 m, followed by the addition of: 0.5 µL M-MLV enzyme (Yekta Tehiz, Iran), 1x RT enzyme buffer, and 10 µM dNTPs. The microtubes with the reaction mixture underwent sequential incubation at 25°C for 15 m, 37°C for 15 m, 42°C for 45 m, and 75°C for 10 m in a thermal cycler system. The mixture was stored at −20°C, and gene expression was analyzed using Amplicon SYBR Green Master Mixes RT-qPCR kit (Yekta Tajhiz, Iran). GAPDH and Snord-47 were used as reference genes for the normalization of BDNF and Rno-miR-124-3p expression levels, respectively. The RT-qPCR reaction mixture included 6.25 µL Amplicon SYBR Green, 2.5 µL cDNA, 0.5 µL of each specific forward and reverse primer, and 2.75 µL DEPC-treated water. All reactions were performed in triplicate. Thermal cycling conditions involved an initial denaturation at 95°C for 10 m, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 10 s, and extension at 72°C for 30 s. Primer sequences are listed in Table 1.

Statistical analysis

RT-qPCR data were analyzed using REST (2009) software. A significance threshold of $P < 0.05$ was applied. Experimental procedures and statistical analyses were conducted blindly to prevent bias. For continuous variables that met normality and homogeneity of variance assumptions (e.g., BBB scores, ImageJ measurements), a one-way analysis of variance followed by Tukey's post-hoc test was used for multiple comparisons ($P < 0.05$ considered statistically significant).

Result

Morphology of SCI

Following a defined post-injury interval, the rat's spinal cord was rendered immobile, and tissue blocks were processed for Nissl staining. The histological examination of the affected regions re-

vealed distinctive pathological features, highlighting discernible changes in cellular morphology induced by SCI (Fig. 2). These alterations clearly differentiated the injured areas from the normal tissue, allowing distinction from the sham or control group. Semi-quantitative analysis using ImageJ software revealed a significantly larger cavitation area at the injury epicenter in the SCI group (1.25 ± 0.35 mm^2) compared to the sham group (0.08 ± 0.02 mm^2) and control group (0.05 ± 0.01 mm^2; $P < 0.001$, analysis of variance with Tukey's post-hoc test). Furthermore, the density of inflammatory cell infiltration was significantly increased in the SCI group (525 ± 75 cells/mm^2) compared to the sham (75 ± 15 cells/mm^2) and control groups (60 ± 10 cells/mm^2; $P < 0.001$) (Table 2).

These values indicate that in the SCI group, the cavitation area and inflammatory cell infiltration were significantly greater than in the sham and control groups. These findings are consistent with the pathological hallmarks of SCI and its associated inflammatory response.

At each designated time point, the SCI group exhibited visible depressions at the injury site, resulting in noticeable changes in behavioral patterns. In contrast, the sham group, despite undergoing surgical intervention within the same region, exhibited no detectable changes, as no injury was inflicted. The lesion area at T9-T10, accessed dorsally in all subjects, provided a clear view for observing cellular structural alterations.

Behavioral observations

Throughout the study, the assessment of locomotor function using the BBB test unveiled a marked difference between the control and sham groups compared to the SCI group. Analysis of the movement test scores from the beginning to the end of the study revealed no significant difference between the control and sham groups, indicating that the laminectomy alone did not affect the animals' mobility. However, the SCI group exhibited significantly different motor scores compared to both the control and sham groups, indicating impaired motor function ($P < 0.05$) (Fig. 3).

Expression of Rno-miR-124-3p and BDNF in rat SCI

The RT-qPCR analysis demonstrated a significant downregulation of miR-124-3p expression levels in the SCI group compared to the sham and control groups at four distinct time points: one hour (0.73), one day (0.66), three days (0.43), and seven days (0.52), with a statistical significance of $P < 0.05$. Conversely, BDNF expression was significantly upregulated in the SCI group relative to the sham and control groups at the same time points: one hour (6.41), one day (3.94), three days (8.21), and seven days (7.86), with a statistical significance of $P < 0.05$ (Fig. 4).

Discussion

The intricate involvement of miRNAs in various biological processes has rendered them a focal point of extensive investigation concerning SCI.[19–21] The occurrence of SCI initiates perturbations in the expression levels of miRNAs, implying their significant regulatory role in diverse aspects such as inflammation, oxidative stress, cell death, glial scar formation, and axonal growth. These aberrant alterations highlight the crucial contribution of miRNAs in the pathophysiology of SCI.[22,23]

SCI is anticipated to induce changes in the expression levels of approximately 2,841 genes at different time intervals.[24] Extensive scientific investigations have consistently demonstrated a strong association between the dysregulation of miR-124-3p, a ubiquitous molecule in the central nervous system, and the pathogenesis and progression of disorders affecting this specific physiological system.[25] Conversely, the luciferase assay results provided compelling evidence indicating a direct regulatory influence of miR-124-3p on the expression of BDNF.[26,27]

This study has several limitations that should be acknowledged. Firstly, the sample size, particularly in the control group (n = 8), was relatively small, which may limit the generalizability of our findings. Secondly, while we observed changes in miR-124-3p and BDNF expression levels, we did not perform a luciferase assay to directly confirm the regulatory relationship between miR-124-3p and BDNF. This assay could have provided additional evidence for the direct interaction between these molecules (addressed by referencing studies that have employed this assay). Thirdly, our study focused on a specific time frame (up to seven days post-injury), and longer-term effects were not investigated. Finally, while we used a rat model of SCI, the translation of these findings to human SCI may require further investigation. Future studies should address these limitations to strengthen the conclusions drawn from this research.

Our investigation focuses on elucidating the involvement of miRNA-124-3p in the regulatory mechanisms underlying SCI through the modulation of BDNF expression. Our findings demonstrate a significant decrease in miR-124-3p levels, concomitant with an upregulation of BDNF gene expression in the rat spinal cord following injury at multiple time points. Notably, our study establishes a direct correlation between miR-124-3p expression and BDNF in the context of SCI. Previous bioinformatics studies have identified two specific regions of the BDNF molecule as targets for miR-124-3p.[27,28] Leveraging this prior knowledge, we incorporate these findings into our current study, utilizing the outcomes of previous research to inform and guide our investigation.

The research encompassed three distinct stages. Leveraging previous bioinformatics analyses, the initial stage involved establishing an animal model of SCI through surgical induction of injury at the T9–T10 region in the dorsal aspect of the spinal cord. Subsequent confirmation of SCI was accomplished using histological methodologies and behavioral assessments. Post-injury, it was anticipated that the SCI group would exhibit a significant reduction in BBB scores, indicative of impaired function. Conversely, the sham group was expected to display stable BBB scores akin to the control group. Indeed, this linear representation serves as a visual depiction elucidating the temporal alterations in function in response to SCI and treatment interventions. In the third stage, the assessment of BDNF and miR-124-3p expression levels in spinal cord tissues was conducted using RT-qPCR. The findings unveiled a notable reduction in the expression of Rno-miR-124-3p and an increase in BDNF mRNA expression subsequent to the induction of SCI.

In a relevant context, Cheng *et al*.[29] identified that the SRY-box transcription factor Sox9 serves as a physiological target of miR-124-3p during the transition from transit-amplifying cells to the neuroblast stage. Consequently, the suppression of Sox9 by miR-124-3p plays a crucial role in facilitating the progression of the stem cell lineage in the subventricular zone toward neuronal differentiation.[29]

Nagata *et al*.[30] demonstrated the potential regulatory role of miR-124-3p in modulating various injury-associated transcription factors, including STAT3, which are crucial for the appropriate neuronal healing process. Furthermore, Luo *et al*.[31] highlighted the inhibitory effect of miR-124-3p on tumor formation in glioma, a CNS disease, through the targeting of Fra-2, a constituent of the AP-1 transcription factor family.

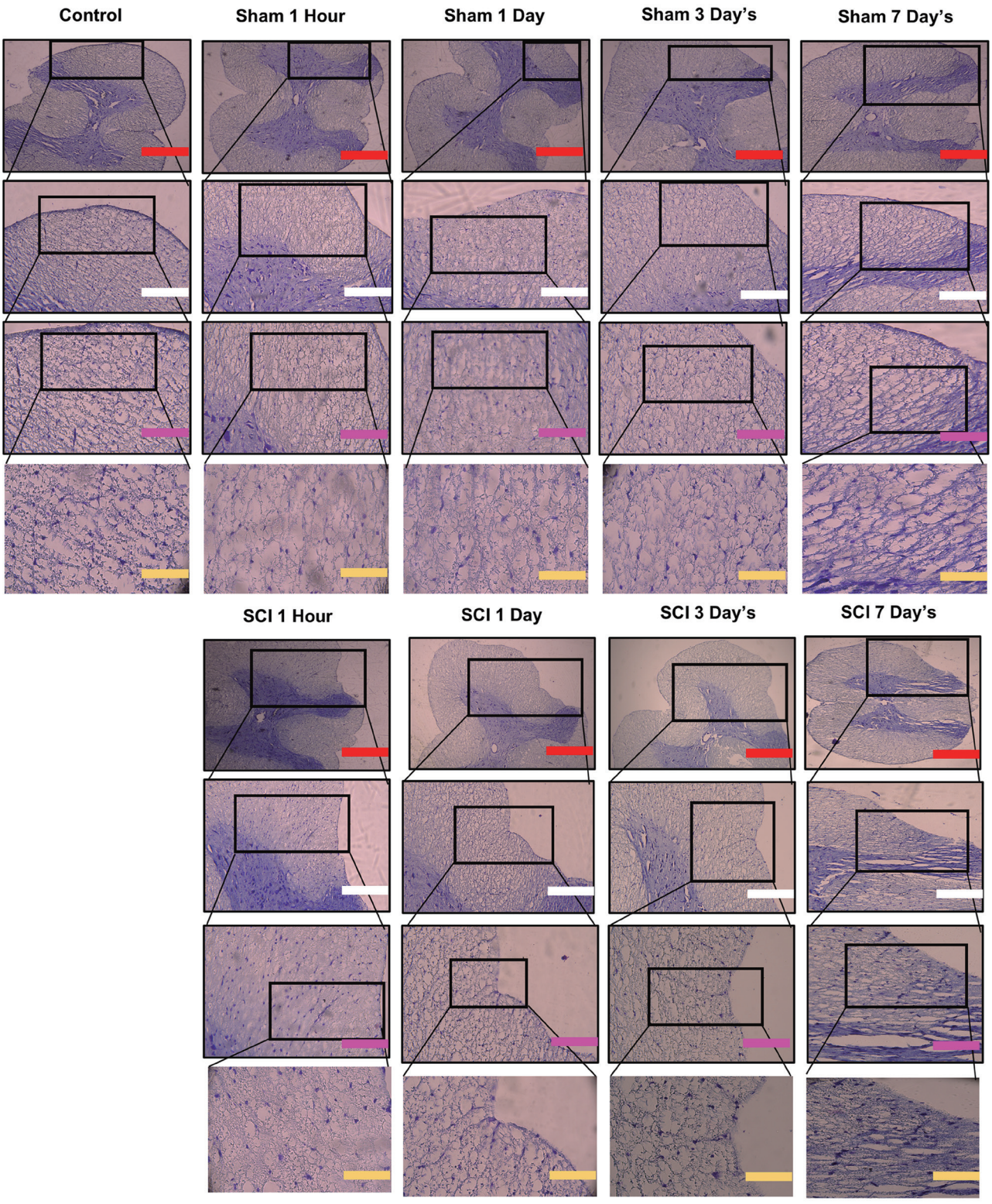

Fig. 2. Representative images of spinal cord sections (T9-10) stained with crystal violet (Nissl staining). The control group (healthy rats) showed normal tissue and cell density. In the sham group (surgery without injury), no deformities were observed at one hour, one day, three days, or seven days post-surgery. In the SCI group, spinal cord injury resulted in depression and deformation at the same time points. The SCI group also showed cavitation and inflammatory cell infiltration at the injury site. Quantification of cavitation area density was performed using ImageJ software. Three biological replicates were used. Scale bars in red, white, pink, and yellow represent 1 mm, 400 µm, 200 µm, and 100 µm, respectively. SCI, spinal cord injury.

Table 2. Results from analyzing histological images using ImageJ software

Test	Intact	Sham	SCI
Cavitation area	0.05 ± 0.01 mm^2	0.08 ± 0.02 mm^2	1.25 ± 0.35 mm^2
Inflammatory cell infiltration	60 ± 10 cells/mm^2	75 ± 15 cells/mm^2	525 ± 75 cells/mm^2

SCI, spinal cord injury.

The aforementioned studies indicate that miR-124-3p exhibits distinct transcription factor targets in neuronal cells or tissues, and any perturbation in the expression of miR-124-3p may contribute to the pathogenesis of CNS disorders. Conversely, these investigations have also demonstrated that the upregulation of miR-124-3p can facilitate the repair of SCI by modulating the expression of various mRNAs in mice. Indeed, compelling evidence indicates that miR-124-3p exerts a significant protective effect by mitigating neuronal loss and suppressing astrocyte activation. Therefore, it is crucial to explore the potential of miR-124-3p expression as a promising prognostic indicator for individuals with SCI.

To the best of our knowledge, our study presents the first documented instance of a decrease in miR-124-3p levels in a rat model of SCI. Based on our research findings, we propose that miR-124-3p holds promise as a diagnostic biomarker worthy of further investigation in clinical settings.

Furthermore, there is a consistent and sustained upregulation of BDNF expression in motor neurons as part of the physiological response to SCI. This heightened expression of BDNF, induced by oxidative stress in neurons, activates apoptotic pathways, leading to cell death.[32] Although targeting multiple transcription factors poses challenges, numerous studies have showcased the efficacy of diverse classes of miRNAs in modulating the expression of the BDNF gene. BDNF holds crucial regulatory functions, and these findings highlight the potential of miRNAs as effective regulators of BDNF.[33,34]

In a separate investigation, it was observed that miR-128-3p exerts a neuroprotective function against neuroinflammation and apoptosis in the context of spinal cord ischemia-reperfusion injury. This protective mechanism is achieved through the downregulation of BDNF expression.[35] Lastly, BDNF, extensively studied and recognized for its multifaceted involvement in various human diseases, particularly in the context of SCI, plays diverse roles.[10] Furthermore, multiple investigations have postulated that BDNF serves as a noteworthy target of miR-124-3p during neurogenesis.[28]

In line with this notion, our RT-qPCR data consistently coincide with these observations, demonstrating that miR-124-3p fosters functional recovery following SCI, whereas BDNF elicits neuroinflammatory responses and prompts apoptosis. Significantly, our study establishes a negative correlation between the expression levels of miR-124-3p and BDNF in the SCI context, aligning with previous research that has recognized BDNF as a viable functional target of miR-124-3p.[36]

Hence, this study elucidated the direct regulatory role of miR-124-3p in modulating BDNF expression in an animal model of SCI. In summary, the targeting of miR-124-3p/BDNF holds considerable promise as a prospective strategy for detecting severe SCI and may serve as a novel diagnostic and prognostic biomarker. Furthermore, our study represents the first confirmation of the impact of miR-124-3p on BDNF in an animal model of SCI. This discovery opens new avenues for future investigations and the potential development of targeted therapeutic interventions for SCI.

Our findings not only contribute to the understanding of molecular mechanisms in SCI but also suggest potential therapeutic strategies that warrant further investigation. The observed inverse relationship between miR-124-3p and BDNF expression in SCI introduces several promising therapeutic interventions. One promising approach could be the development of miR-124-3p inhibitors or antagomirs, which could potentially upregulate BDNF expression and promote neuroprotection and regeneration after SCI. Conversely, BDNF mimetics or direct BDNF administration could compensate for the decreased miR-124-3p levels. Additionally, the temporal expression patterns of miR-124-3p and BDNF observed in our study suggest that the timing of such interventions may be crucial, with early intervention potentially yielding the most significant benefits. Future

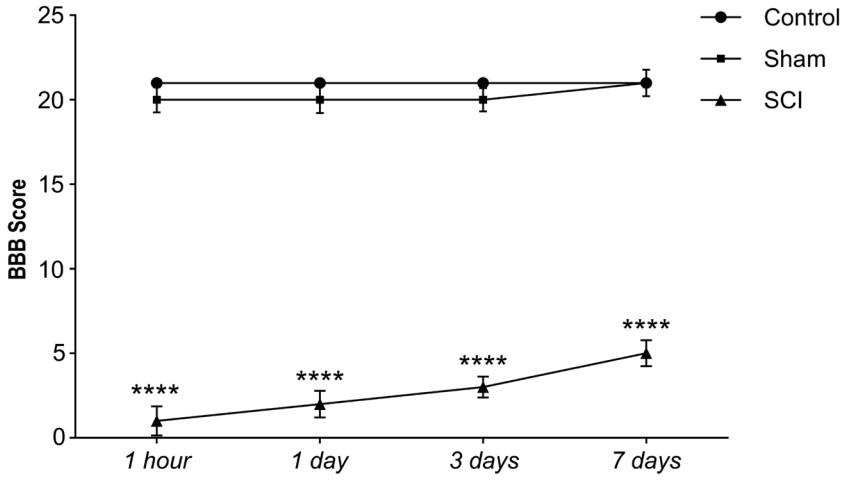

Fig. 3. The linear representation of BBB test results for the control, sham, and SCI groups spans from the initial assessment to the 7th day post-injury, illustrating locomotor function over time. The intact group serves as the baseline for normal locomotor function, the sham group underwent surgery without sustaining a spinal cord injury, and the SCI group experienced a spinal cord injury (***$P < 0.0001$). BBB, Basso, Beattie & Bresnahan; SCI, spinal cord injury.

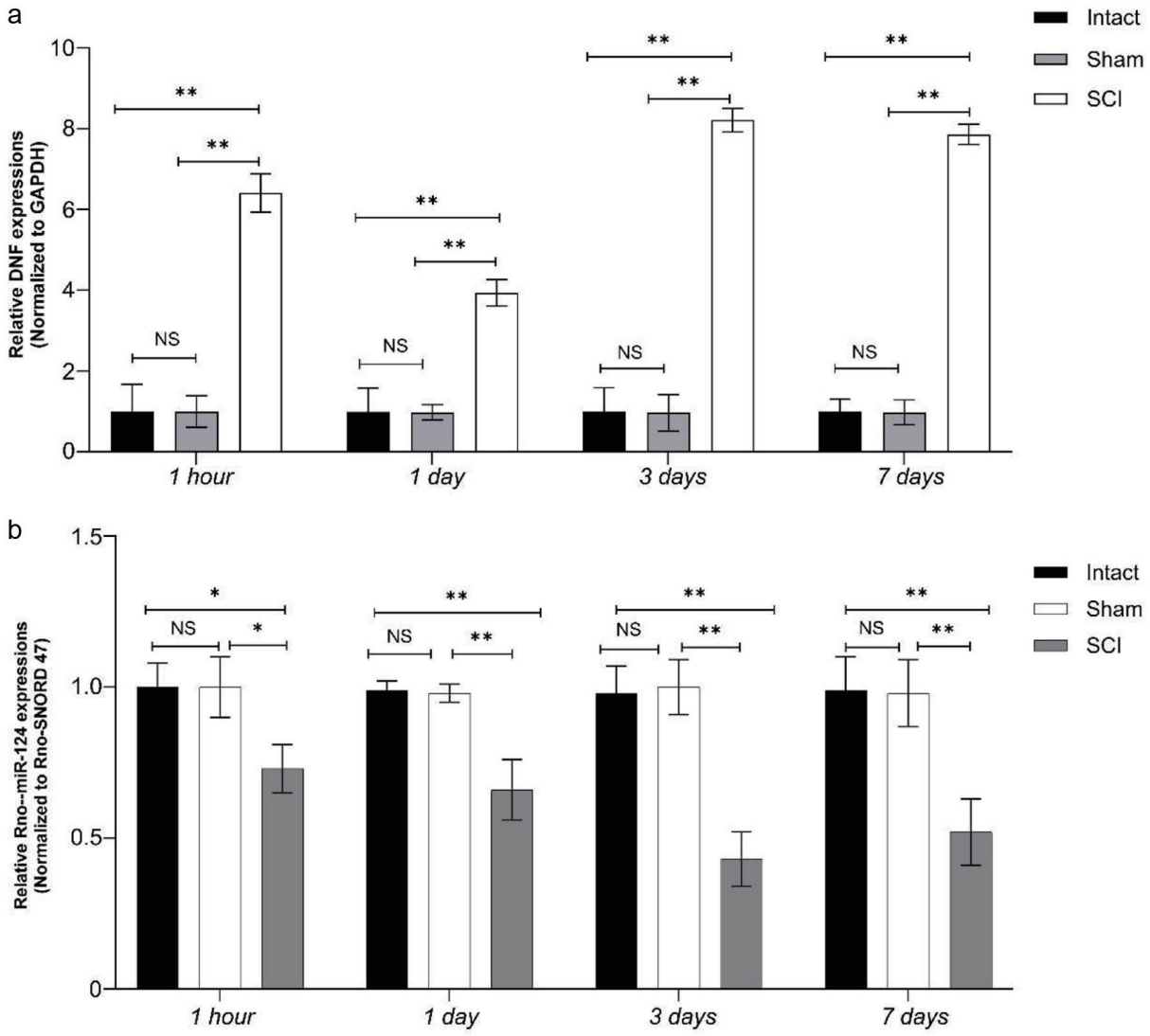

Fig. 4. Assessment of BDNF and Rno-miR-124-3p gene expression in rat spinal cord tissue using RT-qPCR. In the SCI group. (a) Overexpression of BDNF and (b) downregulation of Rno-miR-124-3p in rat spinal cord tissues compared to the control and sham groups. The differences from the control group were found to be statistically significant. The results were normalized using GAPDH and Rno-SNORD 47 (U47) as internal controls (*$P < 0.05$ and **$P < 0.001$). BDNF, brain-derived neurotrophic factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NS, non-significant; RT-qPCR, real time-qPCR; SCI, spinal cord injury.

studies could explore combination therapies that target both miR-124-3p and BDNF pathways simultaneously, potentially enhancing the regenerative response following SCI. Moreover, these findings could inform the development of biomarkers for SCI progression and recovery, aiding in personalized treatment strategies. However, while our results are promising, further research—including *in vivo* studies with miR-124-3p modulation and BDNF augmentation—is necessary to fully elucidate the therapeutic potential of targeting this pathway in SCI treatment.

Conclusions

This study provides valuable insights into the relationship between miR-124-3p and BDNF expression in the context of spinal cord injury. Our findings demonstrate a significant downregulation of miR-124-3p and a concurrent upregulation of BDNF levels following SCI in a rat model. These results, supported by histological examinations and behavioral assessments, suggest a potential regulatory role of miR-124-3p in BDNF expression and SCI pathophysiology. While our study contributes to the understanding of SCI molecular mechanisms, further research is needed to fully explore the therapeutic potential of targeting the miR-124-3p/BDNF pathway. Future studies should focus on *in vivo* manipulation of miR-124-3p levels and explore combination therapies to enhance regenerative responses after SCI. These findings may pave the way for novel diagnostic biomarkers and therapeutic strategies in spinal cord injury management.

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Conflict of interest

The authors declare no conflicts of interest in this work.

Author contributions

Investigation, validation, formal analysis, data curation, software, methodology (MZ, MS, BK, MM), resources (MS, BK, MM), visualization (MZ, MS, MM), writing - review & editing (MZ, MM), writing - original draft (MZ, MM), project administration, and supervision (MM). All authors read and approved the final manuscript.

Ethical statement

All research procedures and animal experiments adhered to the guidelines outlined in the Manual for the Proper Care and Utilization of Laboratory Animals. Ethical approval was obtained from the Review Board and Ethics Committee of Arak University of Medical Sciences (IR.ARAKMU.REC.1401.019).

Data sharing statement

All data generated in this study are available upon reasonable request.

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